Fluorescent Multiplex PCR and In-lane Fragment Analysis

Typhoon 8600 Variable Mode Imager

Production of fluorescently labelled DNA fragments using the polymerase chain reaction (PCR)* method is accomplished directly through the use of modified oligonucleotide primers or deoxynucleotide triphosphates (dNTPs). A wide selection of fluorochrome tags is available for oligonucleotide end-labelling. Use of an end-labelled PCR primer ensures that the amplified PCR product has an equimolar relationship between the label and the DNA molecule. Alternatively, PCR using fluorescently modified dNTPs result in products that are internally labelled at multiple sites per molecule and deliver the greatest sensitivity. The use of fluorescence detection offers the advantages of sensitivity, a wide linear dynamic range for quantification, and the option to use multiple tags in analysis.

The CyDye™ series of fluorochromes are bright, photostable molecules with high water solubility and pH insensitivity. The labels are available in a range of intense colours with narrow emission bands, making them ideal for multi-colour detection. Two of these, Cy™3 and Cy5, are popular labels for two-channel fluorescent experiments such as gene expression arrays. Fluorescent imaging instrumentation with 532 nm and 633 nm laser excitation sources are ideally suited for CyDye imaging.

Products used

Product	Part Number
Typhoon™ 8600	63-0027-96
Hoefer™ MiniVE™ Electrophoresis System	80-6418-77
EPS 301 Power Supply	18-1130-01
Acrylamide	US75820
N, N′-methylene-bis-acrylamide	US75821
TBE buffer, premixed powder	US70454
Bromophenol blue (BPB)	17-1329-01
Cy3-dCTP	PA53021
Cy3.5-dCTP	PA53521
Cy5-dCTP	PA55021
Cy2 mono-Reactive Dye Pack	PA32000
Cy3.5 mono-Reactive Dye Pack	PA23501
PCR Nucleotide Mix	US77212
dNTP Set, 100 mM Solutions	27-2035-01
Taq DNA Polymerase (cloned)	T0303Y
TE Buffer, 50x	US75834

Other materials required

- Oligonucleotides (unlabelled)
- Oligonucleotides modified with 5' terminal CyDye label
- Oligonucleotides modified with 5' terminal amine
- 6× DNA sample buffer (40% sucrose in water, bromophenol blue)
- Human genomic DNA
- Thermal cycler instrument

Protocol

Performing PCR amplification

1 Prepare reactions in one of the following ways:

A. PCR with CyDye 5' end-labelled oligonucleotide primer

Stock	Volume	Final
10× PCR Buffer†	5 µl	1×
25 mM $MgCl_2$	3 µl	1.5 mM
10 mM dATP, dGTP, dTTP, dCTP	1 µl	200 µM each
Forward primer (labelled)		0.5 µM
Reverse primer		0.5 µM
DNA template		70 ng
Taq Polymerase [5u/µl]	0.2 µl	1 unit

sterile ddH_2O to 50 µl final reaction volume.

B. PCR with CyDye labelled dCTP

Stock	Volume	Final
10× PCR Buffer†	5 µl	1×
25 mM $MgCl_2$	5 µl	2.5 mM
2 mM dGTP, dATP, dTTP	1.25 µl	50 µM each
1 mM dCTP (CyDye-dCTP:dCTP, 1:10)	2.5 µl	50 µM dCTP
Forward primer		0.5 µM
Reverse primer		0.5 µM
DNA template		70 ng
Taq DNA Polymerase [5u/µl]	0.2 µl	1 unit

sterile ddH_2O to 50 µl final reaction volume.

†10× PCR Buffer: 500 mM KCl, 100 mM Tris-Cl (9.0)

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2 Using a thermal cycler, denature samples initially at 95 °C for 1 minute and then cycle 30 times [95 °C for 15 seconds, 57 °C for 15 seconds and 72 °C for 30 seconds] with a final incubation at 72 °C for 2 minutes.

Performing gel electrophoresis

1 Prepare a 10% polyacrylamide gel in Tris-Borate-EDTA (TBE) buffer using the miniVE system (1).

2 Mix between 1 µl and 5 µl of amplified product with TE buffer and 6x sample buffer to a final volume of 6 µl.

3 Perform electrophoresis of prepared samples using EPS 301 power supply for 1.5 h at 100 V.

Imaging and analysing gel

1 Remove gel from the glass electrophoresis plates and position on the clean Typhoon glass platen in a small amount of water. Add additional water to the edges of the gel to keep it from drying during the scan.

2 Set up the Typhoon Scanner Control software as shown in Table 1. Choose platen focal height, 100 µm pixel size, appropriate PMT voltage, and normal sensitivity settings.

3 Display and analyse the gel image(s) using ImageQuant™ software, FluorSep™ software, or Fragment Analysis software, as appropriate (refer to user documentation for details).

CyDye	Laser	Emission Filter
Cy2	Green (532)	526 SP
Cy3	Green (532)	555 BP 30
Cy3.5	Green (532)	610 BP 30
Cy5	Red (633)	670 BP 30

Table 1. Typhoon instrument setup for CyDye labels. Laser and emission filter choices are made using the Scanner Control software.

Results

A 268 bp fragment of the human β-globin gene was amplified using either a CyDye labelled oligonucleotide primer or a CyDye labelled dCTP substrate. The results of PCR using a labelled primer are shown in Figure 1. The Cy3 and Cy5 end labelling was done by a supplier of synthetic DNA using phosphoramidite chemistry. Primer labelling with Cy2 and Cy3.5 was accomplished using 5' terminal amine-modified synthetic oligonucleotides and reactive Cy2 and Cy3.5 dyes. To account for different labelling efficiencies, as well as the Typhoon detection limits for each of these labels, different amounts of the four reactions were analysed by gel electrophoresis to yield similar signal levels (Fig 1). Using Typhoon 8600, the limits of detection for these four fluorochromes were determined separately in denaturing polyacrylamide gels by measuring signal to noise ratios from doubling dilutions of end-labelled oligonucleotides (Table 2).

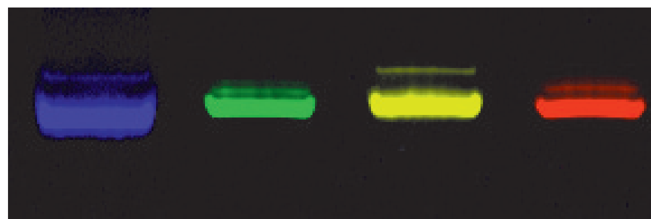

Fig 1. CyDye labelled PCR products generated using end-labelled oligonucleotide primers. The forward primer in each reaction was labelled at the 5' end with one the following: Cy2 (blue), Cy3 (green), Cy3.5 (yellow) and Cy5 (red). The labelled 268 bp products from human β-globin gene amplification were resolved in a 10% polyacrylamide gel and imaged in four separate channels using Typhoon.

CyDye	Limit of Detection (fmol/band)*
Cy2	7.5
Cy3	0.2
Cy3.5	0.2
Cy5	0.2

Table 2. Typhoon 8600 limits of detection (LOD) for CyDye labelled oligonucleotides in a denaturing polyacrylamide gel.

**Values are obtained from signal to noise measurements of a doubling dilution series of end-labelled oligonucleotides analysed in 0.4 mm thick denaturing polyacrylamide gels (15%) and imaged directly, sandwiched between low fluorescence glass electrophoresis plates.*

Incorporation of fluorescently labelled dCTP during PCR amplification of the human globin gene fragment was also studied. The dCTP used in these reactions consisted of CyDye-dCTP (Cy3, Cy3.5, and Cy5) mixed with unlabelled dCTP at a 1:10 ratio. PCR products were resolved in a polyacrylamide gel and detected in three channels using the Typhoon 8600 (Fig 2).

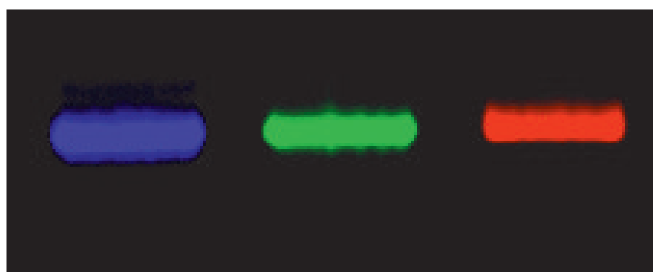

Fig 2. PCR products amplified in the presence of CyDye labelled dCTP. Cy3-dCTP (blue), Cy3.5-dCTP (green), and Cy5-dCTP (red) were used in the production of a 268 bp fragment from the human β-globin gene. Equal amounts of Cy3, Cy3.5 and Cy5 labelled PCR products were analysed.

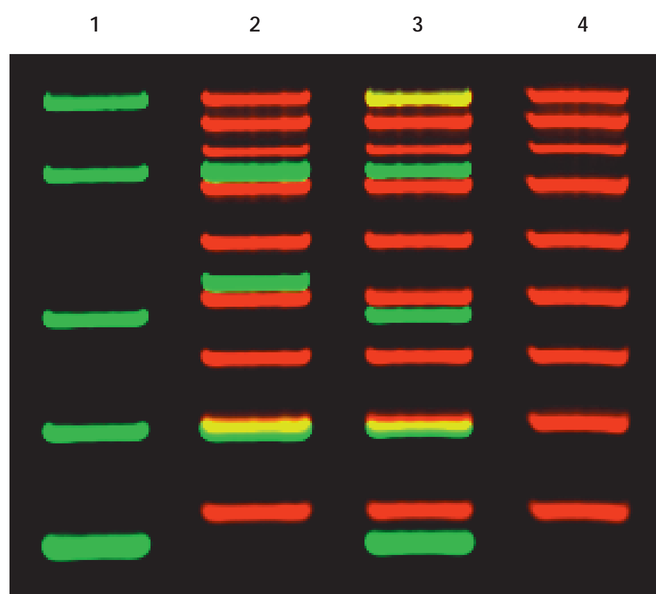

Fig 3. Cy3- and Cy5-labelled DNA fragments. Lane 1, Cy3 (green) labelled fragments (500 bp, 365 bp, 230 bp, 150 bp, 88 bp); lane 2, Cy5 (red) size ladder (500 bp, 450 bp, 400 bp, 350 bp, 300 bp, 250 bp, 200 bp, 150 bp, 100 bp) with Cy3 labelled fragments (365 bp, 268 bp, 150 bp); lane 3, Cy3-labelled fragments with Cy5 size ladder; lane 4, Cy5 size ladder. The presence of both Cy3 and Cy5 signal in the same region of the gel is displayed as yellow by the ImageQuant software (lanes 2 and 3).

The ability to resolve Cy3- and Cy5-labelled DNA in the same gel, and in the same gel lane, was tested. A Cy5-labelled size standard and various Cy3-labelled fragments were combined and resolved in a polyacrylamide gel (Fig 3). Following Typhoon two-channel imaging of this gel, fluorescent signal from Cy3 and Cy5 labels was fully resolved even in the event of overlapping or co-migrating bands. This is illustrated in the merged lane profiles from lane 3 for both the Cy3 and Cy5 channels (Fig 4). The lengths of five different Cy3-labelled fragments were determined using the Cy5 size standards resolved in the same lane (Fig 3, lane 3). Using Fragment Analysis software, mobility of the Cy5 fragments was used as a reference to determine the sizes of the Cy3 fragments (Fig 5). These Cy3 fragment sizes were calculated accurately to within 2.5 percent of their known values. This approach delivers highly reliable results, since lane-to-lane variability in electrophoretic migration is eliminated.

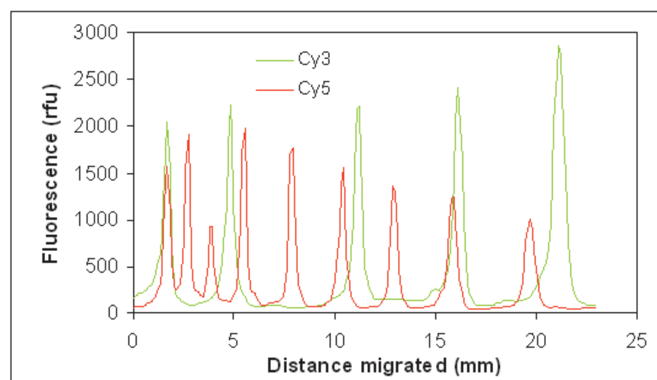

Fig 4. Spectral resolution of Cy3 and Cy5 labels in the same gel lane. Fluorescent signal through lane 3 of Fig 3 is plotted for both the Cy3 (green) and Cy5 (red) channels using ImageQuant software. Full spectral separation of the Cy3 and Cy5 tags is evident in the detection and resolution of the separate Cy3- and Cy5-labelled DNA size ladders (no fluorochrome separation processing was used). The highest molecular weight 500 bp fragments in both ladders appear to co-migrate in this gel lane.

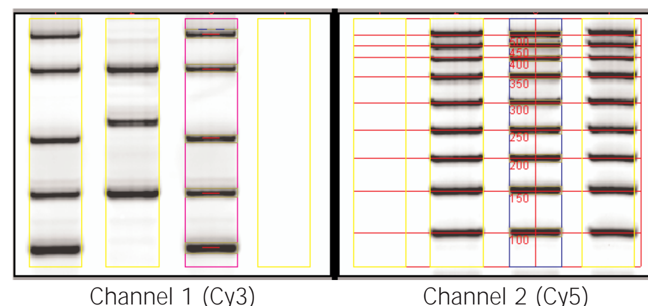

Fig 5. Estimation of Cy3 fragment lengths using an in-lane Cy5 size standard and Fragment Analysis software. The image is a window from Fragment Analysis software showing the Cy5 size standard ladder (channel 2, lane 3) used to estimate the sizes of five Cy3-labelled fragments (channel 1, lane 3) resolved in the same gel lane (lane 3). Estimated sizes of the five Cy3 fragments with percent deviation from the known size values are tabulated (Table 3).

Known (bp)	Estimated (bp)	Deviation (%)
500	505	1.0
365	370	1.4
230	235	2.2
150	147	2.0
88	86	2.3

Table 3. Cy3-labelled DNA fragment sizes.

Conclusions

The CyDye labels offer a versatile range of labelling options for nucleic acids. The strong fluorescence signal from these dyes can give highly sensitive results. Good separation between the fluorescence emission spectra of Cy3 and Cy5 labels makes them an excellent choice for two-channel fluorescent experiments.

In order to obtain the best data from two-channel experiments, the detection system should have the following characteristics:

- Optimal excitation of each label using separate laser sources
- Highly efficient fluorescent optical collection system to enrich the signal strength and purity in each channel
- Perpendicular scanning mechanism to eliminate image spatial distortion
- Optimal emission filters to effectively separate fluorescent signals
- Software capable of eliminating unfiltered spectral crosstalk

The Typhoon 8600 in combination with CyDye fluorochromes facilitates the transition to multiplex PCR assays, offering savings of cost and time. Multicolour fluorescent detection also delivers improved throughput and accuracy in size analysis through the use of in-lane size standards.

References

1. Ausubel, F.M., R. Brent, R. Kingston, D. Moore, J. Seidman, J.A. Smith and K. Struhl (1998) Current Protocols in Molecular Biology, John Wiley and Sons, NY.

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