Mutation Typing Using Electrophoresis and Gel-Immobilized Acrydite™ Probes

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BioTechniques 25:516-521 (September 1998)

ABSTRACT

A new electrophoresis technology for hybridization-based sequence detection and mutation typing is described. Intrinsic to this approach is copolymerization of specially modified oligonucleotide probes directly into polyacrylamide gels. Electrophoresis of single-stranded samples through gels containing specific immobilized probes results in hybridization-mediated capture of complementary targets. By increasing gel temperature or including denaturants in the buffer, the method can be used to type single-nucleotide polymorphisms. The method can easily be adapted to type mutations in PCR-amplified samples. Acrydite Ô gel technology will also be useful for many other applications, including hybridization-based diagnostics, analysis of gene expression and purification of nucleic acids from biological samples.

INTRODUCTION

We have developed a flexible new approach for nucleic acid detection, isolation and sequence analysis. The basic idea is to perform electrophoresis in gels containing nucleic acid probes that are immobilized in the gel matrix. Figure 1 illustrates an Acrydite™ gel assay for detection of specific single-stranded nucleic acid targets, such as cDNA or denatured polymerase chain reaction (PCR) products. Probes complementary to the target of interest are immobilized within a centrally located capture layer. As complementary single-stranded nucleic acids enter the capture layer, they hybridize with the probes and become immobilized. Noncomplementary nucleic acids pass through the layer unimpeded. Thus, the novel feature of the Acrydite gel approach is that electrophoretic separation is based on the sequence of the target molecules rather than on their molecular size.

For the type of nucleic acid detection assays shown in Figure 1, efficient hybridization is the key to high sensitivity. Acrydite gel hybridization reactions are efficient because the applied electrical field forces the sample to interact with the capture probes in the gel. High probe concentration in the capture layer also drives the reaction. Consequently, complementary targets hybridize as soon as they enter the capture zone. This applies even to dilute, large-volume samples. By careful choice of target size, gel dimensions and voltage, short assay times can be achieved.

This technology was made possible by development of Acrydite attachment chemistry (Mosaic Technologies, Boston, MA, USA). Acrydite is a proprietary phosphoramidite that is capable of free-radical copolymerization with acrylamide. Acrydite can be used in standard DNA synthesizers to introduce copolymerizable groups at the 5′ terminus of any oligonucleotide probe. During gel preparation, Acrydite oligonucleotide capture probes are mixed with acrylamide solutions and polymerized into gel layers using standard methods (see Materials and Methods). The Acrydite groups on the oligonucleotides are efficiently incorporated into the gel matrix. Acrydite oligonucleotides are also efficiently used in PCR, and the resulting PCR products can be copolymerized into gels for use as capture probes.

MATERIALS AND METHODS

Oligonucleotides

Oligonucleotides containing 5′-terminal Acrydite groups were obtained from Research Genetics (Huntsville, AL, USA), Eurogentec (Seraing, Belgium) or Operon Technologies (Alameda, CA, USA). Unmodified and 5′-fluorescein-

labeled oligonucleotides were obtained from Ransom Hill Biosciences (Ramona, CA, USA). Lyophilized oligonucleotides were dissolved in TE buffer (10 mM Tris-HCl, pH 8.3, 1 mM EDTA) and stored frozen at -20°C. Concentrations were determined from A_{260} nm readings (assuming 33 µg/mL oligonucleotide per 1 optical density [OD] unit). All concentrations refer to oligonucleotide strands.

Sequences of oligonucleotides used are listed below. Fl and Ac represent fluorescein and Acrydite modifications, respectively. In Figure 2, the capture probe used was 5′-Ac-GTA CCA TAA CAG CAA GCC TCA-3′, and the target oligonucleotides were as follows: *(i)* left complementary lane, 5′-Fl-TGA GGC TTG CTG TTA TGG TAC-3′; *(ii)* right complementary lane, 5′-Fl-TGA GGC TTG CTT TTA TGG TAC-3′; and *(iii)* noncomplementary, 5′-Fl-ATT ACG TTG ATA TTG CTG ATT A-3′. The two complementary oligonucleotides differ at a single position, 12 bases from the 5′ end (underlined). Under the non-stringent gel conditions used for Figure 2 (23°C gel temperature), the sequence difference between the two complementary oligonucleotides is not discriminated. In Figure 3, the capture oligonucleotide was the same as in Figure 2, and the target nucleotides were based on the fluorescein-tagged complement (compl.): 5′-Fl-TGA GGC TTG CTG TTA TGG TAC-3′. The various mutant targets are labeled above Figure 3 by the type of mispair with the capture probe and distance from the 5′ end of the target strand. The mispairs are identified by the base in capture probe followed by the base in target oligonucleotide, separated by a colon. For example, the target used in the c:t,5 lane is: 5′-Fl-TGA GTC TTG CTG TTA TGG TAC-3′, and the target used in the c:t,5;a:a,7;a:c,14 lane is: 5′-Fl-TGA GTC ATG CTG TCA TGG TAC-3′. The underlined positions differ from the fully complementary target. The noncomplementary target in Figure 3 was the same as that used for Figure 2 (see above). In Figure 4, the capture probe used to identify the human immunodeficiency virus (HIV) reverse transcriptase codon 41 mutation was 5′-Ac-GT ACA GAG CTG GAA AAG G-3′ (the mutant position is underlined). The capture probe used to identify the codon 215–219 mutations was 5′-Ac-GG CTC TAC ACA CCA GAC CAA-3′. In Figure 5, the capture probes were: *(i)* A′, 5′-Ac-GTT TTC AGC TCC ACC TAC CAC AAG T-3′; *(ii)* B′, 5′-Ac-GTT TTA GCT CCA ACT ACC ACA AGT T-3′; *(iii)* C′, 5′-Ac-GTT TTG CAC CTC AAA GCT GTT CCG T-3′; and *(iv)* D′, 5′-Ac-GTT TTG TTC ATG CCG CCC ATG CAG G-3′; and the target oligonucleotides were: *(i)* A, 5′-Fl-AC TTG TGG TAG TTG GAG CTG-3′; *(ii)* B, 5′-FL-AA CTT GTG GTA GTT GGA GCT-3′; *(iii)* C, 5′-Fl-AC GGA ACA GCT TTG AGG TGC-3′ and *(iv)* D, 5′-Fl-CC TGC ATG GGC GGC ATG AAC-3′. Targets A and B share a common sequence of 19 bases.

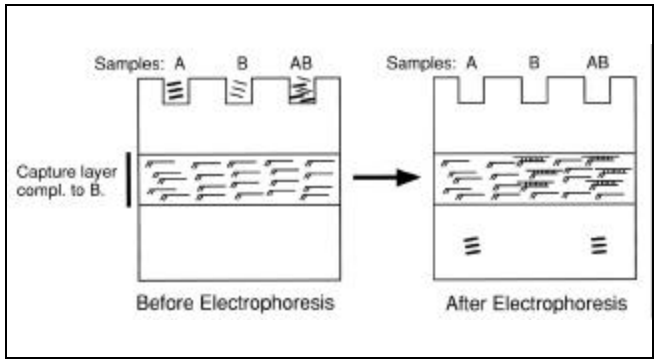

Figure 1. Schematic of Acrydite gel hybridization process. At left, the gel contains multiple layers, one of which contains immobilized Acrydite single-stranded probes, indicated as lines with "vinyl" tails. The gel is loaded with single-stranded nucleic acid samples that are noncomplementary (A) or complementary (B) to the capture probes. The samples are electrophoresed and are forced into contact with the capture probes. The complementary strands (B) hybridize with the capture probes and are immobilized in the capture layer. The noncomplementary samples (A) will not hybridize with the capture probe and migrate through the layer. Hybridization is detected using luminescent or radioactive label.

Gel Casting

Premixed solutions of acrylamide and bis-acrylamide were obtained from Bio-Rad (Hercules, CA, USA). Acrylamide gels (5%–20% total acrylamide, 29:1 monomer to bis-acrylamide) were prepared in 0.5× TBE buffer (45 mM Tris-borate, pH 8.3, 1 mM EDTA) and polymerized by addition of 7 µL 10% aqueous ammonium persulfate and 2 µL *N,N,N¢,N¢*-tetramethylenediamine (TEMED) per milliliter of gel solution. Standard minigel systems were used (Mini-PROTEAN® II, 8- × 10-cm plates [Bio-Rad] and Penguin™, 10- × 10-cm plates [Owl Scientific, Woburn, MA, USA]) with spacers ranging in thickness between 0.8 and 1.5 mm.

For most of the work presented, gels were cast in three sections. First, the lower unmodified gel was poured and allowed to polymerize. Next, a capture layer containing Acrydite oligonucleotide capture probe was polymerized over the unmodified gel. To ensure even polymerization of the layer, the volume of the capture layer was adjusted so that the height of the capture layer was approximately 0.5 cm. Acrydite capture probes (10 µM final concentration) were mixed with the unpolymerized gel solution before catalyst addition. Follow-

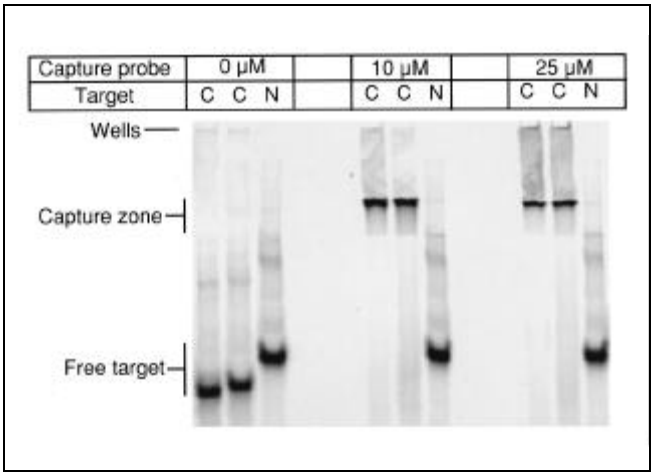

Figure 2. Efficiency of Acrydite gel hybridization. A 21-base Acrydite capture probe was immobilized at concentrations of 10 (center) and 25 µM (right) in discrete central layers (ca. 0.5-cm-tall × 2-cm × 0.15-cm-thick) of a 0.5× TBE, 20% polyacrylamide gel (29:1 monomer:bisacrylamide). Fluorescein-labeled oligonucleotides 21 bases in length were used as model target samples (25 pmol per lane). C and N indicate samples that are complementary and noncomplementary, respectively, to the capture probe. Two different complementary targets were used (see Materials and Methods). The total amount of capture probe that is available to interact with a sample is based on the probe concentration, cross-sectional area of the well and the depth of the capture layer. The amount used here is approximately 250 and 625 pmol for the 10 and 25 µM capture layers, respectively. Fluorescein-labeled targets were visualized using a FluorImager® 595 (Molecular Dynamics, Sunnyvale, CA, USA) .

ing polymerization of the capture layer, the gel surface was rinsed thoroughly with 0.5× TBE, and the remainder of the gel cassette was filled with unmodified gel.

Electrophoresis

Non-denaturing acrylamide gels were run in 0.5× TBE buffer at applied voltages of 100–150 V (gel lengths 8–10 cm). Gels were pre-run for 15–20 min before sample loading. Fluorescein-labeled oligonucleotides were mixed with sucrose-containing buffer and loaded without preheating. Labeled PCR products, usually 5 µL from a 50-µL reaction, were brought to 75% formamide (vol/vol) and heated to 90°C for 2 min before loading.

PCR for Analysis of HIV Reverse Transcriptase

Plasmids containing mutant (pKRT67/70/215/219, pKRT41/215/219) and wild-type (pKRTwt) subclones of the HIV reverse transcriptase gene (HIV-RT) were generously provided by Dr. Richard D'Aquila (Massachusetts General Hospital, Boston). Plasmid samples were restricted with *Hin*dIII, and 10^6 copies of the restricted material were used as initial PCR targets. Amplification was carried out for 30 cycles consisting of 10 s at 94°C, 10 s at 50°C and 1 min at 72°C. The reaction mixture contained 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2.5 mM $MgCl_2$, 0.5% Tween® 20, 50 µM dCTP, dTTP and dATP, 32 µM dGTP, 16 µM Fluorescein-dGTP (NEN Life Science Products, Boston, MA, USA), 0.05 U/µL AmpliTaq® DNA Polymerase (Perkin-Elmer, Norwalk, CT, USA) and 1 µM amplification primers. A 321-bp target containing the codon 215–219 region of HIV-RT was amplified using the primers 5′-ATG AGA CAC CAG GGA TTA GA-3′ and 5′-TAG GCT GTA CTG TCC ATT TAT-3′. A 249-bp target containing the codon 41 region of HIV-RT was amplified using primers 5′-GGA TGG CCC AAA AGT TA-3′ and 5′-CCT GCG GGA TGT GGT ATT C-3′.

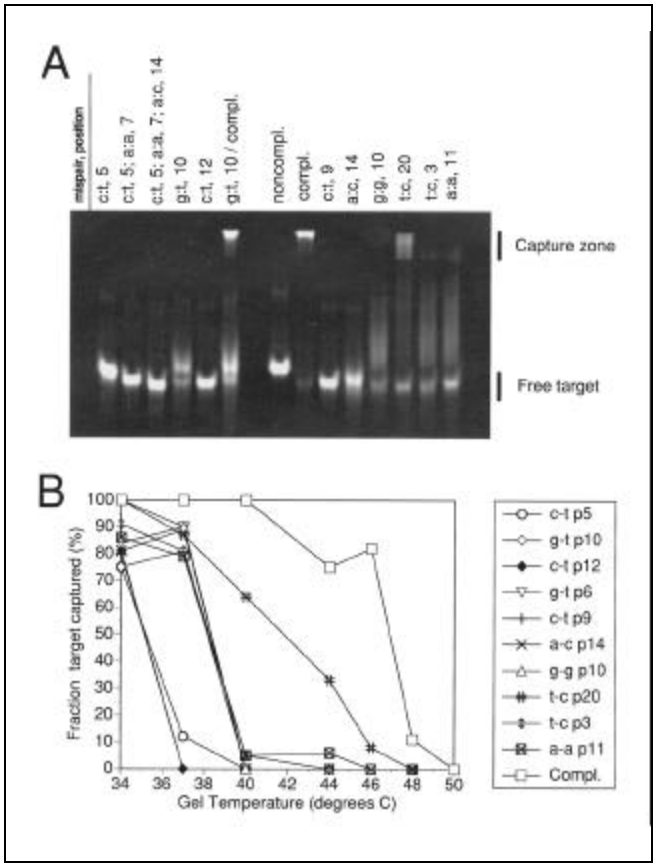

Figure 3. Acrydite gel analysis of mutations. (A) Fluorescein-labeled oligonucleotide targets with different levels of sequence complementarity to the Acrydite capture probe in the gel were electrophoresed in a 20% polyacrylamide 0.5× TBE gel at 40°C. Fluorescein-labeled targets were visualized using UV transillumination and a CCD camera (Biophotonics, Ann Arbor, MI, USA). Samples that interact weakly with the capture probe are retarded and smeared out as they traverse the capture layer (see the four right lanes). The extent and position of the capture layer are clearly seen in the lane with the "t:c,20" mismatch. (B) Acrydite gel "melting" profiles for 11 different oligonucleotide targets. The experiment shown in Panel A was repeated at different gel temperatures (X-axis), and the fraction of each sample that was retained in the capture layer (% retained, Y-axis) at each temperature was quantified. The legend at right identifies the mutant targets that were analyzed.

RESULTS

Efficient Hybridization Using Electrophoresis in Acrydite Gels

Figure 2 illustrates the efficiency of hybridization using fluorescein-labeled oligonucleotides as model samples for capture by complementary capture probes (both 21-bases-long). The gel was polymerized in three sections as described in Materials and Methods. The top and bottom layers contained polyacrylamide without capture probe. Three capture layers were used: one without Acrydite probe (Figure 2, left) and the others containing Acrydite probe at 10 (center) and 25 µM (right).

Samples of two fluorescein-labeled oligonucleotides that are complementary to the capture probe (Figure 2, C lanes) were electrophoresed through the capture layer at room temperature. As a negative control, a noncomplementary fluorescein-labeled oligonucleotide was also electrophoresed (Figure 2, N lanes). The results show that the complementary samples were completely immobilized on the capture layers that contained the Acrydite probe, while noncomplementary DNA of the same length was not retained. This demonstrates that complementary base pairing between the samples and the capture probe is responsible for sample immobilization. Increasing the concentration of Acrydite capture probe from 10 to 25 µM increased hybridization efficiency, as seen from the decreased thickness of the captured sample band on the 25-µM layer.

Acrydite Gels for Rapid Mutation Typing

In the Acrydite gel process, target capture is an extremely efficient hybridization reaction and is therefore affected by changes in electrophoresis conditions in a predictable manner. For instance, electrophoresis at low-ionic-strength, elevated-temperature, high-voltage gradient or with formamide-containing gels reduces the efficiency of target capture (data not shown). For this reason, we have tested Acrydite gel applications for rapid typing of single nucleotide polymorphisms.

A series of oligonucleotide targets containing different multiple and single base mismatches with the capture probe were run in Acrydite gel assays at various temperatures. Figure 3A shows a typical gel run at 40°C, while Figure 3B provides a graphical representation of the entire data set. As

seen in Figure 3B, the mismatches fell into several classes—most were sufficiently destabilizing that a gel temperature of 40°C prevents capture. A few were still partially retained at 40°C, but were efficiently cleared from the capture layer at 45°C. Only one mismatch was not completely eliminated from the capture layer at 45°C. However, that mutant was located at a near-terminal position (20- of a 21-base target), and it is usually difficult to distinguish such near-terminal mismatches without a kinetic melting analysis (2). The high stringency achieved with Acrydite gel assays is demonstrated by the fact that the near-terminal mutant target smears into (and through) the capture layer because of its weakened binding under conditions where the fully complementary target forms a tight band.

Denaturing solvents can also be used to achieve single nucleotide stringency. For instance, results similar to those shown in Figure 3A were obtained from 10% formamide gels run at room temperature (data not shown).

The process can also be used to type mutations in PCR products. Figure 4 shows Acrydite gel assays used to type two sets of mutations in HIV reverse transcriptase, which cause resistance to the drug 3′-azido-3′-deoxythymidine (AZT). The codon 41 mutation is a single nucleotide change, while in the codon 215–219, mutation involves substitution of four nucleotides (see Materials and Methods). Fluorescein-labeled PCR products were prepared from plasmid targets carrying HIV-RT fragments, denatured with formamide and then loaded onto gels containing capture probes specific for AZT-resistant, HIV-RT mutants. In both cases, the mutant probe-complementary strands are efficiently captured, while the wild-type (wt) products are not. Thus, in both cases, the mutant genes can be clearly distinguished from wt genes.

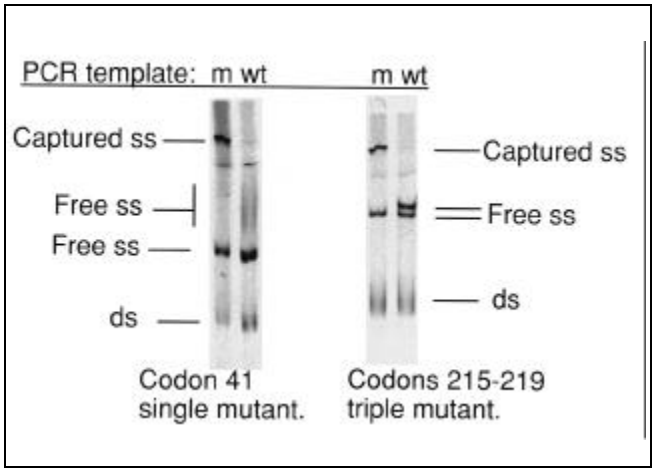

Figure 4. Mutation detection in PCR products. Fluorescein-labeled PCR products were generated using mutant (m) or wild-type (wt) plasmid HIV-RT targets. The PCR products were brought to 75% formamide, denatured for 2 min at 90°C and loaded on gels containing capture layers (50 μM in probe), which were specific for the mutant state of the HIV-RT gene. The PCR products undergo strand separation in the non-denaturing 8% polyacrylamide 0.5× TBE gels used. A small fraction of the PCR products re-associate to give the original double-stranded product, which runs below the single-stranded material as a smear. The probe-complementary PCR product strands are quantitatively captured under these conditions. Weak interaction between the codon 41 capture layer and the wt HIV-RT PCR product is evident from the smearing of the free single-stranded wt product. Image of fluorescein-labeled PCR product was obtained with a FluorImager 595.

Multiplex Analysis Using Acrydite Gels

The number of polymorphisms that can be tested in Acrydite gel experiments can be increased by using multiple capture layers. Figure 5 illustrates this capability, where a series of four capture zones were used in the same gel to assess probe cross-hybridization. Because targets A and B share a common 19-base sequence, both are efficiently captured by the first A′ capture layer. Complementarity of targets A and B with the B′ capture layer can be observed from target spillover caused by a slight overloading of the A′ capture layer, as is best seen in the lanes where both A and B targets were loaded simultaneously (Figure 5, far left and right lanes). Targets C and D display complete specificity for layers C′ and D′, respectively.

DISCUSSION

Acrydite-based mutation assays offer high-throughput, high-resolution sequence information with little more effort than most researchers already spend on PCR assays. Thus, Acrydite gel-mutation assays should find a broad range of applications in molecular biological, clinical and pharmaceutical research. The technology should be especially useful for high-throughput applications where many samples must be typed for a defined set of mutations. Acrydite-modified oligonucleotides are now commercially available through distributors in North America and Europe (see Materials and Methods).

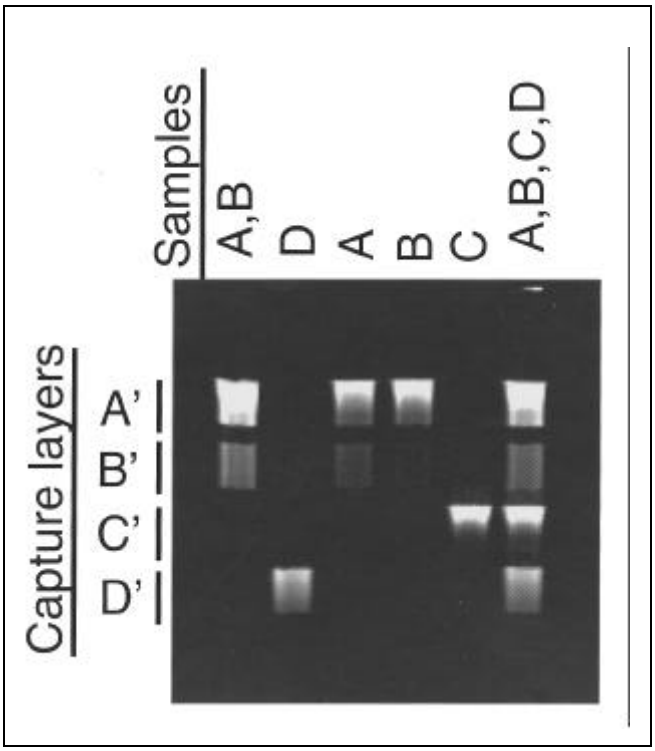

Figure 5. Acrydite gel with multiple capture layers. Four different fluorescein-labeled oligonucleotide targets were electrophoresed in different combinations through a gel with four different capture layers to assess probe cross-hybridization. The direction of electrophoresis was from top to bottom. Image of fluorescein-labeled oligonucleotide targets was obtained with a CCD camera (Biophotonics) and UV transilluminator.

In addition to using multiple capture layers, another way of increasing the number of screened mutations is to use more than one probe in each capture layer. In such assays, any signal in the capture layer indicates that at least one of the test sequences is present in the sample. An example of an application where this approach would be useful is HIV drug-resistance screening. For HIV-RT and HIV-protease, any one of several mutations can make the enzymes drug-resistant. An Acrydite gel assay using multiple capture layers (each layer containing multiple probes specific for mutations that confer resistance to a particular drug) would provide a rapid, cost-effective method for selecting an appropriate patient-specific drug therapy.

Acrydite gel assays are also attractive for hybridization-based diagnostics. Using commercially available fluorescence technologies, rapid Acrydite gel-diagnostic assays capable of detecting 10^5–10^6 fluorescently labeled target molecules are feasible. This level of sensitivity is suitable for a variety of microbiological testing applications, including blood-product contamination screening.

Other research applications that require rapid, quantitative hybridizations will also find Acrydite gel technology useful. Assays using capture probes to specific mRNAs could be used to analyze labeled cDNA products and labeled RNA generated in run-off transcription assays for study of gene expression. Acrydite gel technology can also be used to purify specific nucleic acids from complex RNA and DNA samples, cloned libraries and crude biological samples. The method should also be useful for improving genetic subtraction protocols.

Finally, Acrydite gel technology may be useful for purification and analysis of sequence-specific DNA binding proteins. It should be possible to construct Acrydite gel purification devices with double-stranded capture probes that contain a desired protein-binding site. The high capture-probe concentrations achievable with Acrydite probe copolymerization and the ease of Acrydite gel casting suggest that this method should offer some advantages over traditional sequence affinity purification methods (1).

ACKNOWLEDGMENTS

We gratefully acknowledge Dr. Richard D'Aquila, Massachusetts General Hospital, Boston, MA, for the gift of plasmids carrying mutant and wild-type forms of HIV-RT.

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