Self-Seal™ Reagent: Evaporation Control for Molecular Histology Procedures without Chambers, Clips or Fingernail Polish

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ABSTRACT

Sensitive nucleic acid based detection methods such as in situ PCR, in situ RT-PCR and PRINS have great potential in the areas of developmental biology, pathogenesis and diagnostics. However, control of evaporation from in situ reactions is critical to ensure reliable data. Self-Seal® Reagent, a component added directly to the in situ reaction mixture, effectively controls evaporation during in situ procedures by creating an evaporation-limiting barrier around the periphery of a standard cover glass as the reaction proceeds. At the end of the procedure, the cover glass is easily removed by soaking in an aqueous solution.

A model is presented for how Self-Seal Reagent controls evaporation while maintaining reagent concentrations. Self-Seal Reagent is shown to be effective in the detection of HIV sequences in cells by in situ PCR.

INTRODUCTION

The application of molecular biological techniques to tissues affixed to microscope slides provides exciting new methodologies to investigate processes in situ. Development, pathogenesis and diagnostics are just three of the areas where in situ techniques are being widely applied. These procedures include in situ nucleic acid hybridizations (ISH) (6), in situ polymerase chain reaction (IS-PCR) (2) and its variants (e.g., in situ reverse transcription PCR (IS-RT-PCR), isothermal amplifications such as in situ self-sustained sequence replication (3SR) (13), oligonucleotide primed in situ DNA synthesis (PRINS) (5) and antigen-based detection of tissue features (12).

The aqueous test solutions, usually less than 50 µL on the slide, are often held for minutes or hours at or above ambient temperatures or are rapidly cycled among a variety of temperatures ranging up to nearly 100°C. Without a means to control evaporation, the test solutions can quickly dry out, or, perhaps worse, component concentrations can change, thereby altering the specificity of the reaction.

Various methods have been devised to control evaporation from microscope slides. Incubation chambers, humidified with the appropriate solutions, can be used for procedures where the temperature is held constant (e.g., nucleic acid hybridizations). If the procedure requires a changing temperature regimen, humidified chambers generally are not appropriate due to problems associated with condensation and evaporation during thermal transitions, especially at elevated temperatures.

An obvious method of eliminating evaporation is to create a small chamber directly on the microscope slide over the tissue. Traditionally, chambers have been made by positioning a standard cover glass over the tissue with a layer of the test solution between the cover glass and the slide.

To seal the chamber, some practitioners immerse the slide (with the test solution over the tissue and a cover glass in place) in a small volume of a non-water-miscible fluid (e.g.,

mineral oil). This method has a number of disadvantages: it makes rapid thermal cycling difficult, and it also requires rinsing with nonaqueous solvents to remove the mineral oil from the equipment (and often the operator) after the procedure is completed.

More commonly, the edges of the cover glass are sealed to the slide by a variety of substances, including fingernail polish, rubber cements and various commercially available glues. These sealing methods, although quite popular, are tedious, messy and can involve the inhalation of organic solvents. They are also prone to failure, or worse, they can compromise test results if not applied correctly (2). When the test procedure involves thermal cycling reactions (e.g., IS-PCR), the only one of the above sealing methods that can withstand the resulting high internal pressures is nail polish.

Another problem with these sealants is experienced when removing them and the cover glasses after the procedure is completed. Removal often requires soaking in solvents (e.g., ethanol or xylene) and/or careful manipulation with a razor blade followed by scraping to remove residual sealant from the slide.

Various commercially available devices are designed to provide evaporation control by affixing small chambers directly to the slide or sandwiching the slide between rigid plates. However, each of these devices have limitations, such as excessive thermal mass, limited temperature range, limited area of slide covered, size or design incompatibility with some equipment or the need for dedicated instrumentation and disposables.

A factor common to all of the above methods is the need for skilled user intervention for sealing and unsealing slides. Any improvement in evaporation control methods that minimizes this requirement would be beneficial, not only for learning and performing techniques reliably, but also for future development of automated slide-handling systems.

We have developed a novel approach to sealing slides that totally eliminates the need for exogenous sealants, disposable chambers or special equipment. Instead, a novel reagent (Self-Seal™ Reagent; MJ Research, Watertown, MA, USA) is mixed directly with the in situ reaction components. Slides are assembled with a cover glass and placed directly on the appropriate thermal control instrument, without additional manipulations. At the end of the procedure, the covers are easily removed by soaking the slide assembly in an appropriate aqueous buffer. Here we characterize the mode of action of Self-Seal Reagent and show that this material is compatible with in situ PCR.

MATERIALS AND METHODS

General

A complete discussion of the in situ PCR protocols used here (excluding Self-Seal Reagent) can be found in Bagasra et al. (2). The following summarizes some of these protocols and includes other information specific to Self-Seal Reagent.

Conductivity Measurements

Conductivities of test solutions were determined with a Model B-173 Conductivity Meter (Horiba Instruments, Irvine, CA, USA), which allowed accurate measurements on 50-µL samples.

Mock PCR Solutions

To characterize the sealing performance of Self-Seal Reagent visually, by weight loss and by conductivity, mock reaction mixtures were used, which contained many of the components of the standard PCRs but lacked polymerase, primers, dNTPs and template DNAs. In addition, xylene cyanol was added in sufficient concentration to easily visualize the thin liquid layer between the slide and a cover glass. The mock reaction mixture contained 20 mM Tris-HCl (pH 8.2 at room temperature [RT]), 50 mM KCl, 2 mM $MgCl_2$, 0.1% bovine serum albumin (BSA; Catalog No. A-7284; Sigma Chemical, St. Louis, MO, USA), 0.1% xylene cyanol, and, when present, Self-Seal at 1× concentration.

Recovery of Reaction Mixtures from Slides

For conductivity determinations, mock PCR solution was recovered from a slide after quick-freezing the assembled slide on dry ice. Using dry ice temperature razor blades, the cover glass was removed, and the frozen reaction mixture was carefully scraped from selected areas as shown in Figure 1B. The crystals were rapidly thawed and transferred to a capped 0.2-mL tube. For conductivity measurements, the recovered material was diluted, in duplicate, 1:50 or 1:75 in deionized water (diH_2O).

To recover PCR products for agarose gel analysis, the Self-Seal peripheral barrier was softened by placing the assembled slide between sheets of damp filter paper at RT for a few minutes. The covers were then easily lifted with the aid of a razor blade. As the cover was lifted, the liquid gathered in the angle between the cover and the slide, and was collected in a micropipet tip.

Standard PCRs

The standard liquid PCR (the in situ PCR is described below) consisted of the following components: 10 mM Tris-HCl, pH 8.2; 50 mM KCl; 1.5 mM $MgCl_2$; 200 µM dNTPs; 0.5 µM each primer; 5×10^3 λ genomes/µL; 0.05–0.1 U/µL *Taq* DNA Polymerase (Life Technologies, Gaithersburg, MD, USA). The primers were designed to anneal at about 70°C and to amplify a 497-bp product. For reactions done on microscope slides, 0.1% BSA was included as a blocking agent. Reactions including Self-Seal Reagent were prepared by substituting the reagent for the same volume of water. Liquid reactions were usually 20 µL, and reactions on slides under 22- × 50-mm covers were usually 50 µL. Liquid PCRs (with λDNA) were performed on a Model PTC-200™-16/16 or 96 V, or on a Model PTC-100-96V or -16MS (both from MJ Research). The basic thermal cycling protocol was as follows: denaturation at 92°C for 2 min followed by 30 cycles of (94°C for 30 s; 70°C for 1 min) and a final extension of 70°C for 5 min.

Cell Lines and Slide Preparation

The cell lines used in this work are routinely used in the authors' laboratory (L. Bobroski and O. Bagasra) as controls for in situ PCR detection of human immunodeficiency virus Type 1 (HIV-1) sequences. The HIV-1 positive cell line "U1"

(3) has stably incorporated a single copy of HIV-1 into its genome. The negative cell line is the uninfected lymphoma cell line U-937 (10). Characterization of these cell lines as in situ PCR controls is described in Reference 1. For the experiments described here, these cells were mixed at various ratios of positives to negatives, then placed on the slides, air-dried overnight and heated at 105°C for 30 s. Proteinase K digestion was done at 6 µg/mL for approximately 12 min at room temperature, after which the slides were heated at 92°C for 2 min (2), rinsed in phosphate-buffered saline (PBS) then water (10 s each) and air-dried. The timing of the proteinase K digestion is critical and must be optimized for each tissue type and lot of proteinase K. The 12-min digestion used here had been empirically determined to be optimal.

In Situ PCR

The reaction mixture (with or without Self-Seal Reagent) contained the following components: 1.25 µM each primer; 200 µM each dNTP; 10 mM Tris-HCl (pH 8.3 at RT); 50 mM KCl, 1.5 mM $MgCl_2$; 0.1%–0.2% BSA; 0.1 U/µL *Taq* DNA Polymerase (Perkin-Elmer, Norwalk, CT, USA or Life Technologies). The primers were SK38 and SK39, specific for sequences in the gag region (p24) core protein of HIV-1 (8). Twenty microliters of the appropriate solution were placed over the cells on the slides, and the cover glasses were carefully lowered into place without trapping air bubbles.

To seal slides with fingernail polish, a thin layer of nail polish (Wet and Wild Clear Nail Protector; Pavion, Nyack-on-the-Hudson, NY, USA) was applied to the periphery of the cover glass, allowed to dry, then followed by a thicker layer to complete the seal. Slides assembled with Self-Seal Reagent in the reaction mixture required no additional sealing procedure.

Slides were placed in the slide chambers of a thermal cycler (Model PTC-100-16MS or PTC-200-16/16; MJ Research). The in situ thermal cycling conditions were as follows: (94°C for 1 min; 45°C for 1 min and 72°C for 1 min) × 30.

After thermal cycling, the cover glasses sealed with fingernail polish were removed by soaking the slide assemblies in 100% ethanol for about 5 min, followed by careful lifting of the cover glasses off of the slides with a razor blade. Residual fingernail polish was carefully removed from the slides with the razor blade. Cover glasses were removed from the Self-Seal Reagent slides by immersing the slides in 2× standard saline citrate (SSC) for a few minutes at RT. After immersion, the covers easily slipped off when the slides were dipped up and down then lifted out of a Coplin jar.

The uncovered slides were heated to 95°C for 1 min to help immobilize the amplified signal, then immersed 5 min in 2× SSC at RT before hybridization.

In Situ Hybridization

The standard hybridization solution contained 50% (vol/vol) deionized formamide, 2× SSC, 10× Denhardt's solution, 1 mg/mL sonicated denatured calf thymus DNA, 0.1% sodium dodecyl sulfate (SDS) and 20 pmol of probe. The probe, SK19 (8), was labeled with biotin and is specific for a sequence within the region amplified by the SK38 and SK39 primers.

Twenty-five microliters of hybridization solution were placed on the slides. Cover glasses were placed over the hybridization solutions. All slides were placed in the slide thermal cycler at 95°C for 5 min and then incubated in a humidified chamber at 42°C for 4 h.

Signal Detection

After hybridization, the cover glasses were removed and the slides were immersed twice, 5 min each, in 2× SSC at RT, washed in 1× PBS, then incubated with streptavidin-peroxidase. Color development was performed with aminoethylcarbazole (AEC). These procedures are fully described in Bagasra et al. (2).

RESULTS AND DISCUSSION

Self-Seal Reagent is a novel approach to controlling evaporation from microscope slides. The goal is not to eliminate, but rather to *control* evaporation. Evaporation must be controlled so that air does not intrude over the sample, and there

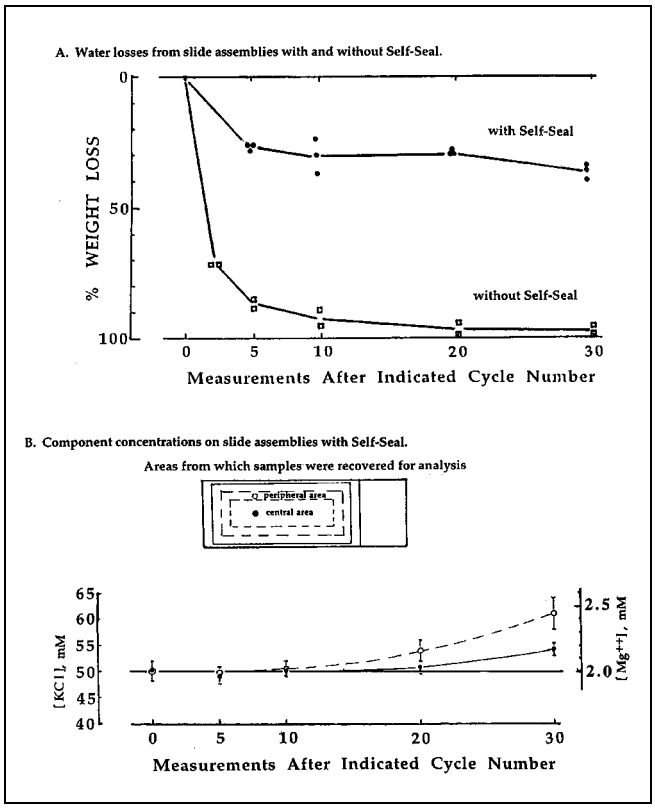

Figure 1. Characterization of water loss (A) and component concentrations (B) during slide cycling, with and without Self-Seal Reagent. Slides and cover glasses were assembled with 50 µL of mock PCR buffer with or without 1× Self-Seal in the solution. All slides were thermally cycled through the following protocol: 94°C for 1 min; 45°C for 1 min and 72°C for 1 min. Each slide assembly was weighed before cycling, and slides were removed and re-weighed after each of the indicated cycles. Panel A presents the water losses as percent weight loss after various cycles, using 50 mg as the initial solution weight. After weighing, the slides containing Self-Seal Reagent were quick-frozen on dry ice. Material recovered from selected areas of each slide was diluted in water for conductivity measurements. The changes in conductivity were converted to equivalent changes in KCl (left axis) and $MgCl_2$ (right axis), using starting concentrations of 50 and 2 mM, respectively (the starting concentrations in the mock PCR buffer). These data are presented in Panel B. Two dilutions were made of each area from each of three slides at each cycling time. Duplicate conductivity measurements were made on each dilution. The error bars are standard deviations of all pooled measurements.

is little, if any, component concentration change in the reaction cocktail over the sample on the slide. As long as these conditions are met, a significant amount of evaporation can occur without compromising the reaction.

Model of Self-Seal Reagent Action

When a slide, reagents and cover glass are assembled, the cover glass floats on a homogeneous layer of reaction mixture. During the first few thermal cycles, evaporation occurs at the edge of the cover glass. The Self-Seal Reagent near the edge becomes increasingly concentrated, until a nearly impermeable barrier has formed. Of critical importance, this barrier traps solutes that concentrate around the edge as the water evaporates. The trapped solutes can not easily move back into the interior liquid, where they might alter reagent concentrations. During further thermal cycles, water evaporation and solute trapping continue, but at a much reduced rate. Thus, Self-Seal Reagent produces a dynamic evaporation-control system, quite unlike traditional static systems that attempt to eliminate evaporation totally.

Evaporation Control and Component Concentrations During a Cycling Protocol

In the absence of any means to control evaporation, water is rapidly lost from the periphery of a cover glass during thermal cycling until the slide is nearly dry. Slides with Self-Seal Reagent in the mixture also lose some water but then stabilize with a low level of continuing evaporation. The extent of the evaporation is determined by a number of factors, most importantly, the level and duration of the highest temperature at each cycle. Figure 1 presents data from slides that were cycled under rather intensive denaturation conditions, 94°C for 1 min at each cycle. Figure 1A shows the water losses from slides with mock PCR buffer with and without Self-Seal Reagent. Slides without Self-Seal Reagent have lost 85% to 90% of their reagent weight by the 5th cycle and are nearly dry. Slides with Self-Seal Reagent in the reagent cocktail have lost 25% to 30% of their weight by the 5th cycle and are slowly approaching 35% to 40% loss by the 30th cycle. Given the observed water loss, the next question is: what are the concentrations of components in the remaining liquid?

The slides of Figure 1A containing Self-Seal Reagent (•) were quick-frozen, and the recovered materials were diluted for conductivity measurements. The conductivities were converted to the equivalent concentration of mock PCR buffer and presented in Figure 1B as KCl and $MgCl_2$ concentrations vs. cycle number. Through the first ten cycles, when the specificity of the PCR is most critical, there was no change in the reagent concentrations, even though the slides had lost about 30% of their weight (Figure 1A, •). By the 20th cycle, little concentration change had occurred, especially in the central area of the slides. By the 30th cycle, the concentration of magnesium had increased from 2.0 mM to about 2.2 mM in the central area and to about 2.5 mM in the peripheral area, which extends to about 3 mm from the edge of the cover glass.

The maintenance of initial reagent concentrations through the first 10–20 thermal cycles indicates that the specificity of

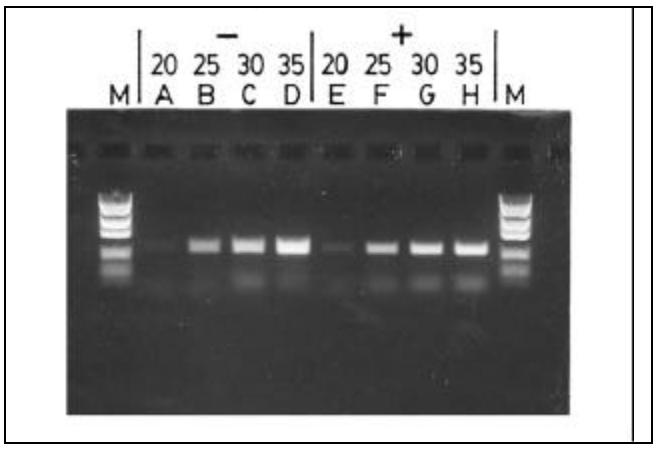

Figure 2. Products of PCRs performed in the presence and absence of Self-Seal Reagent. Standard reactions were assembled with and without Self-Seal Reagent. Each mixture was split into four 20-µL volumes, and all were thermally cycled through the following protocol: 92°C for 2 min; (92°C for 30 s; 68°C for 1 min.) × n cycles. Individual tubes of each mixture were removed after 20, 25, 30 and 35 cycles, as indicated on the figure. Before loading on the 1% agarose gel, reactions were diluted such that all samples, including the standards, were loaded in 1/2 × Self-Seal Reagent. Five microliters were loaded in each lane (= 2.5 µL of the original reaction). The - and + indicate reactions done in the absence or presence of Self-Seal Reagent, respectively. The numbers above the lanes, 20–35, refer to the cycle numbers after which the reactions were assayed. The size and mass standards (Life Technologies) were (top to bottom): 2000 bp, 100 ng; 1200 bp, 60 ng; 800 bp, 40 ng; 400 bp, 20 ng; 200 bp, 10 ng; 100 bp, 5 ng.

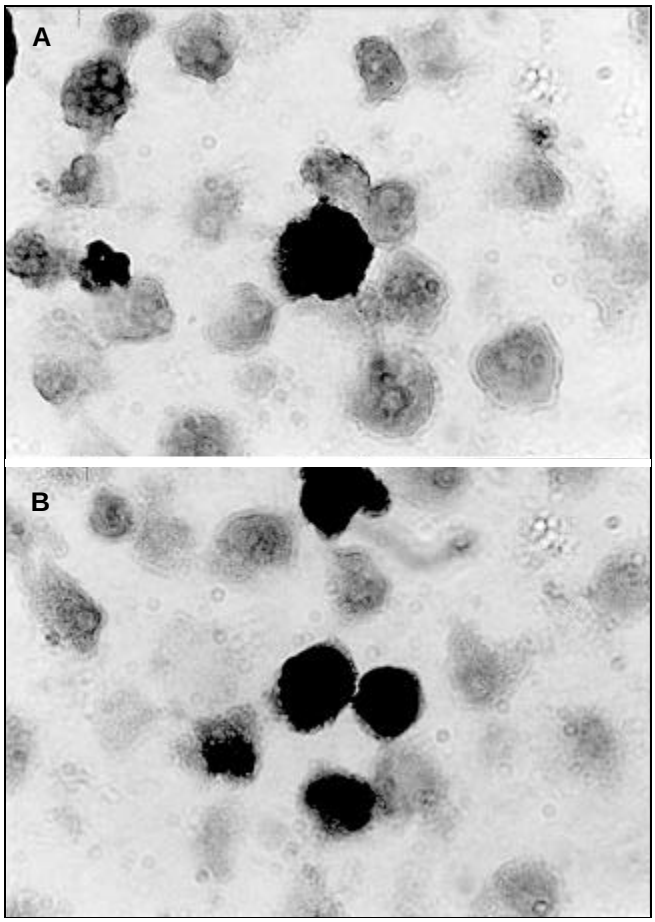

Figure 3. IS-PCR assay for HIV-1 sequences in mixtures of HIV-negative and HIV-positive cells, sealed using nail polish (A) or Self-Seal Reagent (B). The dark red-brown cells are positive for HIV-1. The slides were lightly counterstained with hematoxylin, rendering the HIV-negative cells light gray. (A) Standard amplification solution under a cover glass sealed to the slide with fingernail polish. (B) Standard amplification solution but containing Self-Seal Reagent as the only means to control evaporation from under the cover glass.

amplification reactions containing Self-Seal reagent will not be compromised. The data in Figure 1, A and B, also strongly support the model of action of Self-Seal Reagent discussed above.

Self-Seal Reagent in PCRs

To assess the effect, if any, of Self-Seal Reagent on a standard PCR, amplification reactions were performed with bacteriophage λDNA in the presence or absence of Self-Seal Reagent. Samples were removed after various cycles, held at 70°C for 5 min and analyzed by agarose gel electrophoresis and ethidium bromide fluorescence. All samples were diluted 1:1 and loaded on the gel in 1× Self-Seal Reagent to equalize the slight effect of Self-Seal Reagent on mobility and band appearance. As shown in Figure 2, the presence of Self-Seal Reagent in the PCR (lanes E–H) had little, if any, effect on either the time of first detection of the product or the quantity produced. In addition, there is no detectable change in product appearance, mobility or background levels.

Additional work with seven primer pairs designed for λDNA targets, with predicted annealing temperatures ranging from 47° to 71°C, has shown that Self-Seal reagent had no effect on the broad annealing temperature optima of six of the primer pairs and only a minor effect (<2°C) on the optimum of one of the primer pairs (predicted annealing temperature, 59°C) (data not shown).

In-Situ PCR with Self-Seal Reagent

Data comparing the use of Self-Seal Reagent in the IS-PCR to the traditional slide-cover glass sealing method (fingernail polish) are shown in Figure 3. HIV-positive cells and HIV-negative cells were mixed and prepared on standard microscope slides, and IS-PCR was performed as described above. The slides were sealed either with fingernail polish (3A) or by the inclusion of Self-Seal Reagent in the reaction mixture (3B). After thermal cycling, the slides were processed through the standard hybridization and detection protocols described in Materials and Methods.

The HIV-positive cells in both fields are stained red with the AEC dye. The appropriate ratios of positive cells were observed in both samples. Furthermore, the signal distribution and intensities and the background levels were equivalent in both samples. The essential identity of the two images shows the lack of adverse effects of Self-Seal Reagent on the in situ PCR procedure.

Other In Situ Applications

Self-Seal Reagent also has been used successfully for IS-RT-PCR with murine leukemia virus reverse transcriptase, PRINS and ISH (10).

SUMMARY

The impetus for the development of the Self-Seal Reagent system was the clear need for an easier way to seal slides for IS-PCR other than the traditional method of fingernail polish. We have shown here that Self-Seal Reagent is effective in controlling evaporation, is very easy to use and has little, if any, adverse effect on amplification reactions, either in tubes or on microscope slides. With Self-Seal Reagent there is no need for glues, cements, fingernail polish, disposables, special slides or expensive, dedicated equipment.

As molecular histology techniques become ever more important in research and diagnostics (see, for example, References 4 and 7), methodology and equipment will need to keep pace. We feel that Self-Seal Reagent is a significant improvement over currently available methods of evaporation control.

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