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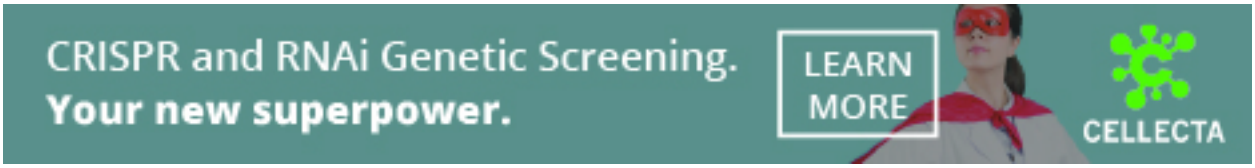

Issues of Variability, Carryover Contamination, and Detection in 3SR-based Assays

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The detection and identification of nucleic acids present at very low levels have been greatly facilitated by the development of in vitro target amplification techniques that exploit the enzyme-mediated processes of DNA replication,[1,2] DNA ligation,[3,4] and RNA transcription. The first reported transcription-based amplification system (TAS) utilized the abilities of avian myeloblastosis virus reverse transcriptase (AMV RT) and T7 RNA polymerase to make multiple RNA copies of target sequences.[5] This strategy, involving a thermocycling step to separate cDNA strands from their RNA templates, has been applied successfully to the detection of human immunodeficiency virus type 1 (HIV-1)-infected cells.[6,7] More recently, isothermal versions of the TAS protocol, known as the self-sustained sequence replication (3SR)[8,9] and nucleic acid sequence-based amplification (NASBA)[10] reactions, have been developed by using the concerted enzymatic activities of *Escherichia coli* RNase H, AMV RT, and T7 RNA polymerase to amplify RNA targets with both high efficiency and specificity. The isothermal characteristic of the 3SR reactions permits the specific amplification of single-stranded RNA target molecules even in the presence of double-stranded DNA molecules containing the same sequence.[9] This ability to target and to amplify RNA specifically using the 3SR method has led to several clinical applications including the detection and characterization of nucleoside[11] and nonnucleoside[12] drug-resistant strains of HIV-1, and the detection of HIV-1 in the plasma of pediatric patients.[13] Importantly, the major reaction product of 3SR reactions is single-stranded RNA that can be directly cloned and sequenced[8] and conveniently detected either by heterogeneous isotopic,[14] nonisotopic,[15] or homogeneous[16] hybridization methods.

Each of these areas of clinical application in which the 3SR reaction has been used has evolved to require more absolute rather than relative quantitation. To achieve this requirement, several critical challenges in current 3SR-based assays need to be addressed. The aspects essential in the establishment of a 3SR quantitative assay are (1) determination of reaction conditions that allow for the least variability, (2) control of potential carryover contamination, and (3) characterization of either heterogeneous or homogeneous detection systems used to quantitate the 3SR RNA products in an assay.

INFLUENCE OF BUFFER CONDITIONS ON 3SR ENZYMES

Initial efforts to develop the 3SR amplification reaction focused on the issue of increasing the sensitivity of the reaction. The use of organic additives and the alteration of the concentration of ribonucleotide triphosphates resulted in an increase of the productivity of the 3SR reaction typically from 10^6-[8] to $\geqslant 10^9$-fold.[17,18] Interestingly, the addition of 15% sorbitol and 10% dimethylsulfoxide (DMSO) enabled the 3SR reaction to proceed in certain amplification reactions without the need of *Escherichia coli* RNase H.[17] In these reactions, the additives presumably enhanced the intrinsic RNase activity of AMV RT to levels otherwise supplied by *E. coli* RNase H. However, in either a two- or three-enzyme protocol, the nature of the 3SR amplification reaction remains a multienzyme concerted reaction. The consequence of failing to coordinate the concerted activities of the reverse transcriptase, RNA polymerase, and RNase H activities is an increase in the variability of the 3SR reactions. Analogously, this has been exemplified for the two-enzyme RT–*Taq* DNA polymerase-mediated PCR reactions in which 2- to 10-fold ranges in reproducibility have been noted.[6,19–21]

Variability in the 3SR reaction can be measured as either the lack of precision or reproducibility. Precision is the measure of agreement in the results of multiple amplification reactions performed repeatedly with a homogeneous target and a consistent pool of amplification reagents (enzymes, buff-

ers, and primers). Reproducibility is the measure of agreement observed in the results of multiple amplification reactions performed repeatedly with heterogeneous target and various preparations of amplification reagents. The lack of agreement in either measurement is usually expressed as the coefficient of variation (cv = S.D. × 100/mean). The precision of the 3SR reaction was measured by targeting a 382-nucleotide region of the third exon of the major immediate early (MIE) mRNA from human cytomegalovirus (HCMV). Using the 3SR reaction conditions described previously,[17] a collection of 5 amplification experiments, totaling 50 3SR reactions (10 amplifications per experiment), was performed on the same day using 0.1 attomole (amole) of a prequantitated 1066-nucleotide RNA transcript[22] encoding the 382-nucleotide amplification region. Of the 50 reactions performed, no 3SR product could be detected using a bead-based sandwich hybridization (BBSH) assay in 26 reactions (Table 1, column I). The cv for this collection of experiments was >200%. Removal of the 26 negative reactions from this data set resulted in a cv of 137%, still indicating a low degree of precision (Table 1, column II). The precision of the heterogeneous BBSH detection assay itself contributed a cv of only 5.6% (Table 1, column VI) to the variability observed in the 3SR amplification experiments. These results and others like these precipitated a search for the causes and solutions for such amplification variability.

Effect of Chloride Salts on the Enzymes in 3SR Reaction

The notion that DNA–protein reactions are highly sensitive to chloride salts has been noted previously in in vitro assays of both *E. coli* RNA polymerase[23] and restriction endonucleases.[23,24] Specifically for 3SR enzymes, the effects of chloride on T7 RNA polymerase activity was measured by Chamberlin and Ring[25] to be highly repressive as compared with its effect on *E. coli* RNA polymerase. Greater than 90% of the activity of T7 RNA polymerase is lost as the concentration of KCl reaches 150 mM, and at concentrations >200 mM KCl there is virtually no T7 RNA polymerase activity. This effect was determined to be a function specifically of the anion concentration because both

TABLE 1 Statistical Analysis of Variability of 3SR Amplification of HCMV–MIE RNA

Assay reaction conditions[a]	I[b]	II[c]	III[d]	IV[e]	V[f]	VI[g]
Mean (fmole/μl)	47.0	98.0	3781.2	3781.2	3781.2	4434.0
Standard deviation	104.2	133.9	1618.2	1216.9	860.8	257.7
Samples	50	24	60	20	10	20
Coefficient of variation (%)	221.6	136.7	42.8	35.3	26.0	5.6

[a]Description of MIE reaction conditions as listed in Ref. 17 and Fig. 1.
[b]CMV–MIE RNA (0.1 amole) was amplified by 3SR in five experiments on 1 day, representing a total of 50 reactions. Reaction conditions were as described in Ref. 17 except that spermidine was omitted and DMSO and sorbitol were present at 5% and 10%, respectively. The 3SR reaction time was 90 min.
[c]This column describes a subset of the data listed in column I representing the 24 reactions that gave a detectable signal by BBSH.
[d]CMV–MIE RNA (0.1 amole) was amplified by 3SR in two sets of 10 reactions per day for 3 consecutive days. Reaction conditions were as described in Fig. 1. This column describes the analysis of the entire data set (60 reactions).
[e]This column describes a subset of the data in column III representing experiments performed on the same day (two sets of 10 reactions).
[f]This column describes a subset of the data in column III representing an individual experiment (10 reactions).
[g]Twenty identical aliquots of 3SR product generated from amplification of the CMV–MIE region were assayed by BBSH.

sodium and ammonium chloride salts at the same concentration as the KCl used produced the same inhibitory effects. In contrast, AMV RT and *E. coli* RNase H have broad tolerances for chloride.[26,27] Consequently, the 3SR reaction can be expected to operate with only a small tolerance in the fluctuation of the chloride concentration based on the sensitivity of T7 RNA polymerase. The total concentration of chloride in the previously published 3SR reaction conditions is 110 mM and includes contributions from the Tris buffer used to dissolve the nucleotides, primers, target, and enzymes.[17] Inadvertent increases in the chloride concentration can be introduced by errors in reagent pipetting, a change in nucleotide reagent stocks, and carryover from the extraction and precipitations of nucleic acids from clinical samples. Sample-to-sample and experiment-to-experiment variations can also influence the precision of 3SR reactions. Therefore, replacement of chloride with an alternative anion in the 3SR was explored as a means of increasing the precision of the 3SR reactions. It was anticipated that an alternative anion would more readily tolerate inadvertent fluctuations of salt in the 3SR reaction.

To explore this hypothesis, the Tris–HCl, $MgCl_2$, and KCl components of the previous buffer conditions[17] were replaced with Tris-acetate, magnesium acetate, and K glutamate (Glu), respectively (Fig. 1). The new buffer conditions were not completely devoid of chloride, because the nucleotide, primers, enzymes, and target RNA solutions were all prepared in Tris-HCl buffer (~10 mM final Cl^- concentration). Titrations of KGlu in a 90-min 3SR amplification of the HCMV–MIE 382-nucleotide region present in the 1066-nucleotide HCMV–MIE RNA transcript were performed. Amplification efficiencies in excess of 10^8-fold were obtained in each reaction utilizing a 25–150 mM range of KGlu concentrations. Optimal efficiencies were observed

FIGURE 1 Amplification Protocol

Stock solutions

5× reaction buffer: 200 mM Tris-acetate (pH 8.1), 150 mM Mg (acetate)$_2$
50 mM DTT, 500 mM KGlu
25 mM rNTPs in 50 mM Tris-HCl (pH 8.1)
25 mM dNTPs in 50 mM Tris-HCl (pH 8.1)

1. Add the following to an autoclaved 1.5-ml Eppendorf tube:
   10 μl of 5× reaction buffer
   2.5 μl of each priming oligonucleotide (0.1 mM each, final)
   2 μl of 25 mM dNTP mix (1 mM final)
   12 μl of 25 mM rNTP mix (6 mM final)
   11 μl of 68.2% sorbitol (15% final)
   5 μl of RNA target (use H_2O for negative control reactions)
   It is recommended that a master mix composed of the 5× buffer, NTPs, primers, and sorbitol be made and a 40-μl aliquot of this mix be pipetted into each reaction tube prior to addition of target.
2. Prepare 3SR enzyme mix. Each reaction requires
   15 units of AMV reverse transcriptase
   1 unit of *E. coli* RNase H
   50 units of T7 RNA polymerase
   Add 40 mM Tris-HCl (pH 8.1), 10 mM DTT for a final volume of 5 μl for the enzyme mix.
5. Heat reaction tubes at 65°C for 5 min to denature the target. Transfer tubes to a heating block at 47°C and incubate for at least 1 min.
6. Add 5 ml of 3SR enzyme mix to each tube and gently flick several times. Incubate at 47°C for 90 min. Store reactions at −20°C.

FIGURE 1 Description of the optimized protocol for 3SR amplification using glutamate/acetate anions. All aqueous solutions were prepared using deionized H_2O treated with 0.1% (vol/vol) diethyl pyrocarbonate to suppress nuclease activity. T7 RNA polymerase was obtained from Stratagene. AMV RT and *E. coli* RNase H were purchased from Boehringer Mannheim. Oligonucleotides were synthesized by phosphoramidite chemistry on Applied Biosystems 394 RNA/DNA synthesizer. KGlu was prepared by adjusting the pH of a 1 M solution of L-glutamic acid to 8.1 with KOH.

using 100 mM KGlu (Fig. 2A). This range of KGlu contrasts sharply with the narrow 0–50 mM range noted for 3SR reaction conditions employing chloride buffers (Fig. 2A).

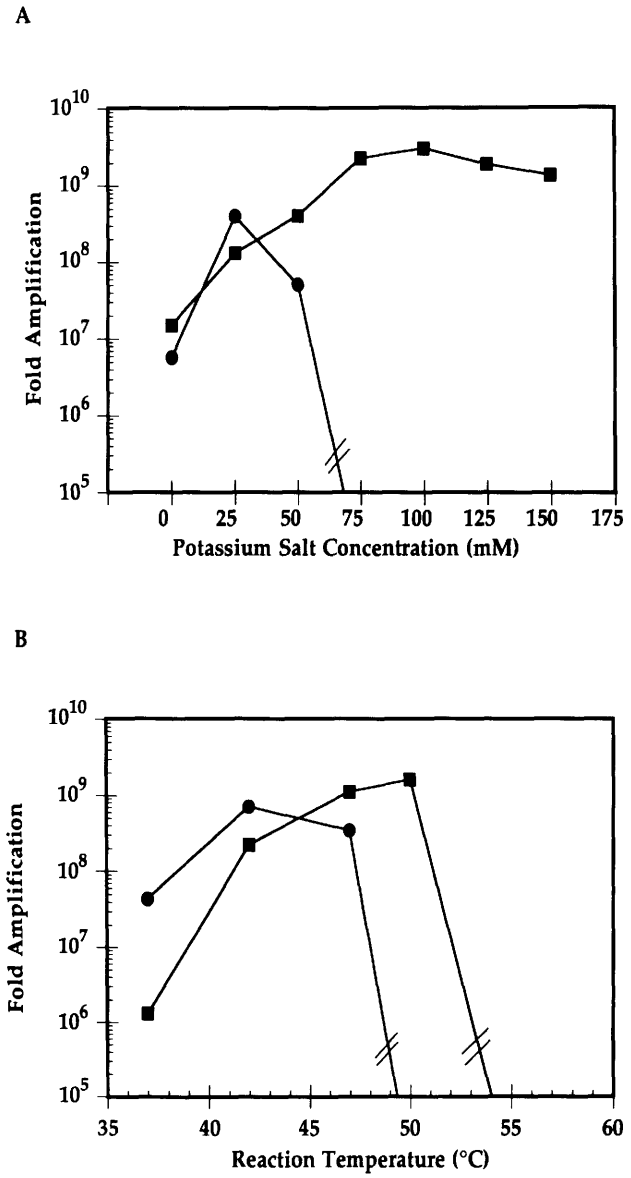

FIGURE 2 (*A*) Titration of potassium salts in the 3SR reaction. Plasmid pACYC–HCMV–*Eco*RI–J, based on pACYC184[28] and containing an *Eco*RI fragment from the genome of the AD 169 strain of CMV was obtained from Dr. Deborah Spector (University of California, San Diego). A 1066-bp *Bgl*II–*Pvu*II fragment from pACYC–HCMV–*Eco*RI–J was cloned into the transcription vector pSP6/T7α19 and transcribed by T7 RNA polymerase to yield a sense RNA product spanning the major immediate early protein (MIE) region of CMV. A 382-base region of 1066-base transcript (0.1 amole) comprising the MIE region of CMV was amplified by 3SR for 90 min with primers 90–635 and 92–135 using a range of KGlu (■) or KCl (●) concentrations. All other reaction conditions were as described in Fig. 1. The reaction products were detected by BBSH using Trisacryl OligoBeads (92–155) and ^{32}P-labeled probe (92–132). (*B*) The effect of reaction temperature on 3SR amplification efficiency under differing buffer conditions. A 382-base region of the CMV 1066-base transcript (0.1 amole) was amplified by 3SR for 90 min with primers 90–635 and 92–135 over a range of temperatures from 37°C to 55°C. Reactions were performed as described in Fig. 1 (■) or by substituting 40 mM Tris-HCl, 30 mM $MgCl_2$, and 20 mM KCl for the corresponding acetate and glutamate salts (●). The reaction products were detected by BBSH using Trisacryl OligoBeads (92–155) and ^{32}P-labeled probe (92–132).

Interestingly, in addition to increasing the salt tolerance of the 3SR reaction, the substitution of chloride with acetate/glutamate in the reaction buffer permitted a shift in the temperature optimum for the 3SR reaction (Fig. 2B). Using the acetate/glutamate buffer, temperatures as high as 50°C could be employed while maintaining amplification levels in excess of 10^9-fold. In these experiments, omission of DMSO from the 3SR buffer was found to be essential to obtain amplification above 45°C (data not shown).

Effects of Glutamate/Acetate Substitution on the Precision of 3SR Reaction

When glutamate/acetate buffers are substituted for chloride buffers and the 3SR reactions are conducted at 42°C, the precision of the 3SR reactions are again characterized by occasional amplification failures (data not shown). However, performing the 3SR reaction at 47°C under the acetate/glutamate buffer conditions has a dramatic effect on the precision of the reaction.

When the 382-nucleotide HCMV–MIE region was amplified in two sets of 10 reactions per day for 3 consecutive days under conditions described in Figure 1, no amplification failures were noted. All 60 reactions produced $>10^8$-fold amplification. The cv of these experiments was reduced to 42.8% (Table 1, column III). When the experiments performed on a single day (two sets of 10 amplification reactions) were analyzed, the cv was reduced to 35% (Table 1, column IV) for both sets of amplification compared with the 222% observed for the single-day experiments performed using the chloride buffers (Table 1, column I). When one set of 10 amplifications was analyzed, a cv of 26% was noted (Table 1, column V). Because 5.6% of this variation is attributable to the BBSH detection method (Table 1, column VI), the net variation compares favorably with that reported recently for RT–PCR amplification assays of HIV-1 RNA performed in competitive[29] and noncompetitive[30] amplification formats.

Although these data refer to the precision of the 3SR reaction, preliminarily data on the reproducibility of the 3SR reaction appear to be similar. Reactions using multiple reagent stocks and with different preparations of targets have been performed without amplification failure. The results of 3SR amplification reactions performed over intervals of 3, 6, and 12 months are being collected and analyzed.

CONTROL OF POTENTIAL CARRYOVER CONTAMINATION

Although the advantages of a 3SR-based amplification assay in terms of excellent sensitivity and specificity are well recognized, its use in routine diagnostic assays can be negated by the potential of carry-over contamination. As with the PCR-based assays,[31] minute amounts of 3SR-amplified products inadvertently transferred to subsequent reactions may be amplified very efficiently, giving rise to false positives. Such an undesirable event can occur through aerosol transfer, contaminated laboratory equipment, and clothing.

In the case of 3SR amplification, both the cDNA and RNA products may act as a source of carryover contamination, making the choice of a suitable sterilization method more complex. Therefore, the design of an effective strategy requires the modification of both the RNA and DNA amplification products to preclude their use as templates in subsequent amplification reactions while ensuring that the modified RNA products can be detected efficiently by hybridization. A postamplification approach using the photoactive agent 4′-aminomethyl-4,5-dimethylpsoralen (IP-10) has been found to modify PCR amplification products.[32,33] More recently, the use of IP-10 has been shown to sterilize 3SR amplicons with a 10^6- to 10^8-fold efficiency.[22] The IP-10-based sterilization method operates by the covalent modification of thymine

and uracil bases of nucleic acids by isopsoralen in the presence of long wavelength UV radiation. An important technical problem encountered in the application of this photochemical strategy was the inhibition of the 3SR amplification process by IP-10. This problem was overcome by segregating the IP-10 from the 3SR reaction by gel encapsulation, as shown in Figure 3. The IP-10 is mixed with low-melting agarose and the gel is solidified in the caps of Eppendorf reaction tubes. The encapsulated IP-10 remains physically separated from the 3SR solution during the amplification reaction. Following amplification, the tubes are heated to 75–80°C to liquefy the gel to enable delivery of IP-10 to the 3SR reaction. A UV-irradiation step completes the sterilization process.

The consequence of IP-10 modification of 3SR amplification products in the hybridization reactions is shown in Figure 4A, in which a 212-base region of the *env* gene of HIV-1 was amplified in the presence of varying amounts of IP-10. The slight decrease in amplification efficiency observed with increasing amounts of IP-10 can be attributed primarily to a decrease in hybridization efficiency. This presumably occurs as a result of chemical modification of the RNA with IP-10. The premise was borne out by the omission of the UV-irradiation step in the presence of 300 μg/ml of IP-10, which resulted in a detection signal equivalent to that of the control reaction containing no IP-10.

The sterilization efficiency of the IP-10 method is exemplified in an experiment in which a 197-nucleotide region of the IEP gene of CMV DNA was amplified and UV-irradiated in the presence of varying amounts of IP-10. In this case, a reverse transcription step was performed prior to the addition of the IP-10-containing gel to the cap of the reaction tube. The amplification products were than quantitated by BBSH, serially diluted, and reamplified with the same primer pair, this time in the absence of IP-10. The results of the reamplification experiment (Fig. 4B) indicated that reamplification of the 3SR product that had contained no IP-10 was very efficient. In contrast, reamplification of the targets that had been photochemically modified was severely inhibited, and the degree of inhibition increased with increasing IP-10 concentration. Even higher levels of sterilization were observed for longer amplicons (300–400 nucleotides),[22] confirming a correlation between the number of reactive sites (thymine/uracil residues) and sterilization efficiency.[32,33]

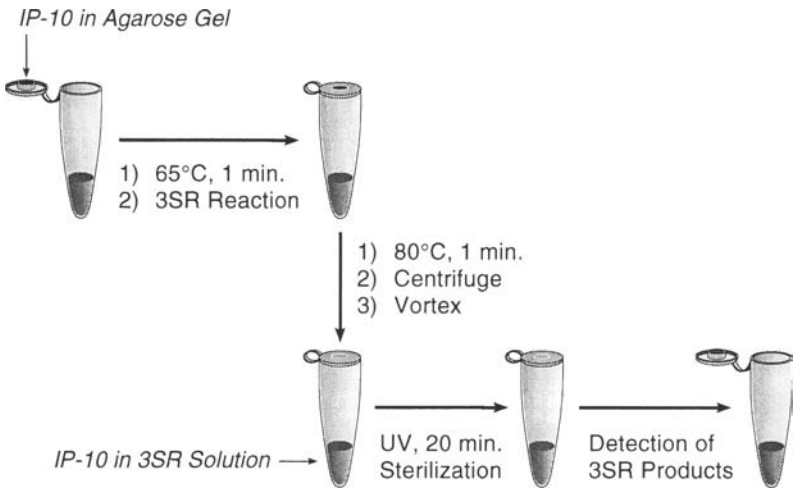

FIGURE 3 Scheme for sterilization of the 3SR reaction using a gel-based delivery of IP-10.

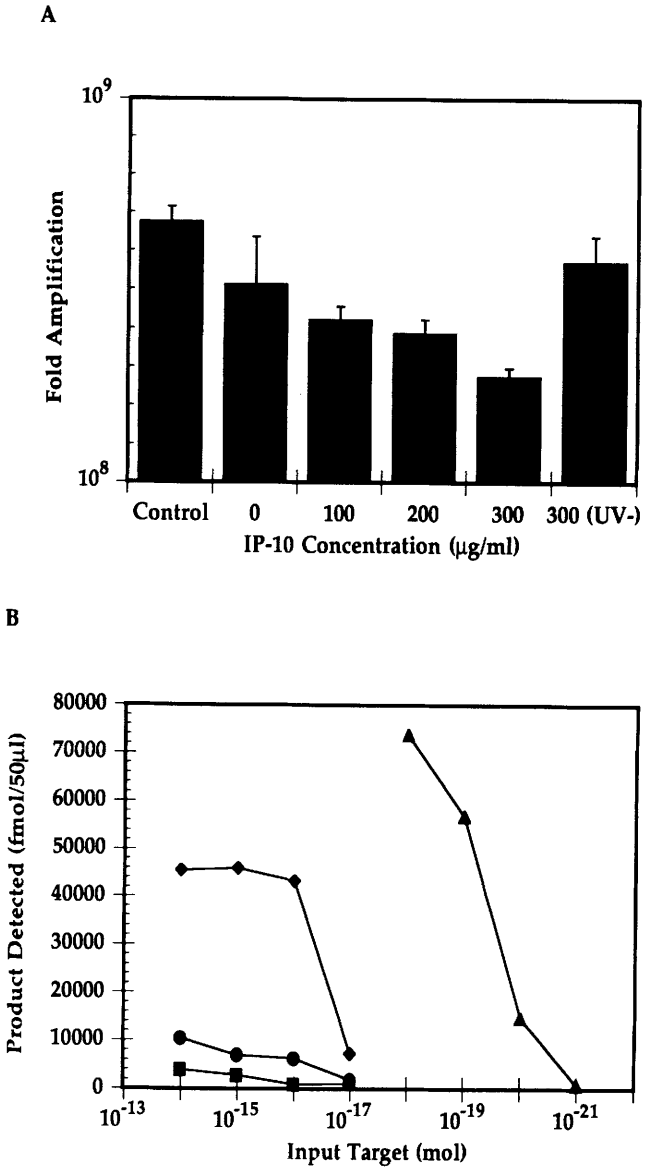

FIGURE 4 (*A*) Effect of 0.5% NuSieve agarose containing IP-10 on 3SR amplification reactions. Ten-microliter aliquots of melted agarose gel containing various amounts of IP-10 were added to the interior of the caps of reaction tubes and allowed to solidify. 3SR reactions were performed in a volume of 50 μl using HIV-1 *env* RNA (0.1 amole) as target and oligonucleotide primers 88–211*/88–347*. The HIV-1 RNA was extracted from CEM cells using the guanidinium thiocyanate/CsCl procedure and quantified by comparative hybridization to predetermined concentrations of pARV7A/2.[5] The control reaction contained no agarose. After completion of the 3SR reaction, all agarose-containing tubes were heated to 80°C for 1 min, vortexed, and centrifuged to give final IP-10 concentrations ranging from 0 to 300 μg/ml. All tubes except the 300 μg/ml (UV) tube were then UV-irradiated for 20 min at 4°C. Antisense reaction products were detected by BBSH with Trisacryl OligoBeads (86–273) and ^{32}P-labeled probe (90–422). (*B*) Relationship between input target and detected product in reamplification of IP-10-treated 3SR products generated with primer pair 90–621/90–626*. Plasmid pACYC–HCMV–*Eco*RI–J, containing the immediate-early promoter-enhancer (IEP) gene of CMV, was used as a double-stranded DNA target (0.1 amole). Irradiated products from 3SR reactions containing 0 (▲), 100 (◆), 200 (●), and 300 (■) μg/ml of IP-10 were diluted in TE and amplified with primer pair 90–621/90–626* in the absence of IP-10 and NuSieve agarose gel. Amplified products were detected by BBSH with Trisacryl OligoBeads (92–212) and ^{32}P-labeled probe (92–214).

CHARACTERIZATION OF HOMOGENEOUS AND HETEROGENEOUS DETECTION OF 3SR PRODUCTS

The products from 3SR reactions are predominantly single-stranded RNA and are amenable to detection by a variety of hybridization methods. The current

assays are predominantly based on heterogeneous hybridization, where the 3SR product is captured on a solid support and detected with a labeled oligonucleotide probe in a sandwich format (Fig. 5A). The capture of the 3SR product is accomplished by hybridization using a complementary oligonucleotide that is end-attached to a solid support such as polyacrylamide, polystyrene, or glass. Hybridization of the 3SR product to a second labeled nucleic acid confers an additional level of sequence specificity to the assay. To date, probes labeled with ^{32}P,[8,9,11,14,17,18,22] alkaline phosphatase,[34,35] and lanthanide chelates[13,15] have been employed to detect 3SR products by heterogeneous hybridization. For detection with ^{32}P-labeled probes, the macroporous Trisacryl resin has been the solid support of choice. The combination of the use of ^{32}P-labeled probe, covalent attachment of the capture oligonucleotides through their 5′ ends, and chemical modification of the surface of the support with anionic groups to reduce nonspecific binding has resulted in detection sensitivities of 0.1–0.5 femtomole (fmole) or 5×10^7 molecules of 3SR-generated RNA products. The dynamic range of this sandwich hybridization assay is a function of probe concentration and the level of nonspecific background. Typically, 0.5–50 fmoles of analyte is detected when 100 fmoles of ^{32}P-labeled probe is used. This sensitivity is a result of a 40–60% efficiency of the BBSH system. When used in conjunction with 3SR amplification, the BBSH system is easily capable of detecting a single HIV-1-infected cell in a background of 10^6 uninfected cells.[8,18]

Although heterogeneous assays afford useful sensitivity, it is difficult to automate the centrifugation and separation steps inherent in this type of detection. Furthermore, hybridization kinetics may be compromised to some extent by steric interference from the solid support. These disadvantages can be overcome by using a homogeneous format that is able to discriminate between hybridized and unhybridized probe. One such method is transient-state polarized fluorescence (TSPF), which has recently been applied to detect 3SR-generated RNA products.[16] The principle of the method is based on the increase in polarized fluorescence resulting from longer rotational relaxation times when a fluorophor-labeled probe hybridizes to its complementary target sequence (Fig. 5B). Fluorescent probes were synthesized by attaching the phthalocyanine dye La Jolla Blue to the 5′ end of an oligonucleotide using a

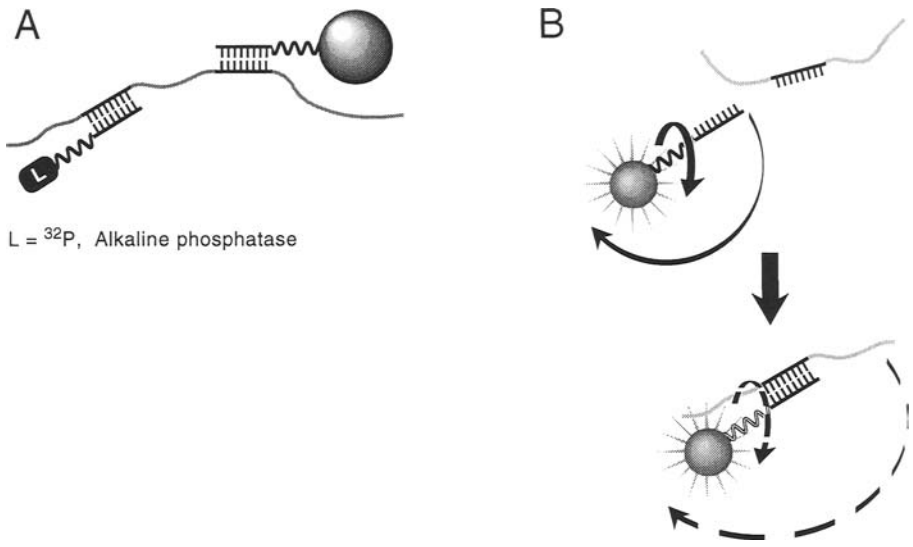

FIGURE 5 (*A*) Scheme for BBSH using radioisotopic or enzyme labels. (*B*) Scheme for detection of 3SR product using TSPF. Following duplex formation, the rotational freedom of the fluorophor label is restricted, resulting in an increase in fluorescence polarization.

flexible alkyl linker. This fluorophor has the advantages of a high molar absorption coefficient and low nonspecific binding characteristics by virtue of its axial polyethylene glycol (PEG) ligands. Furthermore, its emission maximum in the near infrared region (705 nm) ensures minimal interference from background fluorescence of biological molecules. When combined with transient-state detection using single photon counting, the TSPF method is capable of detecting nucleic acids with a sensitivity equivalent to that of the ^{32}P-based heterogeneous assay described above. The performance of these detection systems was compared by assaying a 3SR product generated by amplification of a 382-nucleotide region of the *env* gene of HIV-1. The sensitivity of TSPF is demonstrated in Figure 6A, in which the same concentra-

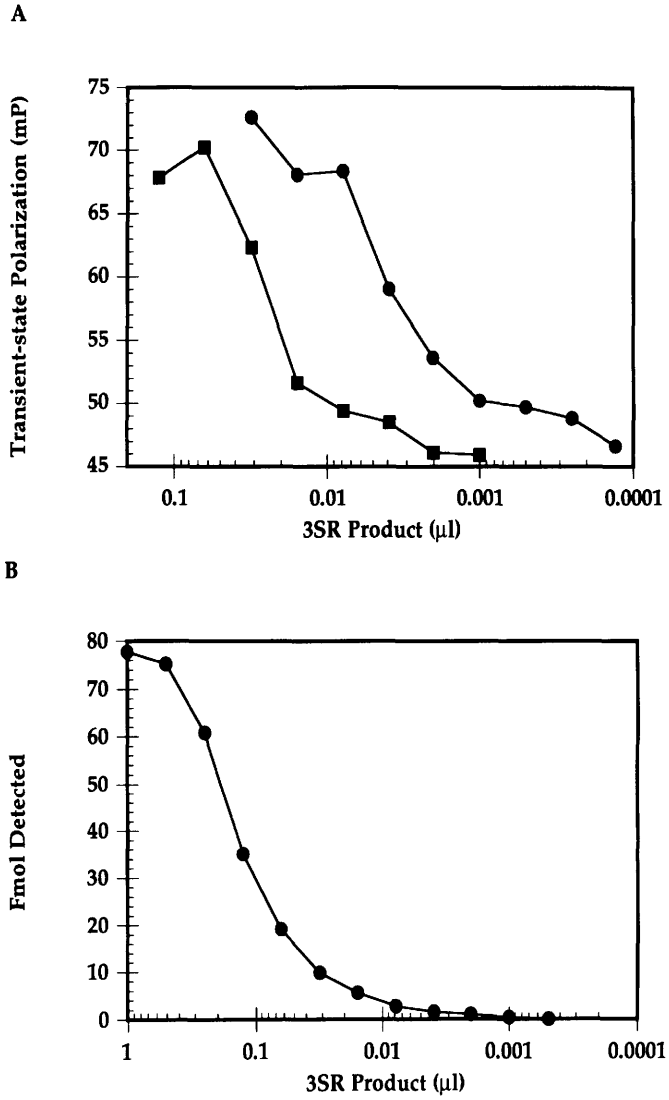

FIGURE 6 (*A*) Sensitivity for TSPF detection of 3SR RNA product with different La Jolla Blue oligonucleotide 90–422 conjugate probe concentrations. Ten fmoles (■) and 0.2 fmole (●) of La Jolla Blue oligonucleotide 90–422 conjugate probe were used in hybridization reactions with 3SR-generated RNA target. The 3SR product was obtained by amplifying HIV-1 RNA with the *env*-specific primer pair 89–255/89–263* to give a 382-base antisense RNA product. A stock solution of this 3SR product (1000 fmoles/µl) was serially diluted to deliver varying amounts of target in the hybridization reactions. Ten microliters of these diluted stock solutions was assayed by TSPF as described previously.[16] (*B*) ^{32}P-Based detection of 3SR product by BBSH. ^{32}P-Labeled oligonucleotide (90–422) and Trisacryl OligoBeads (86–273) were used to detect serial dilutions of the stock solution (1000 fmoles/µl) of 3SR-generated RNA product as described previously.[6,14]

tion range of the 3SR product used in the BBSH method (Fig. 6B) was assayed in a simple "mix-and-read" format.[16] The high fluorescence intensities (>100,000 photon counts/20 sec for 0.2 fmole of probe) obtained in these experiments ensures good precision for the detection measurements. One consideration of the TSPF assay is the relatively poor hybridization efficiency of the 3SR products and the fluorescent probes, requiring 3SR product concentrations considerably higher than that of the probe to drive the reaction to completion. The dependence of the rate of hybrid formation on the concentrations of the RNA target and probe is likely to be the governing factor in determining the ultimate sensitivity of this technique.

CONCLUSION

A 3SR-based assay, like PCR and ligase chain reaction (LCR) assays, is ultimately composed of three components: sample preparation to extract the target nucleic acid of interest, target amplification, and hybridization-detection of the amplified nucleic acid products. This study focused on the developments affecting the last two components of the assay. The influence of sample preparation on the subsequent steps can be profound. The critical feature of sample preparation technologies is the liberation of target nucleic acid with a high degree of reproducibility and efficiency. In addition, the integrity of the target nucleic acid must be appropriate for the target amplification step. A reproducible quantifiable sample preparation protocol is thus an essential requirement for a quantitative clinical assay.

Although the use of additives and buffers allows the 3SR enzymes to function at 50°C, the current reaction is limited to amplifying regions that are <800 nucleotides in length. This constraint can presumably be removed with the discovery of thermostable variants of the 3SR enzymes. It is anticipated that reactions run at more elevated temperatures will alleviate the problem of secondary structure of RNA targets as well as afford greater specificity of the 3SR reaction. A potential application for 3SR amplification of longer target regions is in coupled transcription–translation systems for the expression of functional gene products. An example of such a coupled system has been described recently using a 3SR-like protocol with an in vitro translation system for the simultaneous amplification and expression of brome mosaic virus genomic RNA.[36]

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