Benchmarks

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Enhanced Detection of Multiplex PCR Products Using SYBR® Green I and an Automated DNA Sequencer

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PCR assays have improved our ability to assess the presence and abundance of specific microbial populations in polluted soil. The abundance of target populations that may degrade pollutants can be determined using a competitive PCR approach (2) or a semi-quantitative replicative limiting dilution approach (4). Detection limits of existing assays based on separation of DNA fragments using agarose or polyacrylamide gel electrophoresis followed by staining and visualization with UV light can be improved by using more sensitive detection techniques. We have adapted the fluorescent dye SYBR® Green I (Molecular Probes, Eugene, OR, USA) to laser-induced detection of DNA fragments using an automated DNA sequencer to increase the sensitivity and throughput of

our PCR assays for the detection of pollutant-degrading bacteria in soil.

Automated sequencers such as the ABI PRISM® 377 (Applied Biosystems, Foster City, CA, USA) have increased the ease, sensitivity, and accuracy of detection and quantification of DNA fragments. To enable the detection of PCR fragments by laser-induced fluorescence used by the ABI PRISM377, each DNA fragment must be labeled with an appropriate fluorescent dye. Traditionally, dyes are incorporated via primers synthesized with attached fluorophores or by modified dNTPs during elongation cycles of PCR. These approaches can be costly, reduce product yields because of lower efficiencies of incorporation, and require specific dye-labeled primers for each different PCR target. Nucleic acid stains, such as SYBR Green I, that exhibit a high affinity for DNA in addition to enhancement of fluorescence upon DNA binding may be an inexpensive alternative to labeling during primer synthesis or PCR. SYBR Green I has an excitation/emission maxima of 497 and 520 nm, similar to that of fluorescein.

We routinely perform multiplex PCRs to detect genes involved in aro-

Figure 1. Limit of detection of GeneScan 2500 ROX size standard by laser-induced fluorescence of SYBR Green I versus fluorescent dye-labeled adapter.

Benchmarks

Table 1. Enhanced Detection of PCR Fragments Stained with SYBR Green I by ABI PRISM377

Bacterial Strain	<i>B. cepacia</i> JS150	<i>P. oleovorans</i>	All Cells	<i>B. cepacia</i> DNT	<i>P. putida</i> pWWO	<i>P. sp.</i> ADP
Target gene	<i>merR</i> P	<i>alkB</i>	16S rDNA	<i>ntdAc</i>	<i>xyIE</i>	<i>atzA B</i>
(fragment size in bp)	(1004)	(869)	(515–530)	(330)	(202)	(174)
Actual seeded cell density	1.4 ^{10a}	8.8 ⁹	4.5 ¹⁰	4.8 ⁹	9.0 ⁹	7.9 ⁹
7% acrylamide gel	1.0 ^{4b}	1.0 ⁴	1.0 ³	1.0 ³	1.0 ²	1.0 ⁵
ABI PRISM 377	1.0 ^{6b}	1.0 ⁶	1.0 ⁴	1.0 ⁴	1.0 ³	1.0 ⁴

Detection limit represents detection limit derived from 10 mg soil per PCR assay.
^aAverage number of seeded cells/g soil present in soil inoculated with bacterial seed containing *B. cepacia* JS150, *P. oleovorans*, *B. cepacia* DNT, *P. putida* pWWO, and *P. sp.* ADP.
^bHighest dilution at which gene presence was observed.

matic hydrocarbon degradation from bacteria resident in contaminated soils. Detection limits using existing assays that are based on agarose or polyacrylamide gel electrophoresis separation of DNA fragments followed by staining with ethidium bromide, silver staining, or SYBR Green I are 10 ng, 1 ng, and 20 pg dsDNA PCR products, respectively (6,8,9) (Molecular Probes Product Information Sheet). SYBR Green I has been used in capillary gel electrophoresis and laser-induced fluorescence to detect as little as 80 fg of 200-bp dsDNA (10). Here, we demonstrate that the sensitivity of our assay in comparison with the traditional method of post-staining can be increased by addition of SYBR Green I to the sample loading dye and electrophoresis buffer with subsequent analysis by an ABI PRISM377 sequencer.

The limit of detection with the ABI PRISM 377 sequencer using SYBR Green I stain in loading dye and electrophoresis buffer was assessed using serial dilutions of GeneScan® 2500 ROX (Applied Biosystems), a *Pst*I-digested λ phage DNA molecular weight standard (27 fragments from 55 to 14 097 bp). Twofold serial dilutions of 8 nM of the standard were mixed into SYBR Green I loading dye [17 mg/mL Blue Dextran (Sigma, St. Louis, MO, USA), 8 mM EDTA, and a 1/1000 dilution of SYBR Green I]. DNA were analyzed on a 12 cm long, 0.4 mm thick 5% Long Ranger Hydrolink® nondenaturing gel (BMA, Rockland, ME, USA) using an ABI PRISM 377 automated DNA sequencer. Electrophoresis conditions and data collection were per-

formed using ABI PRISM 377 Sequencer data collection software (version 2.5.1) with the following GeneScan run settings: 12F-2400 run module (using filter set F), 1-h run time, 51°C run temperature, and a NED dye multi-component matrix for subtraction of spectral overlap, created with a NED, 6-FAM, HEX, and Rox Fluorescent Amidite Standards Kit (Applied Biosystems). The NED dye primer matrix set and filter set D are designed to monitor 6-FAM dye-labeled fragments that have excitation and emission characteristics similar to SYBR Green I. Collected data were analyzed using GeneScan analysis software (version 3.1). Where the only dye of interest is SYBR Green I, data were analyzed with baseline adjustments but no spectral overlap (multi-component option) or size standard. Spectral overlap was adjusted when other dyes such as ROX were used. The range analyzed was adjusted to exclude primer and primer dimer peaks that may effect baseline adjustments. Samples were compared by scan number.

Detection limits of SYBR Green I-stained fragments were dependent on fragment size (Figure 1). Nonlinear regression (curve fitting) analyses were performed using GraphPad Prism® version 3.00 for Microsoft® Windows™ (GraphPad Software, San Diego, CA, USA). Signal intensity versus DNA concentration of the standard (all sizes independently) was found to have a linear relationship with a goodness of fit (R^2) equal to 0.9715 (data not shown). Migration of DNA stained with SYBR Green I loading dye was altered by 0.2%–0.5% relative to unstained Gene-

Scan 2500 ROX ladder. The detection limit of a 204-bp fragment was 125 fg, a value similar to reported detection limits with SYBR Green I used in capillary electrophoresis with laser-induced fluorescence (10).

As observed by Miller et al. (5), SYBR Green I dye appears to disassociate from smaller-sized fragments (<300 bp) during electrophoresis, decreasing the efficiency of detection. To compensate for dye stripping during electrophoresis, we examined the effect of adding a 1:100 000 dilution of SYBR Green I to the electrophoresis buffer in the bottom buffer reservoir, in addition to in the loading buffer, on DNA fragment detection limits. Addition of dye to the electrophoresis buffer dramatically increased sensitivity 16- to 30-fold, resulting in detection limits similar to that of ROX dye end-labeled DNA fragments.

The increased detection limits achieved using an ABI PRISM 377 sequencer and SYBR Green I loading dye in comparison to post-electrophoresis SYBR Green I staining and UV visualization of traditional vertical gels are demonstrated by parallel multiplex PCR analyses. We have previously developed a multiplex PCR assay targeting bacterial genes involved in pollutant metabolism: Tn501 mercury resistance genes, toluene 1, 2-dioxygenase, toluene-4-monooxygenase, alkane hydroxylase, bacterial 16S rDNA, NAD(P)H dependent nitroreductase, dissimilatory sulfite reductase, nitrotoluene dioxygenase, biphenyl dioxygenase, catechol 2,3-oxygenase, and atrazine chlorohydrolyase (7). The detection limits of six tar-

Benchmarks

gets in this assay were examined using a surface soil seeded with approximately 10^8 cells/g soil each of *Burkholderia cepacia* JS150, *Pseudomonas oleovorans*, *B. cepacia* DNT, *P. putida* pWWO, and *P. sp.* ADP, bacteria containing the multiplex PCR target genes. Seed cell density was determined by plating of a dilution series onto agar plates containing $2\times$ yeast-tryptone broth. Total DNA was isolated from seeded soil into a final volume of 50 μ L from 500-mg triplicate subsamples of soil as described in Borneman et al. (1) using a mini-beadbeater and a FastDNA SPIN[®] (BIO 101, Vista, CA, USA). Multiplex PCRs were performed as described previously (7).

For analysis of PCR products by laser excitation using an ABI PRISM 377 automated sequencer, the following protocol was used: 20- μ L reactions were precipitated with 5 μ L 10 M ammonium acetate and 75 μ L ethanol. DNA was recovered by centrifugation at $14000\times g$ in an Eppendorf[®] model 5415C centrifuge (Brinkmann Instruments, Westbury, NY, USA). DNA pellets were washed with 70% ethanol to remove residual salts and analyzed on an ABI PRISM 377 with SYBR Green I loading dye. In parallel, multiplex reactions were performed and products were analyzed on 10×10 cm, 1 mm thick, 7% Long Ranger Hydrolink vertical gels. Gels were electrophoresed in TAE buffer (40 mM Tris-acetate/1 mM EDTA, pH 8.0) at 100 V for 30 min. After post-run staining in 100 mL TAE buffer with 10 μ L SYBR Green I, 10×10 cm gels were imaged using a 300-nm transilluminator and CCD camera (ChemiImager[™] 4000; Alpha Innotech, San Leandro, CA, USA). In both systems, multiplexed PCR products were identified on the basis of relative mobility to size standards and gene target standards run in adjacent lanes.

For comparison, the minimum detectable number of target genes was determined by a semi-quantitative replicative limiting dilution approach (2). Each of the three soil subsample DNA extracts was serially diluted 10-fold. Serial dilutions were then amplified by multiplex PCR. Samples were scored for the greatest dilution at which a band is observable. To compensate for heterogeneous distribution of bacte-

ria, a gene was scored as detectable if at least two of the three replicate soil subsample extracts had the correct band. The dilution factor at which a band is last seen is then considered the minimum detectable copy number for the gene in question. Values were not corrected for inhibition of PCR by the soil extract. Detection of multiplex PCR fragments using SYBR Green I loading dye and the ABI PRISM 377 was 10- to 100-fold more sensitive than detection by post-electrophoresis staining with SYBR Green I (Table 1).

We have demonstrated that SYBR Green I is a simple alternative to incorporation of fluorescent dyes using labeled primers or labeled nucleotides when analyzing PCR products by an ABI PRISM automated sequencer. DNA fragment detection limits were comparable to that of DNA end labeled with ROX dye. This technique may be useful in applications when the number of copies to detect can be low such as detection of viral DNA or RNA in blood or BCR-ABL fusions by quantitative competitive reverse transcriptase-PCR after bone marrow transplantation (3). The greater sensitivity achieved using laser-induced fluorescence results in a more accurate quantification of the presence of PCR products than detection using post-staining of agarose gels without the added expense of custom dye-labeled primers. The use of SYBR Green I staining with an ABI PRISM377 sequencer may be most useful in situations where accurate base-pair sizing is not required because internal size standards run with samples will also stain with SYBR Green I, possibly obscuring detection of PCR products. In addition, different multiplex PCR products should have different product lengths to distinguish SYBR Green I-stained products.

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