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Rapid and Convenient Sample Preparation in a Single Tube Using Magnetic Beads for Fluorescence Detection of Single Nucleotide Variation Based on Oligonucleotide Ligation

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We here describe a rapid and convenient sample preparation in a single tube for detecting single nucleotide variation based on oligonucleotide ligation assay (OLA) using a biotinylated primer and a set of four different fluorophore-labeled primers. Only one of the fluorophore-labeled primers that was fully complementary to a DNA template was ligated with the biotin-primer by DNA ligase. We isolated the ligated fragments that carried a biotin at the 3′-end by capture with streptavidin-coated magnetic beads. The distinct fluorescence signature of each ligation product coded the single nucleotide differences.

Single-nucleotide substitutions represent the most common genetic variations found in the human genome. Such variations appear in the human genome with an average density of once every 1000 bp and some of them are directly linked to human disease.[1] Although DNA sequencing and DNA microarray technologies are powerful and straightforward for initial discovery of single-nucleotide variations,[2,3] alternative genotyping methods, which can be conducted simply, rapidly, and more conveniently without expensive instruments, are required in case a massive genotype screening is not necessarily needed in research and clinical labs.

Oligonucleotide ligation assay (OLA) is one of the most robust techniques used for detecting single-nucleotide substitutions. In this assay, two primers (a discriminating primer and a common primer) anneal to a locus of interest in a DNA template. The discriminating primer contains a base at its 3′-end that coincides with the single-base substitution site. The two primers are ligated to form an OLA product by DNA ligase only if the nucleotide at the variable site is complementary to the 3′-end of the discriminating primer. This process then linearly amplifies OLA products during subsequent thermal cycles. Detecting the OLA product reveals single nucleotide differences at the variable sites with high accuracy.

Conventionally, electrophoresis[4–8] or low-density DNA microarray[9–16] is used to analyze OLA products, which makes the postligation reaction processes complex and time-consuming. In contrast to the conventional approach, the method presented here employs neither typical electrophoretic separation nor DNA microarray. A convenient sample preparation technique using a biotinylated common primer and four discriminating primers (each labeled with a different fluorescent dye molecule) in conjunction with solid-phase capture by magnetic beads in the present study enables simple discrimination of single-nucleotide variation by the difference in fluorescence signature of the OLA product generated in a single tube, making the OLA-based genetic analysis more rapid, less expensive, and easier to perform.

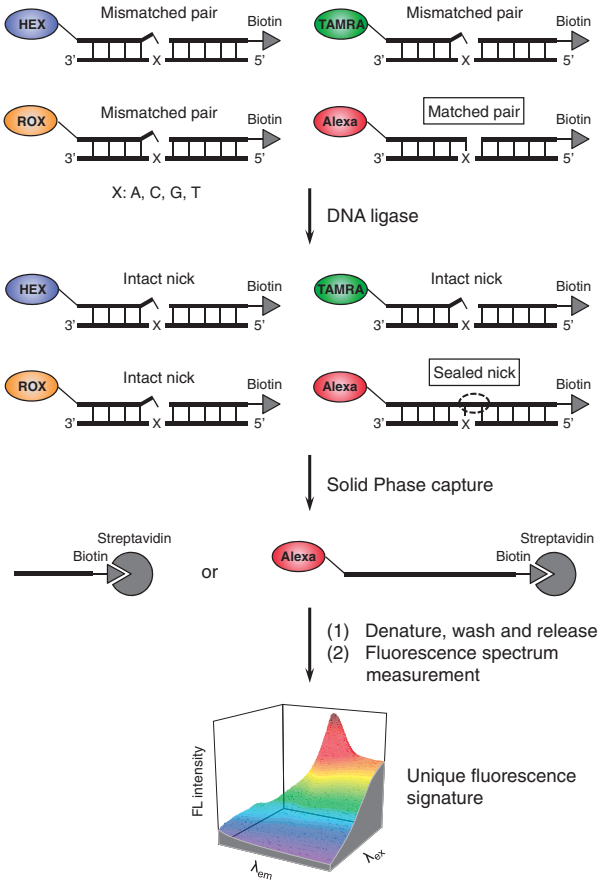

Figure 1. Conceptual schematic for the OLA-based single nucleotide variation discrimination using fluorophore-labeled primers and a biotin-primer.

The OLA-based approach for detecting single nucleotide variations using the fluorophore-labeled oligonucleotide primers and the biotin-common primer is schematically shown in Figure 1. Upon hybridization to the DNA template, one of the four fluorophore-labeled discriminating primers that has a perfect match to the single nucleotide variation site will be covalently joined to the biotin-common primer in the presence of a thermally stable DNA ligase. This process then linearly amplifies the primer ligation products during subsequent thermal cycles. After the thermal cycles, the fluorophore-labeled ligation products were immobilized on streptavidin-coated magnetic beads and the other components were washed away. The extension products were cleaved from the magnetic beads by

Table 1. The fluorophore-tagged and the biotinylated oligonucleotide sequences and the portion of DNA template sequences containing single nucleotide substitutions (substituted nucleotides in bold)

Primer/template	Sequence
HEX-labeled primer	5′-AAACTTGTGGTAGTTGGAGCTGA-3′
TAMRA-labeled primer	5′-AAACTTGTGGTAGTTGGAGCTGT-3′
ROX-labeled primer	5′-AAACTTGTGGTAGTTGGAGCTGG-3′
Alexa-labeled primer	5′-AAACTTGTGGTAGTTGGAGCTGC-3′
Biotinylated common primer	5′-TGGCGTAGGCAAGAGTGCCT-3
Template G12D for HEX primer	5′∼GCCTACGCCA**T**CAGCTCCAAC∼3′
Template G12V for TAMRA primer	5′∼GCCTACGCCA**A**CAGCTCCAAC∼3′
Template G12G for ROX primer	5′∼GCCTACGCCA**C**CAGCTCCAAC∼3′
Template G12A for Alexa primer	5′∼GCCTACGCCA**G**CAGCTCCAAC∼3′

denaturing the biotin–streptavidin interaction with formamide and analyzed with a spectrofluorometer.

Synthetic DNA templates which possess four nucleotide variations, a biotinylated common oligonucleotide, and allele-specific oligonucleotides were obtained from Integrated DNA Technologies, Inc. (Coralville, IA) (see Table 1 for the oligonucleotide sequences). We used the following four fluorescence dyes, which have distinct maximum fluorescence emission wavelengths, for labeling the allele-specific oligonucleotide primers: HEX ($\lambda_{em}{}^{max} = 560$ nm), TAMRA ($\lambda_{em}{}^{max} = 576$ nm), ROX ($\lambda_{em}{}^{max} = 599$ nm), and Alexa 647 ($\lambda_{em}{}^{max} = 670$ nm).

For individual oligonucleotide ligation, 50 μL of reaction cocktail contained 1 × *Taq* DNA ligase reaction buffer {20 mM Tris-HCl (pH 7.6), 100 mM KCl, 10 mM $Mg(CH_3COO)_2$, 10 mM DTT, 1 mM NAD^+ (nicotinic adenine dinucleotide, a cofactor for ligase enzyme), and 0.1% Triton X-100}, fluorophore-labeled primer mix (50 nM each), 200 nM biotin-common primer, 10 nM DNA template, and 0.4 U μL^{-1} of *Taq* DNA ligase enzyme (New England Biolabs, Beverly, MA). The reaction cocktail was preheated to 94 °C for 2 min and then subjected to 20 thermal cycles using the following temperatures: 94 °C for 30 s; 65 °C for 2 min.

Ten microliters of streptavidin-coated magnetic beads (Dynabeads M-270 streptavidin; Invitrogen, Carlsbad, CA) were washed with 100 μL of 10 × SSC buffer twice and then responded in 50 μL of SSC buffer. Fifty microliters of the primer ligation product mixture was then combined with 50 μL of the washed streptavidin-coated magnetic beads suspension at 25 °C for 5 min with gentle mixing by a rotator. The DNA template was denatured from the ligated primer by heating the suspension at 94 °C for 5 min, followed by cooling on ice for 1 min. After collecting the beads from the suspension using a magnet, the beads were rinsed with 10 × SSC buffer three times. The ligated primers were released from the magnetic beads in 50 μL of 98% formamide containing 10 mM EDTA at 75 °C for 5 min. The supernatant was subsequently injected into a microcuvette for fluorescence spectrum measurements by a spectrofluorometer (FP-6500, Jasco, Tokyo, Japan).

The fluorescence signature for each sample was recorded at excitation wavelengths from 530 to 680 nm, and emission wavelengths from 550 to 750 nm with a 1-nm interval. Due to the presence of the Rayleigh scattering in the range of the wavelengths, we set the values above the Rayleigh scattering line to zero so as to reduce its influence.

The ligation reactions were carried out in a single tube with the four fluorophore-labeled allele-specific primers directed to four nucleotide variations in synthetic templates mimicking exon1 mutations of the K-*ras* gene (G12D, G12V, G12G, and G12A). After ligation and solid-phase purification, three-dimensional excitation–emission fluorescence signatures for fluorophore-labeled ligation products were plotted (Figure S1[17]). The unique fluorescence signatures were obtained from the individual fluorophore-labeled ligation products as a result of the different fluorescence spectrum properties of the fluorophore tags. The difference in the maximum fluorescence intensity among the plots does not represent differential in the ligation product yield but shows the difference in the fluorescence quantum yield of the fluorophore tag. The unexpected weak fluorescence signals of the blank experiment sample in Figure S1E,[17] which lacks DNA template, should be Raman scattering and/or autofluorescence from the ingredients contained in the sample.

For clarity, the emission spectra obtained by the excitation with the particular wavelengths (544, 553, 578, and 649 nm), which were 6 nm shorter than the pre-examined maximum excitation wavelengths of HEX, TAMRA, ROX, and Alexa, were extracted from the three-dimensional plots in Figures S1A–S1D[17] and were stacked together in Figures 2A–2D. In each stacked plot, the peak vertexes were observed only at a single emission wavelength; i.e., 560, 576, 599, and 670 nm, each representing the maximum emission wavelength for HEX, TAMRA, ROX, and Alexa, respectively. The several different peaks in Figures 2B and 2C should represent fluorescence signals from the common fluorophores obtained by the excitations at different wavelengths because those peaks possess the identical maximum emission wavelength in each plot. The discrete fluorescence signatures of the labeled-ligation fragments enabled clear discrimination of the corresponding nucleotides of the variable site in the template.

In summary, the OLA-based sample preparation using biotin-common primer and four different fluorophore-labeled discriminating primers in conjunction with solid-phase capture by magnetic beads described here offers a fast and accurate method for single nucleotide variation detection and analysis. Compared to the conventional methods that require gel electrophoresis or DNA–DNA hybridization on a solid support, our method is simple and more convenient since single nucleotide difference can be coded into a distinct fluorescence signature without tedious procedures and expensive instruments. Currently, we are refining the experimental conditions to further reduce the assay time while investigating the detection limit of the present assay.

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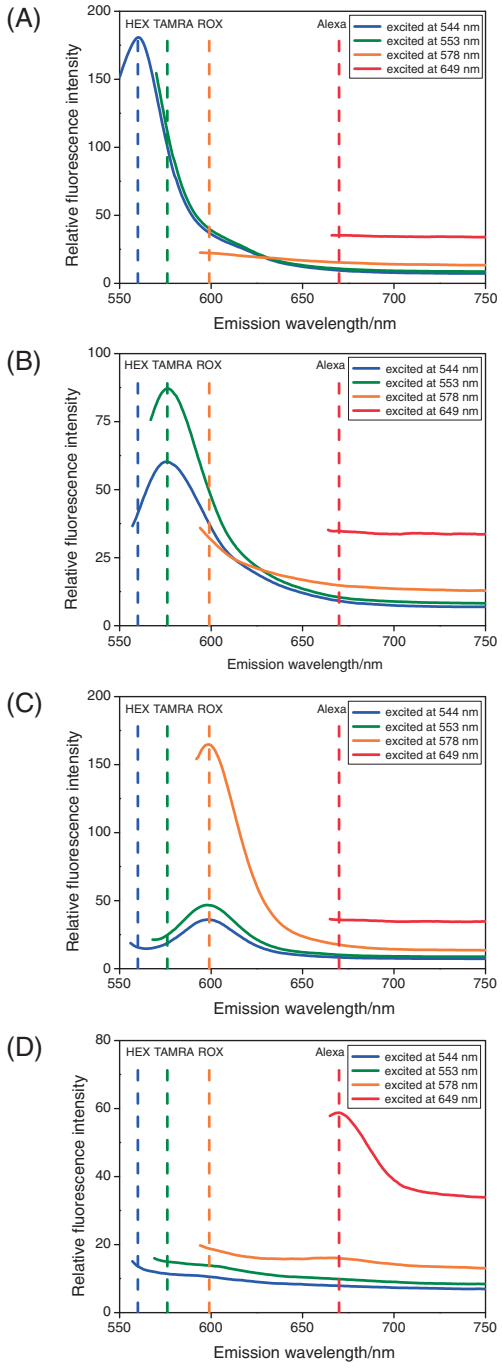

Figure 2. Emission spectra extracted from the 3-D plots of (A), (B), (C), and (D) in Figure S1. The blue, green, orange, and red dashed lines represent the known maximum wavelengths of HEX, TAMRA, ROX, and Alexa, respectively. The particular excitation wavelengths for the emission spectra are described in the inset.

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17 Supporting Information is available electronically on the CSJ-Journal Web site, http://www.csj.jp/journals/chem-lett/index.html.

