SHORT REPORT: A PRACTICAL TECHNIQUE FOR THE CRYOPRESERVATION OF
DIROFILARIA IMMITIS, BRUGIA MALAYI, AND WUCHERERIA BANCROFTI
MICROFILARIAE

LYRIC C. BARTHOLOMAY, HODA A. FARID, EBITESAM EL. KORDY, AND BRUCE M. CHRISTENSEN
Department of Animal Health and Biomedical Sciences, University of Wisconsin-Madison, Madison, Wisconsin; Research and
Training Center on Vectores of Disease, Ain Shams University, Abbassia Square, Cairo, Egypt

Abstract. A technique to cryopreserve microfilariae has been developed. This method was used to cryopreserve microfilariae of Dirofilaria immitis, Brugia malayi, and Wuchereria bancrofti at a controlled rate of 1°C/min by use of a freezing tank. Microfilariae of each of these species retained their ability to infect susceptible mosquito species and develop to the infective stage after cryopreservation. The method presented here is quickly and easily carried out with inexpensive equipment.

Studies of vector-parasite interactions with Wuchereria bancrofti, or those that require third-stage larvae, are complicated by the lack of a convenient animal model in which to maintain the parasite; such studies require constant availability of a microfilaremic host or the use of cryopreserved microfilariae. Unfortunately, standard methods for cryopreservation of filarial larvae require either the use of freezers capable of stepwise freezing rates or extensive incubation steps that do not control freezing rates in a precise stepwise manner. We modified methods from a previous report and used techniques for mammalian tissue culture cryopreservation to develop an inexpensive and convenient method to cryopreserve microfilariae.

Cryopreservation techniques were initially tested on the unsheathed microfilariae of Dirofilaria immitis collected from an infected beagle. According to the protocol described by Lowrie for cryopreservation of Dirofilaria corynodes, aliquots of infected blood were supplemented with 6% dimethyl sulfoxide (DMSO) and polyvinyl pyrrolidone to a final concentration of 0.004 M in 1.0-mL cryotubes. Samples were frozen at a controlled rate of −1°C/min in a Nalgene Model 5100 Cryo 1°C-freezing container (Nalge Nunc International Corporation, Rochester, Rochester, NY) that we use to cryopreserve tissue culture cells.

The container was filled with isopropanol, samples were placed, and the tank was deposited in a −80°C freezer in accordance with the manufacturer’s instructions. After 4 hr, tubes were transferred to liquid nitrogen for long-term storage. Thawing was done as described by Lowrie, with modifications. Blood was thawed by immersing and agitating cryotubes in a 37°C water bath for 2 min and then was immediately added to 11 mL of a chilled wash solution of Aedes saline and 10% fetal bovine serum. The mixture then was centrifuged for 10 min at 200 × g at 4°C. The resulting supernatant was decanted. Pelleted microfilariae were thoroughly resuspended and centrifugation was repeated. Tubes containing thawed microfilariae were kept on ice between centrifugations, and steps were quickly executed to prevent potentially detrimental effects of DMSO on microfilariae.

Motility was assessed before the use of microfilariae in exposures, and cryopreservation was considered successful when parasites developed to the infective stage in a susceptible mosquito strain (Table 1). After 1 month in long-term storage, > 95% of D. immitis microfilariae were motile. Microfilariae were resuspended in uninfected dog blood and presented to a D. immitis-susceptible strain (Table 1) of Aedes aegypti through a Parafilm® (American National Can, Menasha, WI) membrane in a water-jacketed membrane feeder. Fully engorged mosquitoes were separated and maintained as previously described. After a 14-day extrinsic incubation period, mosquitoes were dissected and examined for the presence of third-stage larvae. Mosquito survivorship during this period was comparable to that of mosquitoes exposed to noncryopreserved microfilariae (data not shown). Because this proved successful for D. immitis (Table 2), the procedure was applied to the sheathed microfilariae of Brugia malayi and W. bancrofti.

Blood infected with B. malayi was collected by cardiac puncture from a Mongolian jird (Meriones unguiculatus). Wuchereria bancrofti-infected blood was collected at night from consenting, highly microfilaremic human volunteers from a village in the Gimah Governorate, Egypt. The procedure used to collect blood from volunteers was approved by the Department of Health and Human Services (Assurance S-005012-06). According to conditions standardized by Lowrie for cryopreservation of B. malayi microfilariae, 9% DMSO was added to blood infected with either filarial worm species as a cryoprotectant. Freezing and thawing procedures were performed as described for D. immitis.

After 1 month in storage, thawed B. malayi microfilariae showed > 90% motility and were resuspended in defibrinated sheep’s blood. The infectious blood meal was presented to the Liverpool strain of Aedes aegypti maintained in our laboratory, which is highly susceptible to B. malayi when mosquitoes feed on an anesthetized Mongolian jird (Table 1). Fourteen days later, mosquitoes were dissected for detection of third-stage larvae. Results from 2 B. malayi exposures are summarized in Table 2. Blood containing microfilariae of W. bancrofti was stored at 4°C overnight before being processed. Samples were frozen in a Nalgene Cryo freezing tank, brought back to our United States laboratory on dry ice, and transferred to liquid nitrogen. Thawed microfilariae showed ≥ 93% motility and were presented in a blood meal through a chicken-skin membrane to the Iowa State strain of Culex pipiens pipiens, which is 69% susceptible to W. bancrofti when mosquitoes feed on a microfilaremic human volunteer (Table 1). After 14 days, surviving mosquitoes were dissected and examined for the presence of third-stage larvae (Table 2). Survival of mosquitoes exposed to cryo-
preserved B. malayi or W. bancrofti microfilariae was comparable to that of mosquitoes exposed to non-frozen microfilariae (data not shown).

This method for cryopreserving microfilariae is easily and quickly performed with inexpensive equipment that allows for a controlled freezing rate of ~1°C/min. Additionally, this method may be safer for the researcher because it uses plastic cryotubes rather than glass c overslips or glass ampoules and thus, in the case of W. bancrofti, reduces the risk of exposure to blood-borne pathogens such as hepatitis B or C. The limitation of the method here reported is a controlled freezing rate of ~1°C/min; a previous report showed that optimum freezing rates differ with filarial worm species.

Acknowledgments: We are grateful to Drs. Reda Ramzy and Li-Lin Cheng for technical advice, Linda Christensen and Sherine Kamal for technical assistance, and Drs. Ehab S. Ahmed and Amr M. Kandeel for human blood collection.

Financial support: This work was supported by U.S. National Institutes of Health grant AI 46032.

Authors’ addresses: Lyric C. Bartholomay and Bruce M. Christensen, Department of Animal Health and Biomedical Sciences, University of Wisconsin-Madison, 1656 Linden Drive, Madison, WI 53706-1581; Telephone: 608-262-2373, Fax: 608-262-7420 (email: bartholomay@ahabs.wisc.edu).

REFERENCES


### Table 1

Susceptibility of mosquitoes to noncryopreserved microfilariae

<table>
<thead>
<tr>
<th>Filarial worm species</th>
<th>Mosquito species and strain</th>
<th>Infectious dose (microfilariae/20 μL)</th>
<th>No. mosquitoes examined</th>
<th>Prevalence of infection (%)†</th>
<th>Mean intensity‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dirofilaria immitis</td>
<td>Aedes aegypti, susceptible</td>
<td>110</td>
<td>101</td>
<td>96</td>
<td>6.8</td>
</tr>
<tr>
<td>Brugia malayi</td>
<td>to D. immitis</td>
<td>93</td>
<td>108</td>
<td>85</td>
<td>2.6</td>
</tr>
<tr>
<td>Wuchereria bancrofti</td>
<td>Aedes aegypti, Liverpool</td>
<td>194</td>
<td>81</td>
<td>93</td>
<td>8.4</td>
</tr>
<tr>
<td></td>
<td>Culex pipiens, pipiens</td>
<td>90</td>
<td>27</td>
<td>93</td>
<td>3.6</td>
</tr>
</tbody>
</table>

* Non-cryopreserved microfilariae of Dirofilaria immitis, Brugia malayi, or Wuchereria bancrofti were assessed after exposure via membrane feeding (D. immitis), direct feeding on an anaesthetised pig (B. malayi), or a human volunteer (W. bancrofti).
† Number of infected mosquitoes divided by the number of mosquitoes examined.
‡ Total number of third-stage larvae divided by the number of infected mosquitoes.

### Table 2

Development of cryopreserved microfilariae after long-term storage in liquid nitrogen

<table>
<thead>
<tr>
<th>Filarial worm species</th>
<th>Months in storage</th>
<th>Infectious dose (microfilariae/20 μL)</th>
<th>No. mosquitoes examined</th>
<th>Prevalence of infection (%)†</th>
<th>Mean intensity‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dirofilaria immitis</td>
<td>1</td>
<td>120</td>
<td>34</td>
<td>97</td>
<td>4.6</td>
</tr>
<tr>
<td>Brugia malayi</td>
<td>1</td>
<td>56</td>
<td>25</td>
<td>68</td>
<td>2.1</td>
</tr>
<tr>
<td></td>
<td>1.5</td>
<td>84</td>
<td>76</td>
<td>70</td>
<td>1.8</td>
</tr>
<tr>
<td>Wuchereria bancrofti</td>
<td>2</td>
<td>87</td>
<td>115</td>
<td>43</td>
<td>1.7</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>65</td>
<td>81</td>
<td>41</td>
<td>1.5</td>
</tr>
</tbody>
</table>

* Development of Dirofilaria immitis, Brugia malayi, and Wuchereria bancrofti to third-stage larvae, after long-term storage in liquid nitrogen.
† Number of infected mosquitoes divided by the number of mosquitoes examined.
‡ Total number of third-stage larvae divided by the number of infected mosquitoes.