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Multiplex Detection of DNA Mutations by the Fluorescence Fingerprint Spectrum Technique**

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Detection of DNA mutations is clinically important for diagnostics, prognostics, and disease assessment.[1] A variety of methods have been developed for the detection of DNA mutations, such as DNA sequencing (dideoxy sequencing and pyrosequencing),[2,3] high-resolution melting analysis (HRMA),[4] and qRT-PCR methods.[5] However, these methods are only suitable for mutation detection on a single gene or exon, which cannot achieve multiplexed analysis easily. As the demands of clinical diagnostics, prognostics, and disease assessment increase, it is appealing to develop methods that allow simultaneous detection of multiple genetic alterations. Single-base primer extension assay (such as ABI PRISM SNaPshot Multiplex Kit, Applied Biosystems) is such a highly attractive diagnostic method.[6] However, it needs expensive capillary sequencer as well as sophisticated post-extension treatment. SNaPshot genotyping is suitable for single base substitution detection, but it is not applicable for the detection of complex mutations (such as deletion and insertion mutation).[7] MALDI-TOF (matrix-assisted laser desorption/ionization time-of-flight) mass spectrometry can offer high multiplexing capability for DNA mutation detection; however, it requires expensive instruments and technical expertise. Therefore, it is highly desirable to develop new method for simple, rapid, sensitive, and flexible detection of multiple DNA mutations.

The concept of fingerprint is commonly utilized for developing qualitative detection systems. The dideoxy fingerprint method, which combines the dideoxy sequencing reaction and single-strand conformation polymorphism (SSCP) analysis, has been developed to identify DNA mutations.[8–10] By comparing dideoxy fingerprint patterns of samples with the established standard patterns, DNA mutations can be intuitively and sensitively determined. Nevertheless, this method is also based on electrophoresis separation and is difficult to simultaneously detect mutations on multiple exons or genes. Water-soluble cationic conjugated polymers (CCPs) have attracted a lot of attention in developing highly sensitive DNA biosensors owing to their signal-amplifying effects.[11–19] However, higher multiplex detection capacity for CCP-based systems still needs to be exploited. Herein, we present a proof-of-concept of CCP-based FRET fingerprint spectrum (FFS) assay that can be utilized for multiplex DNA mutations detection. In this method, multi-step-FRET from CCPs to different fluorescent dyes is combined with dideoxy sequencing reaction. In contrast to most present methods for multiplex detection of DNA mutations, the major advantage of our strategy is the "mix and read" manner in homogeneous solution without requiring the electrophoresis separation. This method provides very good multiplex detection capability for homogeneous detection of DNA mutations in one tube. More importantly, multiple DNA mutations originated from clinical samples can be simultaneously and successfully determined using the proposed FFS assay.

The mechanism of the CCP-based FRET fingerprint technique is shown in Scheme 1. First, a dideoxy sequencing reaction is carried out to acquire extension products for different DNA targets. In the basic dideoxy sequencing reaction, an extension primer is annealed to a DNA template, which can be achieved by prior PCR amplification from tested sample, and extended by DNA polymerase in the presence of four dNTPs. The extension reaction can be terminated when four dye-labeled ddNTPs (fluorescein, Cy3, TexRed, and Cy5) are incorporated into the growing strand. The concentration of dye-ddNTPs used in this method is much higher than that of dNTPs, which is different from the traditional dideoxy sequencing reaction.[2] Therefore, all of the extension reactions can be terminated early, and short-strand extension products have a higher yield than long-strand products. For this reason, the base alteration close to the 3′-terminus of primer has a significant effect on the proportion of four dyes incorporated into the extended strands. For wildtype DNA, the degree of chain termination by TexRed-labeled ddATP is higher than other three dye-ddNTPs and follows the sequence: TR-ddA > Fl-ddU > Cy3-ddC > Cy5-ddG. For the case of mutation 1 in Scheme 1 (the nucleotide "T" in the wildtype is replaced by "A" in the mutant target), the degree of chain termination by fluorescein-labeled ddUTP is higher than other three dye-ddNTPs. Thus, the amount of fluorescein-labeled strands increases, while TexRed-labeled strands decreases in total extension products. Similarly, for mutation 2 in Scheme 1, the amount of Cy3-labeled strands increase while that of Cy5-labeled

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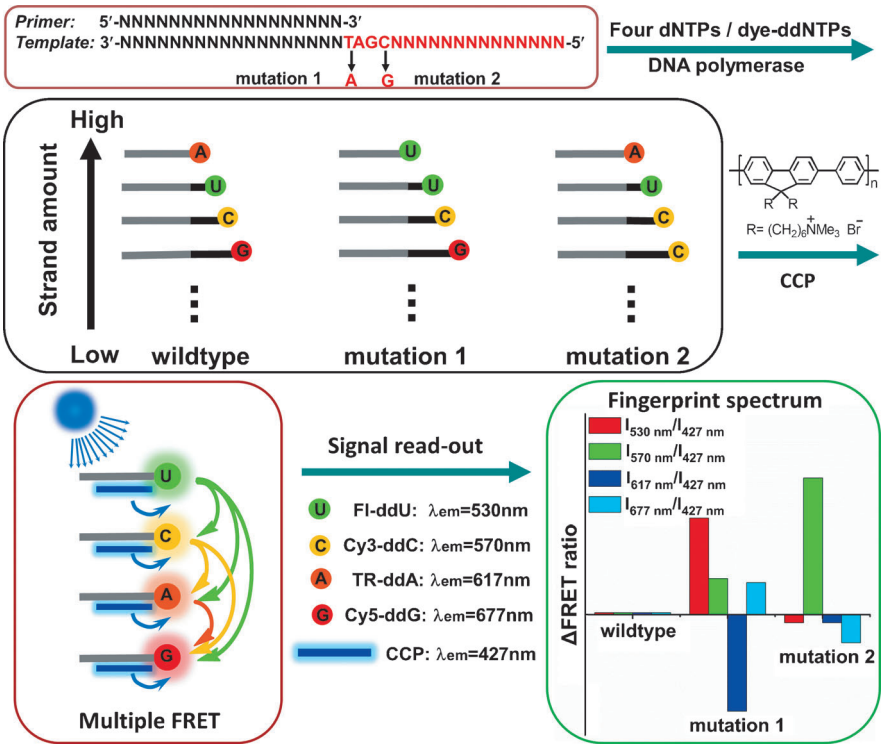

Scheme 1. Presentation of the CCP-based FRET fingerprint technique for DNA mutation detection. The presence of two base alterations results in distinct FFSs, which can be used for multiplex detection of DNA mutations. ΔFRET ratio is the difference value of the FRET ratio in the presence and absence of DNA mutation. Mutiplex FRET can be triggered at a single excitation wavelength of 380 nm. Fl = fluorescein, TR = TexRed.

strands decrease in total extension products. Upon adding CCP to the dye-labeled extension products, the strong electrostatic interactions between negatively charged DNA and cationic CCP bring them close and multistep-FRET between CCP and fluorescence dyes occurs upon selective excitation of CCP with 380 nm. In the multistep-FRET process, CCP acts as donor for four dyes; fluorescein acts as acceptor for CCP but as donor for Cy3, TexRed, and Cy5; Cy3 acts as acceptor for CCP and fluorescein but as donor for TexRed and Cy5; TexRed acts as acceptor for CCP, fluorescein, and Cy3 but as donor for Cy5. Collecting multistep-FRET spectra, FRET ratios are obtained and analyzed, and then the fingerprint spectrum based on FRET ratio change (ΔFRET ratio) is presented, where FRET ratio is defined as the ratio of maximum emission intensity of each dye to that of CCP, and ΔFRET ratio is the difference value of the FRET ratio in the presence and absence of DNA mutations. One single-base alteration close to the 3′-terminus of primer can cause a conspicuous change of FFS. Therefore, FFS patterns can be employed as the basis for multiplex DNA mutations detection.

To demonstrate the multiplex detection principle of the FFS, we established four sets of standard fingerprint spectrum (Figure 1). The first standard fingerprint spectrum is for the detection of non-adjacent mutations. In this case, *PIK3CA* gene is chosen as detection target, which covered four hotspot mutations: E542K, E545K in exon 9, and H1047R, H1047L in exon 20. DNA template containing these four mutations can be achieved by multiplex PCR (Supporting Information, Figure S1). The allele-specific primer extension (ASPE) was designed to detect these four mutations based on the principle that the DNA extension was efficient when the 3′-terminus base of a primer matched its target, but inefficient or nonexistent for mismatched case. Four primers were respectively designed against the four mutations of interest, and extension products could be obtained only if the mutation was present. Downstream DNA sequence of each mutation is unique, so it can be regarded as an ID number and then to give a specific FFS. As illustrated in Figure 1 A, the presence of four mutations results in fluorescence spectrum changes to different extent and four distinct fingerprint spectra are obtained as standards for the detection of multiplex DNA mutations. The second standard fingerprint is for the detection of adjacent mutations; in this case, *KRAS* gene is chosen as detection target, which contains twelve mutations at adjacent codons 12 and 13 on exon 2. A single extension primer was designed to target a sequence at the upstream of these adjacent mutation sites. When a mutation appears in the DNA sequence, it will give a specific FFS. As shown in Figure 1 B, every mutation results in differential standard fingerprint spectra that can be used for discrimination of twelve *KRAS* mutations. The corresponding fluorescence spectra are shown in the Supporting Information, Figure S2. Additionally, the standard fingerprint spectra for the detection of deletion and insertion mutations (Figure 1 C and D) could also be obtained, where identical primer design was utilized as that of *KRAS* mutations. The corresponding fluorescence spectra are given in the Supporting Information, Figure S3 and S4. By comparing FFSs of samples with the standard fingerprint spectra, multiplex detection of non-adjacent and adjacent mutations as well as analysis of deletion and insertion mutations can be achieved easily.

We next investigated the sensitivity and qualitative characteristic of the developed FFS technique for the detection of multiplex DNA mutations. As mutation proportions vary greatly from one sample to another, we acquired the ΔFRET values of tested DNA mutations at various proportions. As shown in the Supporting Information, Figure S5, 5% mutations for E542K, E545K, H1047L, and H1047R are easily differentiated from wildtype targets, which indicates the high sensitivity of our FFS technique. The values of ΔFRET increase as the increase of DNA mutation proportion, which is consistent with more amount of dyes incorporated into the extension products in a higher mutation

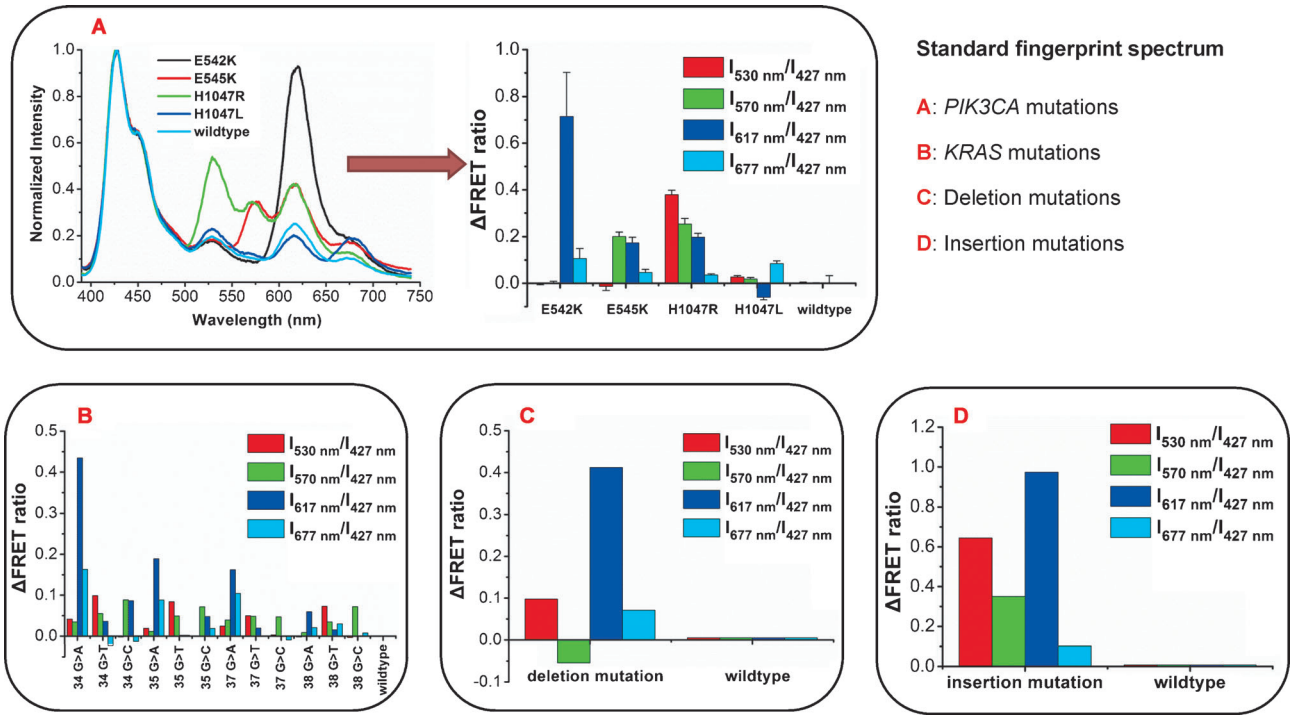

Figure 1. Standard fingerprint spectra for different mutations: A) *PIK3CA* mutations stand for non-adjacent mutations; B) *KRAS* mutations stand for adjacent mutations; C) deletion mutations; D) insertion mutations.

proportion. The profiles of FFS keep identical with different mutation proportions of DNA samples. The same trend was also observed in the corresponding fluorescence spectra (Supporting Information, Figure S6). Thus, we can qualitatively identify *PIK3CA* mutations by comparing the tested results with standard FFS regardless of the change of mutation proportions. To validate the potential use of our FFS assay in clinical diagnostics, we examined *PIK3CA* mutations of thirty formalin-fixed paraffin-embedded breast cancer tissues. As shown in Figure 2, ten mutation samples are detected and the mutation status can be easily identified by comparing tested FFS of samples with standard FFS: one H1047L mutant (sample 6), five H1047R mutants (samples 10, 11, 19, 23, 27), one E542K mutant (sample 9), and three E545K mutant (samples 13, 24, 28). DNA sequencing analysis, a gold standard for mutation detection, was also carried out to verify the accuracy of above results (data not shown). These results exhibit that our FFS technique is a reliable approach for multiplex detection of DNA mutations in clinical samples.

In summary, we have presented a proof-of-concept of CCP-based FRET fingerprint technique that can be used for simultaneous detection of multiple DNA mutations. Our assay possesses several unique features that are summarized in Table 1. First, our detection process is based on multistep FRET of CCP in a homogeneous solution instead of capillary electrophoresis separation, which makes it easier to handle by eliminating the need for denaturalizing extension products and digesting unincorporated dye-ddNTPs. Second, expensive instruments and technical expertise are not required in this method. Third, our method has a universal applicability to detect DNA sequence variation, such as nucleotide deletion, insertion, and substitution. Finally, this method is suitable for the detection of multiplex mutations in different situations, such as non-adjacent and adjacent mutations. Therefore, the present CCP-based FRET fingerprint technique provides a rapid, sensitive, and flexible strategy for multiplex detection of DNA mutations, and also will contribute to other gene analysis, such as DNA methylation and RNA alteration.

Table 1: Comparison of methods for mutation detection.

Method	Sensitivity [%]	Workload	Multiplexing capacity	Expensive equipment	Complex mutation detection
dideoxy sequencing	20	laborious	no	yes	yes
procequencing	5	laborious	no	yes	yes
HRMA	5–20	time sparing	no	yes	yes
qRT-PCR	1	time sparing	no	yes	no
SNaPshot	5	laborious	yes	yes	no
mass spectrometry	5–10	laborious	yes	yes	no
our method	5	time sparing	yes	no	yes

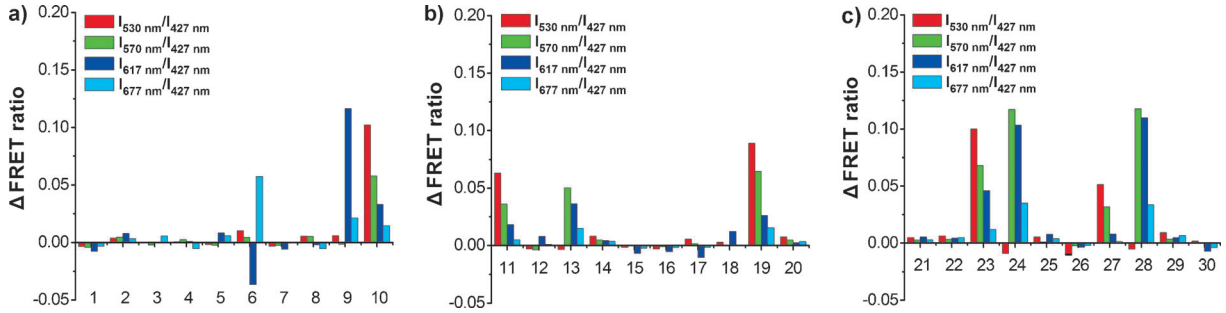

Figure 2. Detection of *PIK3CA* mutations in 30 breast cancer samples by the FFS assay. a)–c) The FFS assay for ten samples: 1–10 (a), 11–20 (b), 21–30 (c).

Experimental Section

Allele-specific primer extension (ASPE) for multiplex detection of *PIK3CA* gene mutations: 1) Clinical samples and controls: Clinical samples were excised from 30 formalin-fixed paraffin-embedded (FFPE) breast cancer tissues. Genomic DNAs (gDNA) of these clinical samples were extracted using a QIAmp DNA kit according to the manufacturer's guidelines. To identify the *PIK3CA* mutation status in these samples, we sequenced their PCR amplified products. Furthermore, positive controls were mutant DNAs prepared by PCR-mediated in vitro mutagenesis[20] and negative control was wildtype gDNA sample isolated from 293T cell line. 2) Multiplex PCR amplification of *PIK3CA* gene: To amplify *PIK3CA* gene including the mutation sites of interest, multiplex PCR was carried out. Two pairs of PCR primers (ex9-FW/ex9-RV and ex20-FW/ex20-RV) involved are described in the Supporting Information, Table S1. 40 ng of DNA was added to 30 μL of a reaction mixture containing 1 × Ex Taq buffer, 2 mM $MgCl_2$, 0.25 mM dNTPs, 0.75 unit of Ex Taq DNA polymerase, 0.2 μM of each primer. Thermal cycler conditions were: 94 °C for 2 min, 45 cycles of 95 °C for 30 s, 60 °C for 30 s, 72 °C for 30 s and finally 10 min at 72 °C. Multiplex PCR products were analyzed for quality and yield utilizing 2.5 % agarose gel electrophoresis. 8 μL PCR products were then taken to be treated with 1 unit of SAP, 10 units of exonuclease I and 0.05 units of pyrophosphatase at 37 °C for 1 h to remove excess primers, dNTPs, and pyrophosphate generated in PCR. 3) ASPE reaction for PCR products of *PIK3CA*: ASPE reactions were conducted in a total volume of 10 μL containing 1 × Thermo sequenase buffer, dNTPmix (0.4 μM dATP, 0.4 μM dTTP, 0.1 μM dCTP, 0.4 μM dGTP), dye-ddNTPmix (2 μM TR-ddATP, 2 μM Fl-ddUTP, 1 μM Cy3-ddCTP, 1 μM Cy5-ddGTP), 1 μM of each extension primer (sequence: Supporting Information, Table S1), 1 unit of thermosequenase DNA polymerase, and 3.4 μL exonuclease/SAP/pyrophosphatase-treated multiplex PCR products. Extension reactions were run in a thermal cycler and the conditions were 94 °C for 2 min, followed by 60 cycles of 94 °C for 30 s and 60 °C for 30 s.

FRET fingerprint spectrum measurement: For fluorescence measurement, 6 μL primer extension products were diluted with 586 μL HEPES buffer (25 mM, pH 8.0), and then 8 μL of CCP (15 μM) was added to the solution. The emission spectra were measured in a 3 mL quartz cuvette with an excitation wavelength of 380 nm.

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[1] T. J. R. Harris, F. McCormick, *Nat. Rev. Clin. Oncol.* **2010**, *7*, 251 – 265.
[2] F. Sanger, S. Nicklen, A. R. Coulson, *Proc. Natl. Acad. Sci. USA* **1977**, *74*, 5463 – 5467.
[3] M. Ronaghi, M. Uhlén, P. Nyrén, *Science* **1998**, *281*, 363 – 365.
[4] C. T. Wittwer, G. H. Reed, C. N. Gundry, J. G. Vandersteen, R. J. Pryor, *Clin. Chem.* **2003**, *49*, 853 – 860.
[5] P. M. Holland, R. D. Abramson, D. H. Gelfand, *Proc. Natl. Acad. Sci. USA* **1991**, *88*, 7276 – 7280.
[6] C. D. Hurst, T. C. Zuiverloon, C. Hafner, E. C. Zwarthoff, M. A. Knowles, *BMC Res. Notes* **2009**, *2*, 66.
[7] Z. Su, D. Dias-Santagata, M. Duke, K. Hutchinson, Y. L. Lin, D. R. Borger, C. H. Chung, P. P. Massion, C. L. Vnencak-Jones, A. J. Iafrate, W. Pao, *J. Mol. Diagn.* **2011**, *13*, 74 – 84.
[8] L. A. Larsen, M. Johnson, C. Brown, M. Christiansen, R. Frank-Hansen, J. Vuust, P. S. Andersen, *Hum. Mutat.* **2001**, *18*, 451 – 457.
[9] D. Martincic, M. J. Koury, K. Gale, *Oncogene* **1999**, *18*, 617 – 621.
[10] Y. O. Shevchenko, S. J. Bale, J. G. Compton, *Biotechniques* **2000**, *28*, 134 – 138.
[11] X. Duan, L. Liu, F. Feng, S. Wang, *Acc. Chem. Res.* **2010**, *43*, 260 – 270.
[12] B. Liu, G. C. Bazan, *Chem. Mater.* **2004**, *16*, 4467 – 4476.
[13] X. Duan, Z. Li, F. He, S. Wang, *J. Am. Chem. Soc.* **2007**, *129*, 4154 – 4155.
[14] S. W. Thomas, G. D. Joly, T. M. Swager, *Chem. Rev.* **2007**, *107*, 1339 – 1386.
[15] B. S. Gaylord, M. R. Massie, S. C. Feinstein, G. C. Bazan, *Proc. Natl. Acad. Sci. USA* **2005**, *102*, 34 – 39.
[16] X. Duan, W. Yue, L. Liu, Z. Li, Y. Li, F. He, D. Zhu, G. Zhou, S. Wang, *Nat. Protoc.* **2009**, *4*, 984 – 991.
[17] Q. Yang, D. Ying, W. Wu, C. Zhu, H. Chong, J. Lu, D. Yu, L. Liu, F. Lv, S. Wang, *Nat. Commun.* **2012**, *3*, 1206.
[18] J. Song, Q. Yang, F. Lv, L. Liu, S. Wang, *ACS Appl. Mater. Interfaces* **2012**, *4*, 2885 – 2890.
[19] F. Feng, L. Liu, S. Wang, *Nat. Protoc.* **2010**, *5*, 1255 – 1264.
[20] R. Higuchi, B. Krummel, R. Saiki, *Nucleic Acids Res.* **1988**, *16*, 7351 – 7367.

