Post-PCR Multiplex Fluorescent Ligation Detection Assay and Flow Cytometry for Rapid Detection of Gene-Specific Translocations in Leukemia

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Key Words: Polymerase chain reaction; Fluorescent thermostable ligase reaction; Southern blot analysis; Leukemia

Abstract

We describe a novel method to detect specific polymerase chain reaction (PCR) target amplicons, involving thermostable ligation of fluorescent and biotinylated oligonucleotides, microparticle bead capture of the ligated products, and flow cytometric analysis. This approach, termed fluorescent ligation detection reaction (f-LDR) is more rapid and cost-effective than oligoprobe Southern blot hybridization (SBH). A standard f-LDR protocol was developed to detect the leukemia-associated chimeric transcripts bcr-abl and promyelocytic leukemia–retinoic acid receptor α (PML-RARα) in 2 multiplex and multicolor assays. The f-LDR platform was 100% specific and demonstrated comparable or better sensitivity than standard oligoprobe SBH. The usefulness of f-LDR was evaluated in 94 posttherapy samples from 13 patients with acute promyelocytic leukemia with the PML-RARα gene fusion. The f-LDR method was highly concordant (93%) with oligoprobe SBH; essentially all discrepancies were noted to be due to the enhanced sensitivity of f-LDR. We conclude that f-LDR is a highly specific and sensitive post-PCR method with wide potential application.

Rapid and accurate identification of prognostically relevant gene fusions arising from chromosomal translocations is critical to establish a specific diagnosis in many pediatric and adult acute leukemias, as well as in chronic myeloid leukemia.1,2 These molecular markers are equally useful for guiding more tailored antileukemic therapy and further provide sensitive, disease-specific targets for posttherapeutic minimal residual disease monitoring.3 Although a variety of specialized methods commonly are used to detect these abnormalities, including cytogenetics and fluorescence in situ hybridization, reverse transcription–polymerase chain reaction (RT-PCR)–based techniques are used widely for their rapidity and the ability to evaluate minimal residual disease. Standard post-PCR analysis by agarose gel electrophoresis may suffice for initial identification of a chimeric gene fusion abnormality; however, many laboratories use additional measures to confirm the specificity of the amplified PCR product, such as Southern blot transfer with oligonucleotide probe hybridization (SBH) for the sequence of interest.

Zhang et al4 described alternative oligonucleotide detection methods, based on simple solution phase binding of a fluorescent probe to a PCR target, and Gaffney et al5 described a novel application of thermostable oligonucleotide ligation termed fluorescent ligation detection reaction (f-LDR). The f-LDR approach was used successfully to distinguish the 2 major transcriptional products of the SYT-SSX chimeric gene fusion resulting from the t(X;18) abnormality in synovial sarcoma. The specificity of this approach was illustrated by the ability of closely related fluorescent ligation primers to accurately distinguish between 2 highly homologous target amplicon sequences, representing the SYT-SSX1 or SYT-SSX2 fusion genes. In the present effort, we sought to apply the f-LDR
approach to more complex leukemia-associated gene fusions, reasoning that this technology would provide rapid and specific detection of these abnormalities following PCR amplification and the ability to potentially multiplex reagents for simultaneous identification of alternative fusion transcripts. This technique emphasizes high specificity, enhanced sensitivity, and the ability to process multiple samples from RNA isolation to final analysis in the same day. Furthermore, this method could be applicable to other molecular diagnostic targets in which rapid and specific detection of PCR products is required.

Materials and Methods

Cell Lines and Patient Leukemia Samples

Five leukemia cell lines were used in this study: K562 and BV-173, respectively, for the B3-A2 and B2-A2 major breakpoint cluster region t(9;22)/bcr-abl fusion transcripts (p210); SUP-B15 for the E1-A2 minor breakpoint cluster region t(9;22)/bcr-abl fusion transcript (p190); and NB4 and UF-1, respectively, for the bcr1/L-form and bcr3/S-form t(15;17)/promyelocytic leukemia–retinoic acid receptor α (PML-RARα) fusion transcripts. In addition, the MOLT-4 T-lymphoblastic cell line was used as a negative control sample and diluted RNA source for sensitivity studies. For clinical validation studies, 94 banked RNA samples from 13 patients with a diagnosis of acute promyelocytic leukemia (APL) were identified. These clinical specimens had been analyzed previously for post-therapy PML-RARα status at sequential time points. This study was approved by the University of New Mexico Health Sciences Human Research Review Committee, Albuquerque.

RNA Extraction and RT-PCR Assays

Total RNA was isolated from cells using the RNeasy method (Qiagen, Santa Clarita, CA), according to the manufacturer’s directions, with yield and purity determined by spectrophotometry. Reverse transcription was performed from 1 µg of total RNA in a 20-µL volume containing a 10-mmol/L concentration of tris(hydroxymethyl)aminomethane (Tris) hydrochloride, a 50-mmol/L concentration of potassium chloride, a 1.5-mmol/L concentration of magnesium chloride, 0.01% wt/vol gelatin (10× PCR Buffer I, Applied Biosystems, Foster City, CA), a 5-mmol/L concentration of dithiothreitol, a 1-mmol/L concentration of deoxynucleotide triphosphates (Invitrogen, Carlsbad, CA), 1× random hexanucleotides (Roche Diagnostics, Indianapolis, IN), 20 U RNAsin (Promega, Madison, WI), and 100 U of Moloney murine leukemia virus reverse transcriptase (Invitrogen).

The reverse transcription reaction was carried out as follows in a PCR Express thermocycler (Thermo Hybaid US, Franklin, MA): 23°C for 10 minutes, 42°C for 30 minutes, 95°C for 5 minutes, and then hold at 4°C. Following complete reverse transcription, the 20-µL reaction was diluted to 50 µL total volume with 1× Tris-EDTA, with each 5-µL aliquot containing a 100-ng equivalent of complementary DNA. PCRs for the various leukemia fusion gene transcripts were each performed in a 50-µL total reaction volume using 100-ng aliquots of sample complementary DNA.

Separate PCR assays were used to detect the variable transcript types for bcr-abl and PML-RARα targets. For bcr-abl, 1 PCR was performed using primers B2 and A2 to detect B3-A2 and B2-A2 major breakpoint cluster region fusions (p210 products), and a separate PCR was performed with E1 and A2 primers to detect the E1-A2 minor breakpoint cluster region fusion (p190). Similarly, for PML-RARα, 2 separate reactions were performed containing either primers P6 and R4 to specifically detect the bcr1/L-form type fusion, or P3 and R4 to detect the bcr3/S-form type fusion. The PCR master mix included final concentrations of reagents as follows: 10 mmol/L of Tris-hydrochloride, 50 mmol/L of potassium chloride, 1.5 mmol/L of magnesium chloride, 0.01% wt/vol gelatin (10× PCR Buffer I, Applied Biosystems), 0.8 µmol/L of deoxyribonucleoside triphosphate mix, 20 pmol of each PCR primer, and 2.5 U of Taq polymerase (Applied Biosystems).

Standard PCR conditions were developed for all assays: denaturation at 95°C for 2 minutes, followed by 35 cycles of 95°C for 30 seconds, 60°C for 30 seconds, 72°C for 1 minute, and final extension at 72°C for 5 minutes and cooling to 4°C. For each sample, the integrity of RNA and fidelity of the reverse transcription and PCR steps were confirmed by amplification of a 173-base-pair segment of the E2A gene product. The E2A amplicons were assessed rapidly using a minichip capillary electrophoresis platform (Bioanalyzer 2100 and DNA-500 chips, Agilent Technologies, Palo Alto, CA), requiring 1 µL of the E2A PCR product. Capillary electrophoresis was performed according to the manufacturer’s directions. All PCR primers were obtained from Integrated DNA Technologies (Coralville, IA), and the primer sequences are given in Table II.

PCG Product Detection by Chemiluminescent Oligonucleotide SBH

PCR products were analyzed by electrophoresis in 1.5% agarose gels, followed by vacuum blot transfer (BioRad 785, BioRad, Hercules, CA) of the gel products under alkaline conditions onto precut nylon membranes (Pall Biodyne, East Hills, NY). Membranes were hybridized stringently with specific 5’ fluorescein-labeled oligonucleotide probes directed to the junctional regions of each fusion gene transcript of interest. Hybridized probes were detected by using an alkaline phosphatase–conjugated antifluorescein antibody and a chemiluminescent dioxetane-phosphate substrate according to the manufacturer’s directions (Chemiluminescent Detection System, DAKO, San Diego, CA). The light signal emitted
from hybridized blots was captured on radiographic film (Kodak XAR, Eastman-Kodak, Rochester, NY) during a standard 5-minute exposure time. All modified hybridization oligoprobe sequences were obtained from Integrated DNA Technologies and are shown in Table 1.

**PCR Product Detection by Multiplex f-LDR Assay and Flow Cytometry**

Ligation primers were designed to bind contiguously to specific sequence regions of amplified chimeric gene transcript targets on 1 strand of the double-stranded DNA amplicon, with some of the primer region encompassing the junctional locus. The upstream ligation primer was synthesized with a 6-carboxy-fluorescein (6FAM) or cyanine 5 (CY5) fluorescent moiety on the 5’ end, whereas the immediately adjacent downstream primer was modified by 5’-phosphorylation and 3’-biotinylation.

To perform multiplex f-LDR, 1 μL of each PCR product (2% of reaction) was placed in a total reaction volume of 20 μL in a 0-2 mL PCR tube containing 5 pmol each of all pertinent ligation primers (see later text in this paragraph), 1X ligase buffer, and 0.5 μL of thermostable Taq DNA ligase (New England Biolabs, Beverly, MA). Of note, while separate RT-PCRs were used to amplify the transcript variants of *bcr-abl* or *PML-RARα* in a given sample (see preceding details of PCR), these individual PCR products subsequently were combined in a single f-LDR reaction tube for the corresponding multiplex f-LDR assay. Thus, for f-LDR detection of *bcr-abl* transcripts in a given case, a multiplex reaction consisting of 1 μL of each of the *bcr-abl* PCR amplification products (ie, p210 and p190) was combined with B3, B2, E1, and A2 ligation primers in a single tube. Similarly, for *PML-RARα* detection in a particular specimen, 1 μL of each of the *PML-RARα* PCR products (ie, bcr1/L-form and bcr3/S-form) was combined in a single tube with PML3, PML6, and RARα ligation primers. The multiplex primers and ligation reactants for *bcr-abl* or *PML-RARα* f-LDR were assembled as a “master mix” and added in aliquots to the tubes containing the combined PCR product templates for each sample. All modified ligation primer sequences were obtained from Integrated DNA Technologies and are shown in Table 1.

The DNA ligase reactions were carried out in the thermocycler as follows: denaturation at 95°C for 5 minutes, 20 cycles of 95°C for 30 seconds and 60°C for 2 minutes, and then final denaturation at 95°C for 5 minutes and cooling to 4°C. Following ligase reaction, a 5-μL aliquot of each multiplex ligation product was mixed with 5 μL of 6.0- to 8.0-μm streptavidin-coated polystyrene microbeads (Spherotech, Inc., Lincolnshire, IL).
Libertyville, IL) in a 12 x 75-mm plastic tube and incubated at room temperature in the dark for 15 minutes. After incubation, the beads were diluted with 500 µL of a 100-mmol/L concentration of Tris/sodium chloride buffer (pH 8.3) for flow cytometric analysis. No bead wash step was required. Analysis of beads was performed on a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA) using 488- and 635-nm dual laser excitation for 6FAM and CY5 fluorophores, respectively, with detection in FL1 and FL4 channels.

Positive results (intensity >10^1 log scale relative fluorescence) indicated coating of beads by ligated and specific fluorescent nucleotide products. The dual-color nature of the multiplex f-LDR permitted simultaneous evaluation for the presence of different transcript types associated with \( bcr-abl \) or \( PML-RAR\alpha \) fusion gene abnormalities. To standardize the evaluation of f-LDR, uniform detection settings were established on the flow cytometer and maintained with each set of samples.

Dilutional Sensitivity Studies

Serial dilutions of total RNA from NB4, U937, K562, and SUP-B15 leukemia cell lines were made into MOLT-4 RNA to produce a range from 100 ng to 100 pg of target leukemic RNA. A constant amount of 1 µg of each dilution was reverse transcribed and subjected to PCR as described. Post-PCR analysis was performed using both gel electrophoresis and oligoprobe SBH (with a standard 5-minute exposure time) and multiplex f-LDR with flow cytometry. In the latter analysis, each RNA dilution was assessed by median channel shift of fluorescence intensity and the magnitude of the “D value,” derived from Kolmogorov-Smirnov statistics. The D value is a measure of the degree of significance between fluorescence spectra of a negative control sample and a test sample.6,7

Evaluation of Multiplex f-LDR vs Oligoprobe SBH in APL Patient Samples

To assess the clinical usefulness of the f-LDR method, 94 cryopreserved RNA samples from 13 patients previously diagnosed with APL were selected. These patient samples were postdiagnostic and had been submitted to the laboratory for molecular monitoring of PML-RAR\(\alpha\) status. RT-PCR for \( PML-RAR\alpha \) was performed on these banked RNA samples, followed by both oligoprobe SBH and multiplex f-LDR for PML-RAR\(\alpha\) detection, using the methods as described.

Results

Development and Validation of f-LDR for Leukemia Translocation Fusion Gene Detection

Leukemic cell lines containing translocation fusion genes of interest were used to establish and validate the f-LDR assay. The f-LDR method was compared with routine chemiluminescent oligonucleotide SBH detection to determine its dilutional sensitivity and clinical usefulness in a set of patient samples. Although separate RT-PCRs were performed to amplify the chimeric transcripts and variants, the PCR conditions were standardized. All PCR primer and SBH oligoprobe sequences are given in Table 1.

For f-LDR, a multiplex platform was developed, such that simultaneous detection of different transcript variants (for a given fusion type) could be accomplished in a single reaction tube. A schematic depicting the f-LDR technique as applied to the detection of \( bcr-abl \) PCR products is shown in Figure 1. Sequences and modifications for ligation oligonucleotides are given in Table 1.

Initially, the multiplex ligation primers for \( bcr-abl \) and \( PML-RAR\alpha \) were evaluated with individual PCR products from the leukemic cell lines, representing 1 fusion target at a time. These assays demonstrated complete specificity, with no evidence of “crossover” false-positive ligations between related but variant transcript types. Furthermore, PCR products from a variety of nonspecific patient and cell line RNA negative control samples did not show any spurious false-positive ligation artifacts in repeated analyses (data not shown).

For all subsequent studies (ie, cell line dilutions and the evaluation of banked patient specimens), the separate \( bcr-abl \) (p190 and p210) or \( PML-RAR\alpha \) (bcr1/L-form and bcr3/S-form) PCR products in an individual sample were combined in a corresponding single tube multiplex f-LDR, as described in the “Materials and Methods” section and Figure 1. Two-color flow cytometry of streptavidin-coated microsphere beads enabled the simultaneous detection and distinction of the major fusion transcript types, if present in the sample. (Note that for \( bcr-abl \), the multiplex f-LDR distinguishes the E1-A2 [p190] fusion from the B3-A2 and B2-A2 [p210] species; however, the latter 2 fusion types are not further delineated, as the upstream B2 and B3 ligation primers are both labeled with 6FAM, as depicted in Figure 1). The background fluorescence range was set and checked with both water blank (no template) and negative (nonspecific template) control samples; these fluorescence values were consistent between experiments using the same cytometer settings. In addition, the assessment of the water blank and negative control samples permitted detection of possible contamination, which was not encountered in this study.

The f-LDR assay was completed in a thermocycler in approximately 1 hour, and subsequent bead incubation and flow cytometric steps were performed within 25 minutes. Thus, the time to a complete analysis by multiplex f-LDR following PCR was less than 2 hours, compared with nearly a full day for each fusion transcript type using the chemiluminescent oligoprobe SBH technique.
Dilutional Sensitivity of f-LDR

Figure 2 shows composite f-LDR data representing dilution studies of RNA from bcr-abl+ and PML-RARα+ cell lines in MOLT-4 RNA. Although the data are derived from endpoint PCR, fusion transcript detection is readily evident at 100 pg of target-specific RNA in each series. There was some variability in the dynamic range of detection (eg, comparing bcr3/S-form with bcr1/L-form PML-RARα dilutions), likely reflecting
differences in PCR and ligation efficiency arising from the application of standardized conditions. Although the fluorescence signal intensities obtained were not linear over the dynamic range, relatively stable and reproducible values were achievable for these cell line RNA dilutions. The highest target amount used in these PCR/f-LDR experiments was 100 ng, as it was noted that greater initial amounts of cell line RNA did not produce higher fluorescence values (indicating saturation of microsphere beads by bound ligated fluorescent product). The median channel fluorescence shift per cell line dilution was generally very reproducible over triplicate measurements, with only minimal variation (<10%) encountered (data not shown). Although not indicated in Figure 2, analysis of the Kolmogorov-Smirnov D value statistic (expressed on a scale 0-1.0) also revealed stable, significant results per dilution vs negative control samples in replicate assays. The f-LDR cell line dilution findings also were evaluated directly against the oligoprobe SBH method.

The data represented in Figure 3 demonstrate dilutions of bcr1/L-form and bcr3/S-form PML-RARα+ cell lines detected with respective junction-specific oligonucleotide probes. The sensitivities of both f-LDR and oligoprobe SBH were similar in these experiments for the range of dilutions studied.

In addition, we briefly investigated whether modestly increasing the amount of PCR product from 1 µL to 2 or 3 µL in the f-LDR assay would improve dilutional sensitivity. Augmenting the input quantity of PCR product even slightly produced relatively dramatic improvements in low-level detection sensitivity (ie, at the 100-pg cell line RNA dilution level), as evidenced by significantly increased shifts in median channel shift (data not shown). The latter observation suggests that f-LDR might be more sensitive than our current oligoprobe SBH method for detecting minimal amounts of target PCR amplicons, although, for the present study, a standard 1 µL of PCR product was used for comparative evaluations.

![Figure 3](en)
Comparison of Multiplex f-LDR and Oligoprobe SBH in APL Patient Samples

We identified 94 cryopreserved RNA samples from 13 patients with a diagnosis of APL in the clinical molecular diagnostic laboratory. These samples represented sequential assessments of patients undergoing standard therapy for APL and previously had been tested in the laboratory by RT-PCR and oligoprobe SBH methods for the presence of residual PML-RARα fusion transcripts. The samples comprised 78 bone marrow aspirates, 15 peripheral blood samples (including one peripheral blood stem cell specimen), and 1 soft tissue biopsy specimen. The average number of sequential samples per patient was 7 (range, 1-14 samples), with molecular evaluations spanning a period from 0 to 41 months. The timing of sample acquisition was variable (ie, postinduction, consolidation, or maintenance), and the laboratory was not always informed of the exact phase of therapy.

All of these samples were reevaluated by using RT-PCR and both oligoprobe SBH and f-LDR analyses. For each sample, integrity of the RNA was determined by the ability to adequately amplify a segment of the E2A gene transcript (data not shown); this criterion was required for inclusion in the sample set.

Concordance results between the 2 post-PCR techniques are summarized in Table 2. The comparison data were correlated highly for the presence or absence of PML-RARα fusion transcripts in these posttherapy APL samples. Overall, there was agreement between oligoprobe SBH and f-LDR assays in 87 (93%) of 94 samples; however, 7 (7%) of 94 samples were discordant.

All discordant results were found to be positive by f-LDR at very low detection levels (which corresponded in each case to <200 pg of a PML-RARα+ cell line dilution), whereas the corresponding oligoprobe SBH results were interpreted as negative. Nevertheless, when the oligoprobe SBH detection was altered by variably increasing the standard blot exposure time on film beyond 5 minutes (and up to 60 minutes in some cases), 5 of 7 discordant samples were weakly SBH-positive, in agreement with the f-LDR data. The remaining 2 cases remained positive by f-LDR with very low relative transcript

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Table 2
Comparison of Oligoprobe SBH and f-LDR in Sequential Assessment of PML-RARα Transcripts in 94 Samples From 13 Patients With Previously Diagnosed Acute Promyelocytic Leukemia

<table>
<thead>
<tr>
<th>Result</th>
<th>No. of Samples</th>
</tr>
</thead>
<tbody>
<tr>
<td>f-LDR+/SBH+</td>
<td>32</td>
</tr>
<tr>
<td>f-LDR+/SBH−†</td>
<td>7</td>
</tr>
<tr>
<td>f-LDR−/SBH+</td>
<td>0</td>
</tr>
<tr>
<td>f-LDR−/SBH−</td>
<td>55</td>
</tr>
</tbody>
</table>

f-LDR, fluorescent ligation detection reaction; PML-RAR, promyelocytic leukemia-retinoic acid receptor; SBH, Southern blot hybridization.

† Of these cases, 5 of 7 samples were reconciled as SBH weak positive on prolonged blot exposure time. Thus, the overall concordance improves to 98% when additional measures are pursued to reconcile method discrepancies.

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Figure 3
Reverse transcription–polymerase chain reaction (RT-PCR) of RNA/RNA dilutions of promyelocytic leukemia–retinoic acid receptor α (PML-RARα)+ cell lines with oligoprobe Southern blot analysis. Serial dilutions of PML-RARα+ cell lines analyzed by chemiluminescent oligonucleotide Southern blot hybridization following RT-PCR. S-form control (or bcr3-type PML-RARα) (A) is represented by the UF-1 cell line, whereas L-form control (or bcr1-type PML-RARα) (B) is the NB4 cell line. These cell line RNAs were diluted serially as indicated in a background of MOLT-4 RNA. Standard blot exposure time was 5 minutes. For conciseness, dilutions above 5 ng of target RNA are not shown. (A faint signal present in the “Blank” lane for L-form amplification is a shadow band, slightly below the positive band location.) The blot images show comparable detection to the range of diluted target RNA obtained by fluorescent ligation detection reaction analysis.
levels but were persistently negative by oligoprobe SBH. Contamination of the water blank and negative control samples was not observed in sample runs containing the discordant cases. **Figure 4A** demonstrates typical oligoprobe SBH data from a subset of patient samples. **Figure 4B** reveals f-LDR data for 2 of these cases designated as negative by SBH. Of these 2 samples, 1 (C2) subsequently was reconciled as SBH-positive by longer blot exposure on film, but the other (G2) was not (data not shown). The 2 “true discordant” samples in this series most likely reflect the improved detection sensitivity of the f-LDR method for low abundance PML-RARα transcript, because no evidence of contamination or nonspecific ligation was evident.

To illustrate the usefulness and sensitivity of f-LDR in assessing sequential patient samples, 3 representative patient cases are shown in **Figure 5**, plotted as sample time lines,
with accompanying fluorescence data for a subset of the respective time points. As shown in Figure 5, the f-LDR method permits a visual depiction of temporal fluctuations in the presence of the \textit{PML-RAR\alpha} transcript in individual patients over time.

**Discussion**

Molecular characterization of recurrent fusion gene abnormalities is an important adjunct in the diagnosis of leukemias. The specificity of PCR detection clearly is
achieved by ensuring stringency in primer design and reaction conditions; however, additional post-PCR manipulations, such as oligonucleotide SBH, might be necessary to attain this goal. These post-PCR procedures have variable compromises in terms of speed, complexity, and ease of interpretation. Moreover, in some cases, the reference laboratory might not be clearly informed about the nature of a given specimen (e.g., diagnostic vs posttherapy sample), requiring sufficient assay sensitivity to exclude false-negative results. Thus, post-PCR detection methods ideally should be rapid, robust, and unambiguous.

Gaffney et al\(^5\) recently described a novel post-PCR method termed f-LDR to detect and distinguish 2 closely related SYT-SSX gene fusion transcripts arising from the t(X;18) abnormality in synovial sarcoma. Extending the initial experience with this technology, we have developed multicolor or f-LDR assays to identify leukemia-associated gene fusions arising from characteristic chromosomal translocations.

As summarized in the schematic of Figure 1, this method relies on ligation of a specific, fluorescently labeled 5′ oligonucleotide to an adjacent biotin end-labeled 3′ oligonucleotide to form a fluorescent probe fragment in situ on 1 strand of a PCR product target. The biotin tag permits capture by streptavidin-coated polystyrene microparticles and fluorescence detection in a flow cytometer. In contrast with the earlier work, the use of different fluorescent dyes on the specific 5′ primers enables simultaneous multiplex/multicolor detection of transcript variants arising from a given leukemic gene fusion.

The multiplex f-LDR is performed essentially like a PCR and is complete in approximately 1 hour. The subsequent detection steps (bead incubation through flow cytometry) are relatively trivial in terms of time and technical labor. Although our protocol requires a cytometer with at least dual-color detection capability, this should not be an impediment to implementation in hematology laboratories performing cell phenotyping and molecular diagnostics. Furthermore, any fluorescent read-out platform likely could be adapted for this analysis, such as plate fluorimetry or scanning fluorescent analysis on an automated slab gel or capillary sequencer. As shown herein, the time required to generate highly specific f-LDR results is significantly shorter than other post-PCR confirmatory techniques, particularly oligoprobe SBH, with comparable or lower overall costs.

Given the nature of this “1-sided” (i.e., single-strand template) f-LDR, it is apparent that a modest linear amplification occurs during cyclic ligation from the PCR product. Thus, the detection sensitivity for specific targets should be at least comparable or enhanced relative to simple oligoprobe hybridization. To this end, we demonstrated that f-LDR is sufficiently sensitive in cell line dilution experiments. From a practical perspective, f-LDR was applied to a set of 94 samples from 13 patients previously diagnosed with APL, and the technique was correlated highly with results of oligoprobe SBH. The fluorescence data for individual patients can be stored electronically and retrieved to permit graphic qualitative comparisons of sequential PCR/f-LDR results. Notably, f-LDR seemed more sensitive in detecting very low abundance amplified PML-RAR\(\alpha\) transcript in a minority of cases initially missed by SBH. Finally, our experience with f-LDR suggests that it is a “tunable” assay in terms of its lower limit of detection sensitivity (e.g., by slightly increasing target template amount), a feature that cannot be achieved easily with typical chemiluminescent SBH methods. However, both PML-RAR\(\alpha\) and bcr-abl gene fusions are characterized by rarely occurring alternative transcript forms,\(^8\)\(^-\)\(^11\) which would not be detected with the ligation primer sequences we used. In particular, the variable or V-form (bcr2) type PML-RAR\(\alpha\) transcript, observed in approximately 5% or less of APLs, or similarly infrequent variants of bcr-abl involving exon a3 of abl, would not have been detected with the oligonucleotides used in this study. To resolve this issue, the use of alternative ligation primers would be required.

Thermostable ligase chain reaction is a well-described non-PCR–based amplification method.\(^12\)\(^-\)\(^13\) Other investigators have described variations of PCR and fluorescent oligonucleotide ligation for identifying genetic point mutations or nucleotide polymorphisms, with subsequent detection of fragment length on a DNA sequencer,\(^14\)\(^,\)\(^15\) or using colored microsphere beads modified with specific oligonucleotide “capture tags” and flow cytometry.\(^16\) A post-PCR LDR method based on fluorescent product generation, denaturing gel electrophoresis, and detection on a DNA sequencer also has been put forth as an alternative to fluorescence in situ hybridization for quantitating HER-2/neu gene status in breast carcinoma.\(^17\) In contrast with these other methods, our approach does not require fragment size discrimination by specialized gel electrophoretic techniques or synthesis and attachment of specific capture oligoprobes on microbeads.

More pertinent to our study though, is a recently described application of fluorescent PCR product detection using multiple, specific oligonucleotide probes and a proprietary high-throughput bead-array platform (Luminex 100, Luminex, Austin, TX), to identify the common translocation fusion genes occurring in pediatric acute lymphoblastic leukemia.\(^18\) Although the latter investigators also used rapid cytometric detection technology, the post-PCR preparative and analytic times were similar to those for our method. In addition, the Luminex technology still requires modification of manufacturer-supplied microparticle beads to attach a number of specific capture oligoprobes to different colored beads, introducing an additional manipulation and the need to evaluate the efficiency of target-probe interactions for each PCR product assayed.

The technique we describe herein can be applied readily to many samples at a time or equally to a single specimen, in
each setting optimizing for reagent and labor costs. Furthermore, our biotin-streptavidin f-LDR product capture is a very robust and reproducible system. In combination with the use of different fluorophores, these features establish a flexible analytic method, which permits the detection of various, specific amplified products on a single type of bead.

Considering the now universal acceptance of quantitative real-time PCR for measuring minimal residual disease in leukemia and lymphoma, our illustration of the capability of f-LDR to detect small amounts of transcript in endpoint PCR samples is not intended to serve as an alternative to the former method in this regard. Rather, it is important that clinical molecular diagnostic assays have complete specificity and sufficient sensitivity to contend with samples that are partially degraded or unknown with regard to sampling time point (eg, phase of disease). We described a novel application of thermostable ligation, f-LDR, and showed that this process of in situ fluorescent probe generation is highly specific and sensitive for the detection of target PCR amplicons. We have demonstrated the usefulness of this technique for identifying leukemia-associated gene fusions in a multiplex format, with particular application to the clinically relevant scenario of qualitative PML-RARα fusion transcript detection in posttherapeutic specimens. These features, in combination with the reasonable costs, rapidity, and relative technical ease, indicate that f-LDR can be used reliably in the molecular diagnostics laboratory.

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