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Two color hybridization analysis using high density oligonucleotide arrays and energy transfer dyes

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ABSTRACT

High density oligonucleotide arrays (DNA chips) have been used in two color mutational analysis of the 3.43 kb exon 11 of the hereditary breast and ovarian cancer gene *BRCA1*. Two color analysis allows competitive hybridization between a reference standard and an unknown sample, improving the performance of the assay. Fluorescein and phycoerythrin dyes were previously used due to their compatibility with a single line 488 nm excitation source. Here we show that an alternative dye combination, containing the energy transfer dye system phycoerythrin•cy5 along with phycoerythrin, provides more evenly matched signal intensities and decreased spectral overlap between the two fluorophores, while maintaining compatibility with a 488 nm excitation source.

In previous experiments, RNA target was generated by PCR amplification of *BRCA1* exon 11 from genomic DNA using primers containing T3 and T7 RNA polymerase promoters, followed by *in vitro* transcription reactions [1× Transcription Buffer (Promega), 1 mM DTT, 1 mM NTP mix, 50 ng amplicon template and 10 U T3 or T7 RNA polymerase in a 20 µl volume] with either 1 mM fluorescein-12-UTP or 0.5 mM biotin-16-UTP (1). Fluorescein standard and biotinylated test targets were subjected to partial chemical hydrolysis (transcription reactions brought to 30 mM $MgCl_2$ and heated at 94°C for 45 min), diluted into 400 µl buffer A (3 M TMAC, 1× TE, 0.005% Triton X-100) and hybridized to the *BRCA1* exon 11 array at 37°C for 4 h. The array was subsequently stained with a phycoerythrin–streptavidin conjugate, producing fluorescein (green) and phycoerythrin (red) hybridization signals, acquired using a scanning confocal microscope equipped with a 488 nm argon laser (GeneChip® Scanner, Affymetrix) and a photomultiplier tube. Arrays were scanned twice using 525DF50 (green) and 565LP (red) emission filter sets (Omega Optical).

Although this two color dye system has been successful in *BRCA1* mutation detection (1), it has presented several technical challenges. The considerable spectral overlap (38% spillover of fluorescein signal into the 'red' emission channel with 2% spillover of phycoerythrin signal into the 'green' emission channel) between the two dyes produces signals that require significant numerical correction (1). Furthermore, the hybridization signal from the fluorescein-labeled sample is generally 8–10-fold weaker than that of the phycoerythrin-stained biotinylated sample, under the conditions described above.

Energy transfer dye systems make robust multicolor spectral analysis possible while using a single line excitation source (2). We used a streptavidin•phycoerythrin•cy5 conjugate (Pharminogen) to stain biotinylated target hybridized to the array [0.4 µg/ml conjugate in wash buffer (6× SSPE, 0.005% Triton X-100, 5 mg/ml BSA) for 30 min]. Phycoerythrin (480 nm local excitation maxima) and cy5 (670 nm emission maxima) serve as energy transfer donor and acceptor dyes, respectively. The phycoerythrin•cy5 streptavidin conjugate produced fluorescent hybridization signals (650LP emission filter, Omega Optical) ~35–45% of the intensity of that of the phycoerythrin–streptavidin conjugate. For two color analysis, it was necessary to use different labeling schemes for the two dyes. As a separate means of staining with phycoerythrin, independent of the biotin–streptavidin system, a monoclonal antibody to digoxigenin (Boehringer Mannheim) was conjugated to phycoerythrin (Molecular Probes). Internally labeled digoxigenin *BRCA1* exon 11 RNA target [transcribed with 0.5 mM digoxigenin-12-UTP (Boehringer Mannheim)] was hybridized to arrays and stained with anti-digoxigenin•phycoerythrin conjugate (0.9 mg/ml conjugate in wash buffer for 30 min). Hybridization signal was collected using a 575DF25 emission filter (Omega Optical). The anti-digoxigenin•phycoerythrin dye system gave a 2-fold averaged increase in the hybridization signal relative to fluorescein-labeled target. This increase was sequence dependent, with some nucleotide tracts having signal intensities equivalent to that of fluorescein target and others several fold higher.

To establish compatibility of the two color red (phycoerythrin) and far-red (phycoerythrin•cy5) dye system, we co-hybridized digoxigenin- and biotin-labeled sense strand targets to *BRCA1* exon 11 arrays. Digoxigenin-labeled target **1** has wild type sequence while biotinylated target **2** is heterozygous for five common *BRCA1* exon 11 polymorphisms (1). Digitized images of sense strand array hybridization signals uncorrected for fluorescent signal spectral overlap from these targets are shown in Figure 1a–f. A family of four 20mer probes per target strand, containing one of the four nucleotides in the 11th position,

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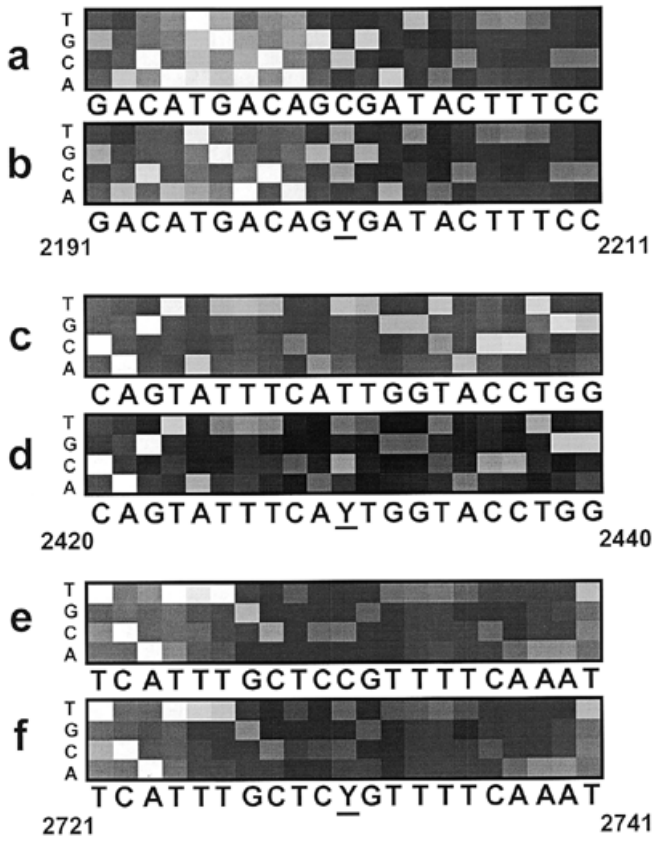

Figure 1. Two color red/far-red chip image comparisons. Magnified digitized grayscale images uncorrected for spectral overlap show co-hybridization patterns of *BRCA1* targets to DNA chips evaluating sense target strands. Contrast and brightness settings are changed in each panel to increase image clarity and signal may be non-linear at extremes of brightness. Nucleotide identities are given under the respective columns with polymorphic sites underlined. (**a**, **c** and **e**) Targets **1** (homozygous wild type). (**b**, **d** and **f**) Targets **2** (heterozygous for five common polymorphisms 2201 C/T, 2430 T/C, 2731 C/T, 3232 A/G and 3667 A/G) are digoxigenin- and biotin-labeled, respectively. (a) and (b) show results interrogating *BRCA1* cDNA nucleotides 2191–2211, (c) and (d) interrogate nucleotides 2420–2440 and (e) and (f) interrogate nucleotides 2721–2741. The numbering of the first and last nucleotides interrogated in (a–b), (c–d) and (e–f), are given below (b, d and f), respectively. In each instance the heterozygous polymorphism is readily apparent with the biotin–streptavidin–phycoerythrin•cy5 system. All other base calls are unambiguous by our standard algorithm (1).

interrogate the identity of each target nucleotide (1,3). By quantitating ratios of hybridization signal in each family of four probes, target sequence information can be determined (1,3). For example, the significant difference in hybridization patterns in Figure 1a and b is the additional signal intensity in the 'T' nucleotide substitution probe (Fig. 1b) interrogating the underlined *BRCA1* coding nucleotide position 2201, in agreement with the 2201 C/T target **2** genotype. In a similar manner, the difference in hybridization patterns shown in Figure 1c and d is the increased underlined 2431 'C' probe hybridization signal in target **2** containing the 2431 C/T alleles. Comparing Figure 1e and f, increased fluorescent intensity at the probe interrogating underlined nucleotide position 2731 (Fig. 1f) indicates the 2731 C/T genotype in target **2**. These results confirm signal specificity in this nucleic acid hybridization-based two color system.

An attractive aspect of this two color system is the minimal spectral overlap between the phycoerythrin and phycoerythrin•cy5 dyes (~4–5% fluorescent emission signal overlap between the 575DF25 'red' and 650LP 'far-red' channels in both directions). This minimizes the influence of numerical signal correction which can potentially skew results due to local changes in background fluorescence. In addition, the more closely matched signal strength between the two channels aids in creating hybridization conditions which maximize the hybridization signal strength and specificity of targets labeled with either of the two dyes. This comes at the expense of lessening the signal strength of the streptavidin–phycoerythrin•cy5 dye relative to the streptavidin–phycoerythin dye. However, this signal loss is partially compensated for by the anti-digoxigenin–phycoerythrin dye providing greater signal than fluorescein, the weaker of the two dyes in the original two color system. The anti-digoxigenin conjugate signal strength could be increased by optimizing phycoerythrin coupling conditions. While the use of multi-line laser excitation sources would further expand the choice of dyes, this dye combination provides a useful alternative for two color analysis using a single line excitation source.

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