# QUICK BLOTS AND NONRADIOACTIVE DETECTION OF DNA PROBES FOR THE IDENTIFICATION OF MOSQUITOES

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ABSTRACT. The quick blot protocol is an improved technique for preparing crude insect homogenates for hybridization to nucleic acid probes. Individual insects are ground in wells of a microtiter plate and transferred to a dot blot manifold. This allows preparation of multiple filters and provides uniformity and an orderly arrangement of samples. The high background detection resulting from use of crude insect homogenates with nonradioactive detection systems was eliminated by incubating quick blot filters in a laundry stain remover containing proteases. We used mosquito species-specific DNA probes to demonstrate the effectiveness of nonradioactive DNA labeling systems with quick blots.

## INTRODUCTION

DNA probes have great potential for the detection of pathogens (Kirkpatrick et al. 1987) and the identification of cryptic species of mosquitoes (Cockburn 1990, Collins et al. 1988). In many ways DNA probes are well adapted to field conditions, since they can be used with crude samples, such as mosquitoes squashed on filter paper (Cockburn 1990), and the state of preservation is not critical. DNA probe detection is roughly comparable to ELISA in difficulty, and ELISA kits are becoming popular for identifying blood meals and detecting parasites. However, DNA probes have not yet become a common tool in applied entomology.

There are 2 major problems with existing DNA probe techniques. First, only one (Kirkpatrick et al. 1987) or two (Cockburn 1990) blots can be made with any group of insects, making it difficult to use multiple probes. For example, with the *Anopheles gambiae* complex, one might want to probe with 4 mosquito species-specific probes and also probes for *Plasmodium* species. This necessitates reprobing of filters, which greatly increases the length of time required to obtain complete results and can cause technical difficulties (Hill et al. 1991). Second, radioactive labeling has been used to detect the hybridized DNA. This is a sensitive method for DNA detection, but it requires a special laboratory for handling the radioactive label and makes field kits impossible.

Nonradioactive DNA labeling and detection kits (similar to ELISA kits) are commercially available, but nonspecific signals with crude insect homogenates make these kits of limited use (Cooper et al. 1991, Hill et al. 1991). In this report, we summarize our recent success in adapting commercial nonradioactive techniques to crushed mosquitoes.

## MATERIALS AND METHODS

*Species of mosquitoes:* Specimens of the following mosquito species were used: *Aedes taeniorhynchus* Wied., *Anopheles albimanus* Wied., *Anopheles quadrimaculatus* species A (ORLANDO strain) and *Culex nigripalpus* Theobald.

*Species-specific probe:* Cockburn (1990) reported the isolation of a bacteriophage clone, Arp2, containing a species-specific repetitive sequence from *An. quadrimaculatus* sp. A. Clone Arp2 hybridized intensely to squash blots of species A individuals, less intensely to *Anopheles* sp. B individuals, and not at all to *Anopheles* sp. C or D individuals. We have subcloned portions of the Arp2 insert to form the plasmid pKA2, which provides specific detection of species A equivalent to Arp2. On squash blots the radioactively labeled subclone does not hybridize significantly to *An. albimanus*, *Cx. nigripalpus*, or *Ae. taeniorhynchus*.

*Quick blots:* Mosquitoes (larvae, pupae or adults) were placed individually into the wells of a 96-well microtiter plate. Denaturing solution (0.5 M NaOH, 1.5 M NaCl) was then added. For standard sized wells (10 mm deep, 13 mm diam), a maximum of 200 µl per well of denaturing solution was used. The mosquitoes were thoroughly ground with a Replaclone® (96 prong model, L.A.O. Enterprises, Gaithersburg, MD)[3] for about 3 minutes. The plate was incubated for 30 min at room temperature, and then neu-

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[3] Names of commercial products are necessary to report factually on available data; however, the USDA neither guarantees nor warrants the standard of the product, and the use of the name by USDA implies no approval of the product to the exclusion of others that may also be suitable.

tralization solution (3 N sodium acetate, 2 N acetic acid) was added (one-fourth the volume of denaturing solution) and mixed thoroughly in the sample wells using the Replaclone.

A dot blot manifold was used according to the manufacturer's (Schleicher and Schuell, Keene, NH) recommendations, except that the larger chunks of tissue and cuticle were filtered through a wet laboratory tissue that was placed over the wet membrane filter (nitrocellulose or nylon). A low level of vacuum was applied, and samples from the microtiter plate were applied to the dot blot manifold wells. After aspiration, 350 μl of 2× SSPE (1× SSPE: 0.18 M NaCl, 10 mM $NaH_2PO_4$, 1 mM EDTA, pH 7.4) was used to complete washing of each well. Several equivalent filters were prepared in the dot blot manifold, until the total sample volume had been used.

*Squash blots and dot blots:* Previously published methods were used for hybridization of mosquito species-specific DNA probes to squash blots (Cockburn 1990) and dot blots (Costanzi and Gillespie 1987).

*DNA isolation and labeling:* Plasmids were prepared by a modification of the alkaline-lysis method of Birnboim and Doly (1979) and cesium chloride purification, or by the boiling method (Holmes and Quigley 1981). Insect genomic DNA was prepared by the method of Cockburn and Seawright (1988). DNA was radiolabeled by a nick translation kit (BRL®) with $^{32}$P-dCTP, and unincorporated label was removed by size exclusion chromatography using Bio-Gel P-60 (BioRad, Richmond, CA).

Unless otherwise noted, filters were subjected to the following treatments after application of the target DNA. Prior to prehybridization, nitrocellulose filters were baked for 20–45 min at 80°C under vacuum (vacuum-baked), and nylon filters were subjected to treatment with 300 nm ultraviolet (UV) light. Filters were prehybridized in 1% nonfat dry milk (NFDM), 0.2% SDS at 55°C for at least 30 min, and hybridized with (denatured) probe in 30% formamide, 5× SSPE, 1% NFDM, 0.2% SDS at 55°C overnight.

*Preparation and use of biotinylated probes:* The preparation of biotinylated probes was achieved by nick translation of double-stranded template DNA for the incorporation of biotinylated nucleotides. The BRL Nick Translation System® (BRL, Gaithersburg, MD) reagents were used, according to the recommendations for the Biotin-21-dUTP Labeling System® (Clontech Laboratories, Palo Alto, CA). Unincorporated nucleotides were removed by gel exclusion chromatography. Detection of hybridized biotinylated probes was accomplished with streptavidin-alkaline phosphatase conjugate (SA-AP) according to the directions in the GENE-TECT® protocol (Clontech Laboratories, Palo Alto, CA).

*Preparation and use of ECL probes:* The directions supplied by the manufacturer of the ECL Kit® (Amersham, Arlington Heights, IL) were followed in the preparation and use of ECL probes, including the prehybridization and hybridization steps, except that SSPE was substituted for SSC in the wash solutions. The labeled probe was stored in 50% glycerol at −20°C until used. The supplied hybridization solution was used for both prehybridization (at least 10 min at 40–42°C) and hybridization (overnight at 40–42°C) after adding NaCl to 0.5 M.

*Preparation and use of digoxygenin labeled probes:* The directions supplied by the manufacturer of the Genius Kit® (Genius Nonradioactive DNA Labeling and Detection Kit, Boehringer Mannheim Biochemicals, Indianapolis, IN) were followed in the preparation and use of Genius probes, except that labeled probes were precipitated with NaCl rather than LiCl, and SSPE was substituted for SSC in the hybridization and detection steps. Prehybridization and hybridization conditions were adjusted to 50% formamide and 42°C. Genius probes were prepared by the random primed incorporation of digoxygenin-tagged nucleotides, and detected by immunoassay. The probe DNA was stored at −20°C until used in a hybridization reaction.

## RESULTS

*Experimental design:* This work focused on reducing the technical problems involved in using nonradioactively labeled DNA probes with crude mosquito homogenates. In this paper we will use "background" to refer to signals in areas where no sample was applied, "nonspecific detection" to denote signals in areas where nonhomologous sample was applied, and "hybridization" to describe binding of probe to homologous DNA sequences. Nonspecific detection has been a persistent problem with nonradioactive detection (Hill et al. 1991, Cooper et al. 1991; also see below). We used nonhomologous probes that did not hybridize at all to the test mosquitoes to focus on nonspecific detection. Therefore any signals were entirely spurious and not due to low levels of authentic hybridization. To generalize our results, we tested one species each of the 3 major genera of mosquitoes (*Anopheles albimanus*, *Culex nigripalpus*, and *Aedes taeniorhynchus*). To ensure that any procedures that we developed did not simply eliminate all signals, we included *An. quadrimaculatus* sp. A mosquitoes as a positive control using the probe pKA2 (Cockburn 1990).

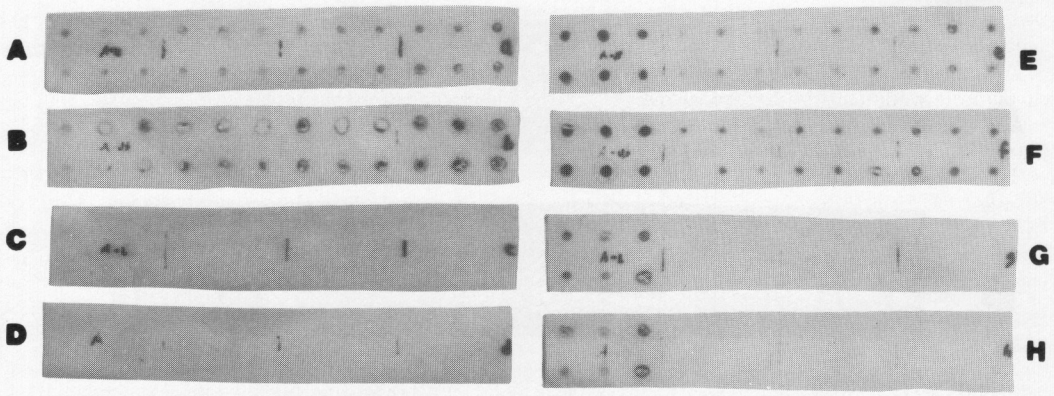


Fig. 1. Effects of various quick blot post-application treatments. Quick blots were prepared using nitrocellulose filters, with each spot receiving 0.1 of the solution from a single mosquito maceration. Each set of 6 (2 rows of 3) spots contained DNA from different individuals of a single mosquito species. Each filter received 4 sets of spots: *An. quadrimaculatus* species A; *An. albimanus*; *Cx. nigripalpus* and *Ae. taeniorhynchus*. Filters were treated between target DNA application and prehybridization at room temperature for 45 min as follows: filters A and E, no post-application wash; filters B and F, post-application wash of urea-SDS (8M urea, 10% SDS); filters C and G, post-application wash of 10% laundry stain remover; filters D and H post-application wash of urea-SDS-stain remover (8M urea, 10% SDS, 10% stain remover). Filters were prehybridized and hybridized without (filters A–D) or with (filters E–H) biotinylated probe pKA2. Detection was according to the GENETECT protocol (Clontech Laboratories), using BRL reagents, except that NFDM was substituted for BSA and SSPE was substituted for SSC.

*Multiple targets using the quick blot procedure:* Previous methods used to squash insects on membrane filters for DNA hybridization were simple, but only gave one or 2 filters from each batch of insects. This number was insufficient for many applications; e.g., identifying the 4 species of the *An. quadrimaculatus* complex requires 3 probes (Cockburn 1990). Squash blots are often hard to interpret due to cross-contamination between individuals and variation in size and shape of signals.

To solve these problems we developed the quick blot procedure. The adaptation of the dot blot manifold provided uniform sample application areas and allowed the use of batch processing techniques. In the first attempts at developing the quick blot protocol, the filters (nitrocellulose and nylon) were probed with radiolabeled pKA2. The new technique was a great improvement over the squash blot method, primarily because of the uniformity of sample application to filters and rapidity of batch processing. There was variation in signal intensity (similar to that seen previously in squash blots) between quick blot spots, but this was reproducible between spots on separate filters that received the same homogenates. The variation was probably due either to variation in the amount of DNA released or to different numbers of target repetitive DNA sequences in the genomes of individual mosquitoes. There also tended to be a concentration of signal in the center of the dot area where samples were applied.

*Nonradioactive detection:* We initially tested 2 methods for nonradioactive labeling and detection of DNA. Using purified mosquito genomic DNA as target and probe, we could detect dot blots using either SA-AP or ECL labeling and detection. High backgrounds seen with nylon filters were reduced by substituting nitrocellulose filters, without sacrificing sensitivity. Despite this success with purified genomic DNA, the SA-AP detection protocol did not work well with DNA probes (Fig. 1A, 1E). Signals were present in the controls with no DNA probes. The nonspecific detection could be caused either by residual streptavidin-binding substance (perhaps biotin bound to protein) or phosphatase activity in the target areas on the filters. The ECL detection protocol was also not useful due to nonspecific detection. The production of signals in the absence of hybridized probes suggested that residual peroxidase activity from the squashed mosquitoes contributed to nonspecific detection.

*Elimination of nonspecific signals:* Several treatments were evaluated to eliminate or minimize nonspecific signals. We washed squash blots for 30 min at room temperature in the following solutions: a) 10% SDS, b) 8 M urea, c) 0.5 M HCl, d) 10% meat tenderizer in 1× SSPE, e) 10% laundry stain remover with protease (LA FRANCE, Dial Corporation, Phoenix, AZ), and f) 8 M urea followed by 10% SDS. A set of treated squash blots were used for the SA-AP detection protocol. The washing step was

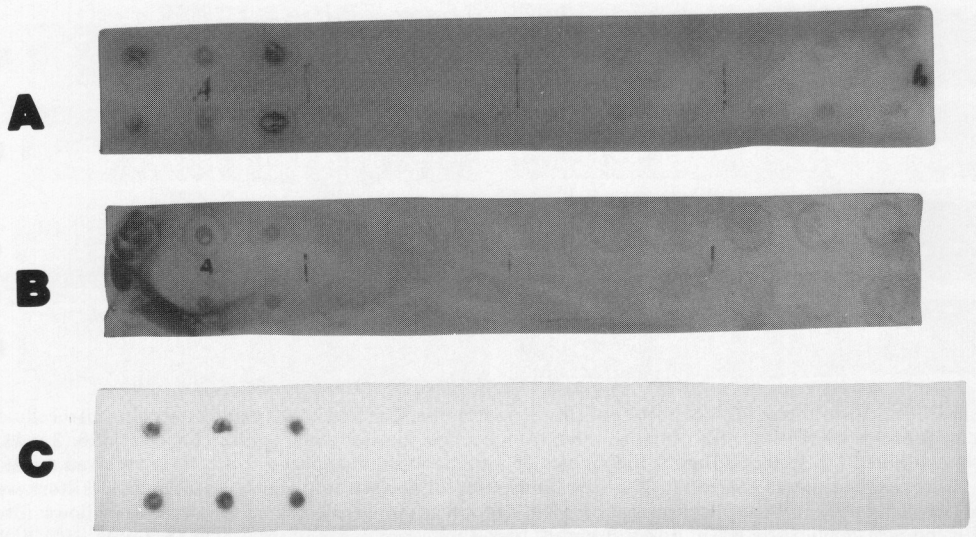


Fig. 2. Three nonradioactive DNA detection systems used with quick blots. All 3 nitrocellulose filters received the urea-SDS-stain remover post-application treatment. Filter A, SA-AP detection; filter B, Genius detection; filter C, ECL detection. Probe was pKA2. See Fig. 1 for sample order.

followed directly by the detection steps, with the prehybridization and hybridization steps omitted, so no signals should have been present. High background levels were observed on most of the nylon filters, and most of the nitrocellulose filters showed low background. The nylon and nitrocellulose filters which received the stain remover treatments showed negligible signals.

When the ECL detection protocol was applied to an equivalent set of squash blots, all the washes were found to be useful in greatly reducing background levels. However, nonspecific detection was still observed in some cases. The lowest nonspecific signals occurred on nylon filters which received the urea and the urea/SDS treatments, and on nitrocellulose filters which received the laundry stain remover or urea/SDS treatments. Nitrocellulose squash blots treated with laundry stain remover could therefore be used with DNA probes to provide specific detection of mosquitoes with either the SA-AP or the ECL systems. Since the post-application treatments of squash blots were effective in lowering nonspecific signals with the ECL and SA-AP detection systems, we tried similar treatments to improve nonradioactive detection of quick blots (Fig. 1). Various post-application washes of the filters were performed in hopes of reducing the level of nonspecific detection, and a nonradioactive probe was hybridized to some of the filters. This probe was omitted from one set of filters to distinguish specific from nonspecific signals. Washes with laundry stain remover containing protease were effective at improving the specificity of detection (Fig. 1C, D, G and H). Even though specific signal strengths were decreased somewhat by the use of the laundry stain remover, the overall effects were valuable due to a dramatic reduction in nonspecific detection caused by the stain remover wash (compare filters E and F with filters G and H in Fig. 1).

The results of using the 3 different nonradioactive detection systems with quick blots are shown in Fig. 2. We obtained reliable specific detection using the ECL and SA-AP systems, but results using the Genius system were quite variable. Figure 2B is typical of our results with the Genius system, as we often observed a patchy distribution of high background which interfered with interpretation of the results.

Dot blots were used with these same post-application and prehybridization treatments to obtain an estimate of the sensitivity of detection. Detection levels were in the 1–10 ng range for ECL, SA-AP and autoradiographic detection.

The effects of using different filter types and DNA binding conditions or methods on SA-AP detection are shown in Fig. 3. Ultraviolet fixation of nylon filters did not affect the detection levels (Fig. 3B and C) with quick blots. A degradation of specificity was seen when alkaline binding was used in preparing a quick blot with a nylon filter (Fig. 3A).

## DISCUSSION

The quick blot protocol is particularly suitable for the preparation of sets of nucleic acid sam-

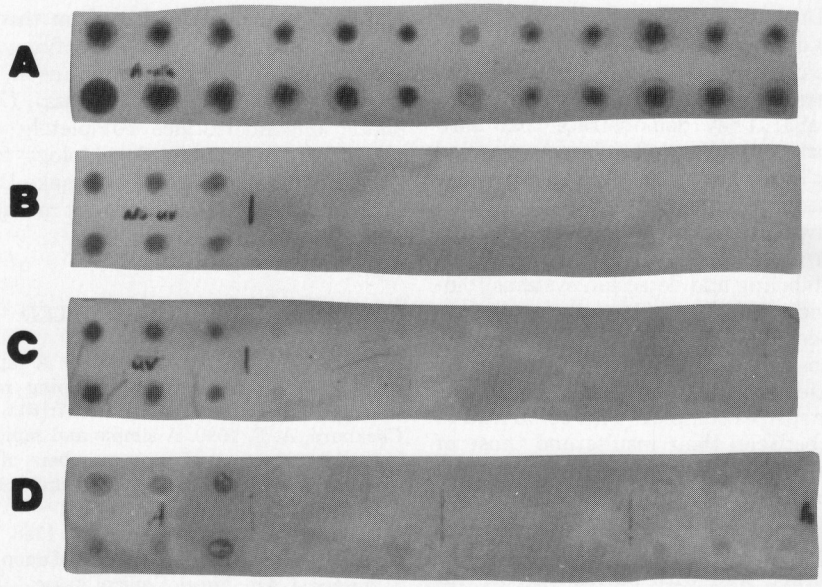


Fig. 3. Effects of filter type and DNA-binding conditions. Filter A (nitrocellulose) was prepared according to the normal quick blot protocol, except that the samples in the denaturing buffer were applied to the blotting filter without being mixed with neutralization buffer. Filters B and C (nylon) were prepared according to the normal quick blot protocol, except that no vacuum-baking step was performed, and filter C was treated with UV light after sample application. Filter D (nitrocellulose) was prepared according to the unmodified quick blot protocol. See Fig. 1 for sample order and abbreviations. All 4 filters were subjected to the urea-SDS-stain remover post-application treatment before prehybridization and hybridization with biotinylated pKA2 probe. Detection was as in Fig. 1.

ples from insects in the 1–20 mg size range, or body parts and isolated tissues from larger individuals. The improvement in signal quality and the possibility of making multiple filters make it a conspicuous improvement over the squash blot method. The signals from quick blots are of uniform size (about 5 mm) and arranged in a perfect grid, with most of the signal concentrated in a central 2 mm dot. With the squash-blot method, the signals are irregular splotches from 10 to 15 mm in diameter (Cockburn 1990). The regularity of the signal makes interpretation easier, so the sensitivity of a quick blot is higher than that of an equivalent squash blot. This could be important for detection of pathogens, since the amount of target DNA can be very low.

The main advantages that the quick blot protocol offers over methods used previously include: 1) minimal cost, because the use of specialty chemicals and complex equipment is minimized; 2) convenience, since all of the equipment, DNA labeling, and detection kits are all commercially available and do not rely on the use of radioisotopes; 3) speed, as quick blots are extraordinarily fast to prepare compared with isolation of purified DNA for dot blots; 4) sensitivity and ease of interpretation, because the uniformity, size, and orderly arrangement of samples on the filters make interpretation simpler than squash blots; and 5) multiple filters, because the evaluation of several DNA probes or ELISA kits for protein antibodies can be done on each insect.

Use of DNA probes with crude insect homogenates is hampered by the presence of large amounts of protein, which can lead to nonspecific binding of DNA probes. It has previously been shown (Sim et al. 1989) that the use of chitinase and proteinase K can greatly reduce nonspecific detection. This problem is much worse when using nonradioactive DNA labeling and detection systems, which rely on enzymatic detection of probe binding (Hill et al. 1991, Cooper et al. 1991). Crude homogenates can have residual enzymatic activity, leading to high levels of detection even in the absence of probe. We have shown that the use of expensive enzymes such as chitinase or proteinase K is unnecessary. Chitin can be excluded by the use of a laboratory tissue as a filter, and the protein can be digested with the inexpensive proteinase in a laundry stain remover.

Since this work was completed, two reports have appeared discussing the use of nonradioactive labeling and detection systems with species-

specific DNA probes from mosquitoes. Cooper et al. (1991) discuss the use of biotin/streptavidin/alkaline phosphatase and ECL with probes for 3 members of the *An. farauti* species complex from Australia. They demonstrate that both methods work well when hybridized to purified DNA on dot blots, but that both give high nonspecific detection when hybridized to squash blots of individual mosquitoes. Hill et al. (1991) conducted a more extensive analysis of 4 nonradioactive labeling and detection systems (biotin/streptavidin/alkaline phosphatase, digoxygenin/antibody, ECL, and an ECl-like kit using alkaline phosphatase). They used a synthetic oligonucleotide specific for several species of the *An. gambiae* species complex. This could lead to differences between their results and those of Cooper et al. (1991) and this paper, since the chemistry involved in coupling labels to oligonucleotides is different from that used with cloned probes. Hill et al. (1991) found that all 4 methods worked well with purified DNA, but both biotin/streptavidin/alkaline phosphatase and digoxygenin/antibody gave nonspecific detection with squash blots. They found that the other 2 kits gave correct signals, which probably indicates that the signals with the *An. gambiae* probe are stronger than those with *An. quadrimaculatus* or the *An. farauti* probes relative to the nonspecific signals. Hill et al. (1991) speculate that the lack of specificity with the streptavidin/alkaline phosphatase system was due to binding of the enzyme complex to residual biotin, but we have demonstrated that unprocessed mosquito homogenates contain both alkaline phosphatase and peroxidase activity which cause the nonspecific detection (data not shown). They also noted that 2 of the kits could not be used to reprobe filters. In summary, these 2 papers demonstrate the need for a method of reducing nonspecific detection with nonradioactive probes hybridized to crude insect homogenates and of producing multiple filters from a group of mosquitoes.

Part of the Arp2 clone has been sequenced which led to the identification of species-specific sequences (Johnson 1990[4]). Radiolabeled synthetic oligonucleotides based on this DNA sequence were effective at identifying individual mosquitoes on quick blots (Johnson, Cockburn and Seawright, unpublished data). Use of synthetic oligonucleotides completely eliminates the need for a molecular biology facility for production of probes and will make DNA-based identification practical for most mosquito workers (Hill et al. 1991).

[4] Johnson, D. W. 1990. Quick blots and nonradioactive detection systems: improvements on methods for DNA hybridizations using mosquitoes. Ph.D. Dissertation, University of Florida.

## REFERENCES CITED

Birnboim, H. C. and J. Doly. 1979. A rapid alkaline extraction procedure for screening recombinant bacterial DNA. Nucl. Acids Res. 7:1513–1523.

Cockburn, A. F. 1990. A simple and rapid technique for identification of large numbers of individual mosquitoes using DNA hybridization. Arch. Insect Biochem. Physiol. 14:191–199.

Cockburn, A. F. and J. A. Seawright. 1988. Techniques for mitochondrial DNA analysis of anopheline mosquitoes. J. Am. Mosq. Control Assoc. 2:261–265.

Collins, F. H., P. C. Mehaffey, M. O. Rasmussen, A. D. Brandling-Bennett, J. S. Odera and V. Finnerty. 1988. Comparison of DNA-probe and isoenzyme methods for differentiating *Anopheles gambiae* and *Anopheles arabiensis* (Diptera: Culicidae). J. Med. Entomol. 25:116–120.

Cooper, L., R. D. Cooper and T. R. Burkot. 1991. The *Anopheles punctilatus* complex: DNA probes for identifying the Australian species using isotopic, chromogenic, and chemiluminescence detection systems. Exp. Parasitol. 73:27–35.

Costanzi, C. and D. Gillespie. 1987. Fast Blots: immobilization of DNA and RNA from cells, pp. 582–587. *In*: S. L. Berger and A. R. Kimmel (eds.), Guide to molecular cloning techniques. Academic Press, San Diego.

Hill, S. M., R. Urwin and J. M. Crampton. 1991. A comparison of non-radioactive labeling and detection systems with synthetic oligonucleotide probes for the species identification of mosquitoes in the *Anopheles gambiae* complex. Am. J. Trop. Med. Hyg. 44:609–622.

Holmes, D. S. and M. Quigley. 1981. A rapid boiling method for the preparation of bacterial plasmids. Anal. Biochem. 114:193–197.

Kirkpatrick, B. C., D. C. Stenger, T. J. Morris and A. H. Purcell. 1987. Cloning and detection of DNA from a nonculturable plant pathogenic mycoplasma-like organism. Science 238:197–200.

Sim, B. K. L., P. Romans and S. Harun. 1989. Use of chitinase to facilitate detection of protozoan, helminth, and single copy genes in squashed whole mosquitoes. Mol. Biochem. Parasitol. 34:127–134.