Improvement of the Mutation-Discrimination Threshold for Rare Point Mutations by a Separation-Free Ligase Detection Reaction Assay Based on Fluorescence Resonance Energy Transfer

Kenta HAGIHARA, Kazuhiko TSUKAGOSHI, Chinami NAKAJIMA, Shinsuke ESAKI, and Masahiko HASHIMOTO†

Department of Chemical Engineering and Materials Science, Faculty of Science and Engineering, Doshisha University, 1-3 Tataramiyakodani, Kyotanabe, Kyoto 610-0321, Japan

We previously developed a separation-free ligase detection reaction assay based on fluorescence resonance energy transfer from a donor quantum dot to an acceptor fluorescent dye. This assay could successfully detect one cancer mutation among 10 wild-type templates. In the current study, the mutation-discrimination threshold was improved by one order of magnitude by replacing the original acceptor dye (Alexa Fluor 647) with another fluorescent dye (Cyanine 5) that was spectrally similar but more fluorescent.

Keywords Point mutation, polymerase chain reaction, ligase detection reaction, fluorescence resonance energy transfer, quantum dot, fluorescent dye

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Introduction

Point mutations are closely linked to the development of various types of cancers, including colorectal, breast, pancreatic, and lung cancer. Detecting such mutations is useful for early cancer diagnosis. A major barrier to detection is that the mutations of interest are often present at low copy numbers within an excess of normal (wild-type) DNA. Direct sequencing is a straightforward method for identifying allelic variations at target loci on a DNA sequence. However, conventional DNA sequencing technology is based on the Sanger reaction, which is impractical for the detection of low frequency mutations because signals from rare mutations are very likely to be out-competed by those from the wild-type DNA.

The ligase detection reaction (LDR) is an effective molecular diagnostic technique that allows for detection of sporadic mutations present within a majority of non-variant DNA. For LDR assays, a primary polymerase chain reaction (PCR) is conducted of the appropriate gene fragments containing the point mutations of interest. Then, the PCR amplicons are mixed with two LDR primers, including a discriminating primer and a common primer. These primers are annealed to the target sequences within the PCR amplicons at an appropriate temperature. The discriminating primer contains a nucleotide at its 3’-end that coincides with the single nucleotide mutation site. The common primer contains the complementary sequence of the template directly adjacent to the point mutation site. If there is a mismatch, no ligation of the two primers will occur. However, a perfect match allows for successful ligation at the two primer ends, which forms a fragment (i.e., LDR product) that is approximately twice as long as each of the original LDR primers. The LDR products corresponding to the presence of a mutation need to be separated before detection, which is conventionally performed by electrophoresis or DNA-DNA hybridization. Although these techniques are effective for the selective detection of LDR products, the procedures involved are generally expensive and time-consuming.

Fluorescence resonance energy transfer (FRET) is the non-radiative energy transfer from a luminescent donor to an acceptor via dipole–dipole interactions over a short distance (approximately 10 – 100 Å). FRET donor-acceptor pairs are not limited to the traditional combination of molecule–molecule, but include other combinations such as nanoparticle–molecule and nanoparticle–nanoparticle. FRET-based DNA probes such as TaqMan probes and molecular beacons allow separation-free detection of target DNA. Use of a FRET-based probe in molecular diagnostics greatly reduces the labor intensity and overall processing time of the assay, as well as the reagent and instrumentation costs.

We previously described a separation-free LDR assay based on FRET between a quantum dot and a fluorescent dye for facile and rapid detection of rare point mutations in a majority wild-type sequence population. Our assay strategy is illustrated in Fig. 1. A 3’-biotinylated common primer and an allele-specific discriminating primer labeled at the 5’-end with a fluorescent dye (Alexa Fluor 647; AF647), which serves as the FRET acceptor, were used in the LDR setup. The enzymatic activity of DNA ligase facilitated formation of an LDR product, but only if the nucleotide at the 3’-end of the discriminating primer was complementary to the nucleotide at the point mutation site (e.g., A-T match as shown in the outcome on the left in Fig. 1). The LDR products generated were then
Fig. 1 Conceptual diagram of the FRET-based LDR assay for the detection of a single point mutation in a target DNA sequence.

captured via biotin-streptavidin interaction on streptavidin-conjugated quantum dots (QDs), which served as the FRET donor. The resulting conjugate brought the QD donor and the fluorophore acceptor into close proximity. Illumination of the QD donor resulted in successful FRET and excitation of the dye acceptor, producing fluorescence emission from the acceptor. With a single-base mismatch between the discriminating primer sequence and the target DNA sequence (e.g., G-T mismatch as shown in the outcome on the right in Fig. 1), no LDR product formed. This meant no FRET occurred between the donor and the acceptor as these two moieties were not sufficiently close together, and the acceptor did not fluoresce. Therefore, fluorescence in the LDR assay specifically indicated the presence of point mutations. Except for LDR products produced in the presence of the target point mutation, none of the DNA fragments remaining in the solution participated in FRET or fluoresced upon excitation of the QDs. The excitation spectrum of AF647 is relatively narrow and does not overlap with the wavelength used to excite the QDs. Therefore, removal of these fragments was unnecessary.

With the developed method, we could detect one mutant DNA in a mixture of 10 normal DNA. While this FRET approach greatly simplified the post-LDR procedures by allowing for selective detection of the LDR products, the mutation-discrimination threshold was low compared to that obtained in our previous LDR study based on capillary gel electrophoresis, which could detect one mutation among 100 normal sequences. In this paper, we report an improvement of the discrimination threshold of the FRET-based LDR assay. This improvement was achieved by simply replacing AF647 as the FRET acceptor with another fluorescent dye, Cyanine 5 (Cy5), which has comparable excitation and emission spectra to AF647. See Supporting Information Fig. S1 for excitation and emission spectra for AF647 and Cy5, as well as those for the QDs used in the present study (i.e., Qdot 605).

Experimental

PCR amplification of genomic DNA

PCR amplifications were carried out to generate 290 bp amplicons using 50 μL of 1× ThermoPol reaction buffer (20 mM Tris-HCl at pH 8.8 containing 2 mM MgSO4, 10 mM (NH4)2SO4, 10 mM KCl, and 0.1% Triton X-100; New England Biolabs, Beverly, MA), 300 μM deoxynucleotide triphosphates (dNTPs, KAPA Biosystems, Wilmington, MA), 0.5 μM forward and reverse primers, and 50 – 200 ng of genomic DNA extracted from cultured human cell lines of a known K-ras genotype associated with the onset of colorectal cancer (LS180 for mutant G12D and HT29 for wild-type G12; ATCC, Manassas, VA). The nomenclature of the mutation (G12D) denotes a DNA base substitution (G → A) within codon 12 (wild-type: GGT) of the K-ras gene, which alters the amino acid at this position from glycine (G) to aspartic acid (D). The gene-specific PCR primers (Integrated DNA Technologies, IDT; Coralville, IA) had the following sequences: forward primer, 5'-TTAAAAAGGTACTGGTGAGATTTTGATA-3′; reverse primer, 5'-AAAATGTCAGAGAAACCTTTATCTGT-3′. After a 2-min initial denaturation at 94°C, 1.25 U of KAPA Taq DNA polymerase (KAPA Biosystems) was added under hot-start conditions, and amplification was achieved by thermal cycling for 35 cycles of 30 s at 94°C and 1 min at 60°C using a commercial thermal cycler (Eppendorf Japan, Tokyo, Japan). PCR products were subjected to electrophoresis on 2% agarose gels, followed by ethidium bromide staining for quantification. After quantification, the PCR products were stored at –20°C until required for the LDR assays.

LDR assays

LDR assays were performed under similar conditions to those described elsewhere, with slight modifications. Briefly, a mixture of the PCR amplicons (wild-type and mutant DNA) was used as template DNA for the LDR. The amplicons were added to a 50-μL reaction mixture containing 1× Taq DNA ligase buffer (20 mM Tris-HCl, 25 mM CH3COOK, 10 mM Mg(CH3COO)2, 1.0 mM NAD+, 10 mM DTT, and 0.1% Triton X-100) at pH 7.6 (New England Biolabs), 0.5 μM forward and reverse primers, and 50 – 200 ng of genomic DNA extracted from cultured human cell lines of a known K-ras genotype associated with the onset of colorectal cancer (LS180 for mutant G12D and HT29 for wild-type G12; ATCC, Manassas, VA). The nomenclature of the mutation (G12D) denotes a DNA base substitution (G → A) within codon 12 (wild-type: GGT) of the K-ras gene, which alters the amino acid at this position from glycine (G) to aspartic acid (D). The gene-specific PCR primers (Integrated DNA Technologies, IDT; Coralville, IA) had the following sequences: forward primer, 5'-TTAAAAAGGTACTGGTGAGATTTTGATA-3′; reverse primer, 5'-AAAATGTCAGAGAAACCTTTATCTGT-3′. After a 2-min initial denaturation at 94°C, 1.25 U of KAPA Taq DNA polymerase (KAPA Biosystems) was added under hot-start conditions, and amplification was achieved by thermal cycling for 35 cycles of 30 s at 94°C and 1 min at 60°C using a commercial thermal cycler (Eppendorf Japan, Tokyo, Japan). PCR products were subjected to electrophoresis on 2% agarose gels, followed by ethidium bromide staining for quantification. After quantification, the PCR products were stored at –20°C until required for the LDR assays.

Post-LDR sample treatment

Streptavidin-coated QDs (Qdot 605 Streptavidin Conjugate; Life Technologies Japan, Tokyo, Japan) were used to capture the biotin-tagged ligation products. These products bound to the QD surfaces through biotin-streptavidin interactions. First,
a 25-μL aliquot of the LDR product solution was diluted with 24 μL of 100 mM Tris-HCl buffer (pH 7.4) and incubated at 94°C for 2 min to denature the primer/template duplexes. This was followed by rapid cooling on ice for 5 min. Next, the treated LDR product solution was combined with 1 μL of a 100 nM Qdot 605 solution and incubated for 30 min. Because the volume of the resultant sample solution was low (i.e., 50 μL), the solution to be measured was injected into a small fused-silica capillary (i.d., 100 μm; o.d., 200 μm; GL Sciences, Tokyo, Japan) rather than a cuvette to avoid diluting the sample; and FRET signal measurements were conducted using the optical setup described below.

### Results and Discussion

#### Evaluation of the proposed detection method

As described in the Experimental section, the volume of the sample solution to be measured was low (i.e., 50 μL). A fluorescence microplate reader is suitable for acquiring fluorescence signals from samples on this scale (tens of microliters). However, a microplate reader was not available for use in this study. Therefore, in the current study, the sample was injected into a small capillary and fluorescence imaging was conducted using a microscope equipped with a camera. This method should be considered in future studies as an alternative to a microplate reader for comparing the fluorescence intensities of different sample solutions. To confirm if this method was sufficiently reliable for practical use, we evaluated the accuracy and linearity of the method. To do this, we prepared a series of diluted solutions of an oligonucleotide labeled with Cy5 at the 5′ end, and performed calibration experiments using these solutions. Briefly, fluorescence images were obtained of sets of 10 separate capillaries, with each capillary in a set containing the same concentration of the labeled-oligonucleotide (0 (blank), 10, 25, 50, 75, or 100 nM) (see Supporting Information Fig. S2(A)). The fluorescence intensity in a square region (50 × 50 pixels) within the image from each capillary was analyzed, and mean intensity values (n = 10) were calculated at each concentration. These mean values were plotted to construct a calibration curve. The calibration plot in Supporting Information Fig. S2(B) shows that there was a linear relationship between the sample concentration and signal readout with high measurement accuracy.

#### Detection of a K-ras mutation in a mixture of majority wild-type DNA

The PCR products (i.e., mutant G12D and wild-type G12) that were amplified independently were mixed, allowing for evaluation of a range of mutant to wild-type ratios. Figure 2A displays the acquired fluorescence images of separate capillaries (samples (i) - (v)) containing various dilutions of mutant DNA in wild-type DNA. Fluorescence of the imaged area indicated in Fig. 2A was integrated across the aligned capillaries and is plotted in Fig. 2B. The blank control sample (i.e., LDR with no template) exhibited a certain level of fluorescence (sample (v)), suggesting slight leakage of the donor (i.e., Qdot 605) emission into the detector. When an LDR assay was performed using the mismatched wild-type template alone as a negative control (sample (iv)), the signal was at a similar level to that obtained for the blank control sample, indicating that few misligation products (i.e., G-T mismatches) were generated. When testing a mixture of mutant and wild-type PCR amplicons at a mutant to wild-type ratio of 1:10 (sample (i)), a fluorescence signal representing matched (A-T) ligation products was obtained that was much larger (approximately 5.6-fold) than that of the negative control. This matched ligation signal decreased in size as the mutant DNA content was reduced in a solution containing a fixed concentration of wild-type template (10 nM). As exhibited in Fig. 2B, the positive control signals could be distinguished from the negative control signals at a mutant to wild-type ratio as low as 1:100. The signal for sample (ii) was approximately 2.5-fold larger than that for the negative control (sample (iv)). This mutant-discrimination threshold was 10-fold superior to that obtained in our previous FRET-based LDR study using AF647 as the acceptor. Because the mutated cell content in primary tumors can be at least 30% of the total cells,
the detection limit realized in this study will be low enough to
detect mutations present in highly developed tumors. In
addition, this method may be applicable to earlier stages of
cancer development.

The fluorescent dye Cy5 is often used in DNA microarray
hybridization, and has an excitation maximum of 648 nm,
emission maximum of 668 nm (according to the manufacturer),
and an extinction coefficient of $250000 \text{ cm}^{-1} \text{ M}^{-1}$. These
properties are similar to those of the relatively new dye AF647,
which has a maximum excitation wavelength of 650 nm,
maximum emission wavelength of 670 nm (information from
IDT’s homepage), and an extinction coefficient of 203000 \text{ cm}^{-1} \text{ M}^{-1}$. According to the manufacturer, the reported fluorescence
quantum yields of Cy5 and AF647 dissolved in water are 0.26 and 0.33, respectively. In view of these values, AF647 appears
to be more fluorescent than Cy5. However, it is well known that
fluorescent molecules are generally sensitive to their microscopic
environment, and the fluorescence efficiencies of many
conjugated dyes differ from those of the corresponding dyes
free in solution (i.e., unconjugated dyes). Previous studies have shown that the fluorescence of some dyes, including
fluorescein,22,23 coumarin,24 and rhodamine,25 are quenched
when they are bound to a nucleobase and/or a DNA chain.
However, it has also been reported that cyanine dyes such as
Cy3 and Cy5 have higher quantum yield when bound to
nucleobases; and, interestingly, the fluorescence of these dyes is
sensitive to not only the closest base but also to additional bases
further away.26 Therefore, we suggest that the Cy5-DNA
conjugate may have higher fluorescence efficiency than that of
the AF647-DNA conjugate. In fact, a study comparing the
performance of AF647 and Cy5 for practical DNA microarray
experiments determined that DNA conjugates of Cy5 were less
photostable over time but more fluorescent than DNA conjugates
of AF647.27 The potentially higher fluorescence intensity of
Cy5-DNA conjugate compared with AF647-DNA conjugate
may have contributed to the improved discrimination threshold
achieved in the current study.

Conclusions
We previously developed a separation-free ligase detection
reaction assay based on FRET from Qdot 605 to AF647, and
successfully detected one cancer mutation in a mixture of 10
wild-type templates using the developed methodology. In the
current study, the mutation-discrimination threshold of this
technique was improved by one order of magnitude (i.e.,
detection of one mutation among 100 normal sequences) by
replacing AF647 with Cy5. The high sensitivity achieved for
detection of infrequent mutations is comparable to that obtained
in our previous LDR study based on capillary gel electrophoresis.2
However, after completion of the LDR protocol, the FRET-
based LDR assay described here involves only simple mixing of
the resultant LDR product solution with the QD solution to create
the FRET donor-acceptor pairs required for detection of
the point mutation. Therefore, post-LDR processing for the
selective detection of the generated LDR products could be
carried out much more rapidly and conveniently compared with
the electrophoresis-based approach.

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Supporting Information
This material is available free of charge on the Web at http://
www.jsac.or.jp/analsci/.

References
1. R. Herr, M. Koehler, H. Andrlova, F. Weinberg, Y. Moeller,
S. Halbach, L. Lutz, J. Mastroianni, M. Klose, N.
Bittermann, S. Kowar, R. Zeiser, M. A. Olayioye, S.
Lassmann, H. Busch, M. Boerries, and T. Brummer,
Cancer Res., 2015, 75, 216.
2. Y. Qi, S. Duhaechek-Muggy, H. Li, and A. Zolkiewska,
PLoS One, 2014, 9, e92536/1.
3. M. Bessarabova, O. Pustovalova, W. Shi, T. Serebriyskaya,
A. Ishkin, K. Polyak, V. E. Velcutescu, T. Nikolskaya,
and Y. Nikolsky, Cancer Res., 2011, 71, 3471.
4. S.-M. Jo, Y. Kim, Y.-S. Jeong, Y. H. Oh, K. Park, and H.-S.
Kim, Biosens. Bioelectron., 2013, 46, 142.
5. F. Barany, Proc. Natl. Acad. Sci. U. S. A., 1991, 88, 189.
6. M. Hamada, K. Shimase, K. Noda, K. Tsukagoshi, and M.
Hashimoto, Electrophoresis, 2013, 34, 1415.
7. M. Hamada, K. Shimase, K. Tsukagoshi, and M. Hashimoto,
Electrophoresis, 2014, 35, 1204.
8. R. Sinville, J. Coyne, R. J. Meagher, Y.-W. Cheng, F.
Barany, A. Barron, and S. A. Soper, Electrophoresis, 2008,
29, 4751.
9. M. Hashimoto, M. L. Hupert, M. C. Murphy, S. A. Soper,
Y.-W. Cheng, and F. Barany, Anal. Chem., 2005, 77, 3243.
10. M. Hommatsu, H. Okahashi, K. Ohta, Y. Tamai, K.
Tsukagoshi, and M. Hashimoto, Anal. Sci., 2013, 29, 689.
11. C. Situma, Y. Wang, M. Hupert, F. Barany, R. L. McCarley,
and S. A. Soper, Anal. Biochem., 2005, 340, 123.
12. M. Lorenz and S. Diekmann, Methods. Mol. Biol., 2006,
335, 243.
13. M. Massey, W. R. Algar, and U. J. Krull, Anal. Chim. Acta,
2006, 568, 181.
14. J. Michaelis, G. J. van der Heden van Noort, and O. Seitz,
Bioconjug. Chem., 2014, 25, 18.
15. M. Xue, X. Wang, H. Wang, D. Chen, and B. Tang, Chem.
Commun., 2011, 47, 4986.
16. C. A. Heid, J. Stevens, K. J. Livak, and P. M. Williams,
Genome Res., 1996, 6, 986.
17. S. Tyagi and F. R. Kramer, Nat. Biotechnol., 1996, 14, 303.
18. M. Hashimoto, K. Yoshida, and K. Tsukagoshi, Anal. Sci.,
2010, 26, 1255.
19. N. P. Gerry, N. E. Witowski, J. Day, R. P. Hammer, G.
Barany, and F. Barany, J. Mol. Biol., 1999, 292, 251.
20. G. P. Anderson and N. L. Nerurkar, J. Immunol. Methods,
2002, 271, 17.
21. U. Schobel, H. J. Egelhaaf, A. Brecht, D. Oelkrug, and G.
Gaughitz, Bioconjug. Chem., 1999, 10, 1107.
22. A. O. Crockett and C. T. Wittwer, Anal. Biochem., 2001,
290, 89.
23. I. Nazarenko, B. Lowe, M. Darfler, P. Ikonomi, D. Schuster,
and A. Rashchitich, Nucleic Acids Res., 2002, 30, e371.
24. C. A. M. Seidel, A. Schulz, and M. H. M. Sauer, J. Phys.
Chem., 1996, 100, 5541.
25. T. Heinlein, J.-P. Knemeyer, O. Pievert, and M. Sauer,
J. Phys. Chem. B, 2003, 107, 7957.
26. C. Agbavve and M. M. Somozoa, PLoS One, 2011, 6, e22177/1.
27. J. L. Ballard, V. K. Peeva, C. J. S. de Silva, J. L. Lynch, and
N. R. Swanson, Mol. Biotechnol., 2007, 36, 175.