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frozen roots on numerous occasions.

Our results suggest that the No Heat method is adequate for the routine extraction of fungal DNA from colonized roots. With a single mixture, 12 samples can be easily processed in under 2 h. In contrast, Method 1 would require nearly 3 h. The rapidity and enhanced safety of extraction and purification using the No Heat method over previously published methods stem from three innovations. First, heating, freezing and thawing, and grinding are not necessary. Apparently, the combination of prior freezing (for storage) and the physical disruption of the fungal cells with garnet beads during the extraction appears to efficiently extract fungal DNA. A common laboratory vortex mixer is sufficient to generate the force for the physical disruption of the fungal cells. Specialized shaking apparatus is unnecessary. Second, the use of the IRS in the extraction mixture effectively counteracts the inhibition of the IRS in the extraction mixture effectively counteracts the inhibition of PCR to circumvent spurious priming during gene amplification. Nucleic Acids Res. 1992. Rapid identification of genetic variation of ectomycorrhizal fungi by amplification of ribosomal RNA genes. New Phytol. 122:289-298.


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Rescue of Completed but Unread HIV-1 Antibody ELISA Microplates by Freezing

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Freezing and thawing (FT) of biological specimens before or after the analysis of various analytes is a common laboratory practice. In general, biological analytes present in specimens are usually quite refractory to FT. For example, in the area of infectious disease testing, studies describing multiple FT cycles indicated no deleterious consequences on subsequent analytical analysis for nucleic acid of hepatitis C virus (10) or of HIV-1 (5–7), for infectivity of varicella-zoster (2) or of HIV-1 (3), or for HIV-1 antibody reactivity in ELISAs (4,11).

Routine laboratory testing for the detection of HIV-1 antibodies is performed with ELISAs (9). This type of testing is generally completed within 3–4 h and is conducive to efficiently testing large numbers of samples at one time. However, an interruption in any step of the ELISA due to such circumstances as power outage would typically require repeating the entire group of specimens from the start of the ELISA procedure. In such unusual circumstances, several steps of the ELISA procedure, including washing, could be performed manually without the aid of automated equipment. The mainte-
nance of proper incubation temperatures for the ELISA during a power outage could also be possible for short periods depending on the heat retention of specific incubators. However, the capability to determine an ELISA endpoint result with a spectrophotometric instrument reading is essential for completion of the assay. Hence, electrical power is critical for this last reading step of the assay procedure. If a completed but unread ELISA microplate could be stored before the determination of results with a spectrophotometric instrument reading in the event of an inability to complete the reading due to an electrical power interruption or equipment malfunction, then the necessity for repeating the assay could be mitigated. In this study, the feasibility of storing completed ELISA microplates by freezing at -20°C was investigated by comparing endpoint results obtained before and after freezing.

The Vironostika® HIV-1 MicroELISA System (bioMérieux, Durham, NC, USA) was used for the study per the manufacturer’s directions. The ELISA is configured as a 96-well microplate coated with HIV-1 antigen derived from cell culture. Specimens (3 µL) were added to individual microwells with a diluent (225 µL). Following incubation for 90 min at 37°C, the microplates were washed four times with a phosphate-buffered wash solution; each microwell was filled completely without overflowing. Goat anti-human antibodies (150 µL) conjugated with HRP were added to the microwells to detect the presence of human anti-HIV-1 antibodies. The microplate was then incubated at 37°C for 30 min and subsequently washed again four times as described above. The substrate 2,2′azino-di-[3-ethylbenzthiazolone-6-sulfonate] (ABTS) (150 µL) was added, followed by a 10-min incubation at room temperature. The reaction was stopped with the addition of 150 µL 0.28% sodium fluoride prepared in water. According to the manufacturer’s directions, the stopped microplate should be read within 2 h after completion. If HIV-1 specific antibody is present, then color develops in the microwell. The amount of color present in an individual microwell was proportional to the amount of antibody present, as determined by spectrophotometric absorbance at 405 nm.

ELISA microplates were processed with an Ultrawash II microplate washer (Dynatech Laboratories, Chantilly, VA, USA) and analyzed spectrophotometrically with a OTC 520 microplate reader equipped with a 405-nm filter (Bio-Tek Instruments, Winooski, VT, USA). The discrimination between non-reactive and reactive specimens was made by calculation of a cut-off using the mean of three negative controls (included in the test kit) + 0.270. Specimens with a signal-to-cut-off ratio (SCR) of 1.0 were considered reactive. Data were obtained using an automated software system, Status Symbol™ II version C10, which is designed to calculate the plate cut-off and the results for individual microwells.

Serum specimens from low-risk blood donors and from HIV-1-infected individuals were used for the study. The specimens were collected into plain VACUTAINER® tubes (Becton Dickinson, Franklin Lakes, NJ, USA) using aseptic techniques (1).

Following completion of the reading at 405 nm, an adhesive plate sealer was attached to the microplate before freezing at -20°C and removed before thawing in a 37°C incubator for 40–60 min. The thawed microplate was re-analyzed with the microplate reader using parameters identical to those used for the initial reading.

Descriptive statistics [means and standard deviations (SDs)] for the data obtained before and after FT with the ELISA kit negative and positive controls and with the clinical specimens were generated with SAS® software (SAS, Cary, NC, USA). The data were further analyzed using Wilcoxon’s signed rank test and the UNIVARIATE procedure (SAS).

Similar qualitative results were obtained upon reading 15 different microplates containing either HIV-1-positive specimens or HIV-1-negative specimens before and after FT (Table 1). With 216 HIV-1-negative specimens reported non-reactive on the initial reading, 215 were reported non-reactive upon subsequent reading following FT. The mean SCR of the HIV-1-negative specimens before FT (0.34; SD = 0.059) was similar to that obtained after FT (0.37; 0.081) (mean difference initial reading - FT reading = -0.035; SEM difference = 0.004). The one specimen reported reactive upon the rereading had an initial SCR of 0.8; upon rereading after FT, the SCR was 1.1, indicating a borderline reactive result; the results of additional testing using another FDA-licensed ELISA for the detection of HIV-1 antibodies also were non-reactive (results not shown).

![Figure 1. Mean SCRs for absorbances of an HIV-1 antibody ELISA microplate incubated at 37°C for various times. The results were obtained with 20 HIV-1 antibody positive specimens with different levels of reactivity and with 45 HIV-1 antibody negative specimens.](image-url)
result further indicates that the individual from whom the specimen was obtained was not HIV-1 infected, and the reactive result following FT was due to experimental conditions. All of the 44 known HIV-1 antibody positive specimens were reported reactive for both readings before and after FT. The mean SCRs for the HIV-1 antibody positive specimens before FT were slightly greater (5.64; SD = 1.899) than after FT (5.50; SD = 1.860). For each of the mi-

<table>
<thead>
<tr>
<th>Specimen Type</th>
<th>n&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Mean SCR</th>
<th>No. with Expected Result</th>
<th>Mean SCR</th>
<th>No. with Expected Result</th>
<th>Mean Difference (Initial FT)</th>
<th>SEM</th>
<th>Significance &lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>HIV-1-Negative Whole Blood Donors</td>
<td>216</td>
<td>0.34</td>
<td>216</td>
<td>0.37</td>
<td>215</td>
<td>-0.035</td>
<td>0.0004</td>
<td>P &lt; 0.0001</td>
</tr>
<tr>
<td>HIV-1-Positive</td>
<td>44</td>
<td>5.64</td>
<td>44</td>
<td>5.50</td>
<td>44</td>
<td>0.145</td>
<td>0.038</td>
<td>P = 0.0005</td>
</tr>
<tr>
<td>ELISA Kit Negative Controls</td>
<td>21</td>
<td>0.34</td>
<td>21</td>
<td>0.37</td>
<td>21</td>
<td>-0.033</td>
<td>0.014</td>
<td>P = 0.0625</td>
</tr>
<tr>
<td>ELISA Kit Positive Controls</td>
<td>11</td>
<td>5.45</td>
<td>11</td>
<td>5.37</td>
<td>11</td>
<td>0.082</td>
<td>0.064</td>
<td>P = 0.2051</td>
</tr>
</tbody>
</table>

<sup>a</sup>n = number of specimens tested.  
<sup>b</sup>Significance by signed rank test.
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croplate analyzes performed, the SCRs for all controls, both negative (n = 21) and positive (n = 11), were within the range specified for the test and were not statistically different before and after FT (P > 0.05).

Experimentation was conducted with HIV-1-positive specimens diluted in negative serum to investigate the impact of the rereading after FT on the expected low-level reactive results. With each of 10 low reactive specimens (SCR range 1.1–3.2), very similar SCR results were obtained before and after FT; all 10 of these specimens were reported reactive after FT. Two additional specimens with SCRs of 0.8 and 0.9 before FT had identical SCRs after FT.

To investigate the potential of 37°C incubation as a factor contributing to increase in SCR observed for the one borderline non-reactive specimen (SCR = 0.8) that was reported reactive follow ing FT, a time course experiment was performed with different microplates incubated at room temperature or at 37°C following the addition of the stop solution. The 37°C temperature was selected because the microplates were incubated at this temperature for thawing following freezing. At room temperature incubation, no significant increase was observed for the SCRs of 45 HIV-1-negative specimens over 150 min with 15-min reading intervals. The mean SCR at the initial time point was 0.4 and, at 150 min, 0.5. Further, no significant increase in SCR with the negative specimens was observed at 37°C (initial SCR = 0.3 vs. 0.4) (Figure 1). In contrast, when the HIV-1-positive specimens were incubated at 37°C after addition of the stop solution, a linear increase in the mean SCR was observed over the 150-min observation period (Figure 1). The mean SCRs of the specimens in the microplate incubated at 37°C were significantly different by the signed rank test (P < 0.001) from the initial reading for each reading at each of the 20-min intervals.

The results obtained in this study indicate that the product of the enzymatic reaction between HRP and the ABTS substrate in the presence of the sodium fluoride stop solution remains stable following FT, thus enabling consistent qualitative results with the results obtained before freezing. For the great majority of specimens tested, FT of the HIV-1 ELISA microplates did not result in significant consequences on proper characterization of either reactive or non-reactive specimens. However, the potential exists for non-reactive specimens with a SCR close to the cut-off to be erroneously reported reactive because of the development of more intense coloration of the colored enzymatic product following a 37°C incubation to thaw a frozen microplate. This potential drawback is mitigated by the elimination of the need to repeat the procedure for an entire microplate of specimens. With ELISAs used for HIV-1 testing, an initial reactive specimen is repeat tested in duplicate, and this practice performed without FT would result in the proper non-reactive characterization of such a specimen. Different incubation regimens could possibly be used to thaw the frozen microplate. However, our observation that complete thawing of a frozen microplate incubated at room temperature required at least 2 h led to the conclusion that incubation at lower temperatures was inefficient. Our observations were made with completed ELISA microplates that were held at -20°C before thawing and rereading. In the event of a laboratory power outage, maintenance of completed ELISA microplates in a frozen state could be accomplished with the addition of dry ice to the storage freezer (8).

In conclusion, a technique is described that can be used to rescue completed ELISA microplates in the event of a power outage or malfunction that would prevent obtaining a final result with a microplate reader.

REFERENCES


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