Sodium Vanadate Treatment as a Shortcut Following Alkaline Phosphatase Cleavage

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Alkaline phosphatase (AP) has been widely used in the dephosphorylation of restriction enzyme-digested plasmid vector to eliminate the nonrecombinant background that results from the transformation of recircularized vectors. However, the highly active phosphatase must be completely inactivated or removed after the reaction if subsequent ligation are to work efficiently. This is generally achieved by digesting with protease K at 37°C for 30 min or by heating to 65°C for 1 h in the presence of 5 mM EDTA (pH 8.0), followed by extracting several times with phenol-chloroform. The procedure is quite tedious, and the loss of dephosphorylated vector after the process may reduce the cloning efficiency. To overcome these lengthy and inefficient manipulations following the treatment with AP, we have found that sodium vanadate (Na$_3$VO$_4$), a potent transition-state, analog-based inhibitor for most phosphatases (5), can be used to effectively inactivate AP. Furthermore, with no additional manipulation, the resulting solution can be used directly for ligation. Thus, the low-background subcloning process using AP can be drastically simplified.

In a routine cloning process using Na$_3$VO$_4$, we usually started with 1 µg of vector plasmid. Following digestion with one or two restriction enzymes in the suitable buffer, the digested vector was phenol-chloroform-extracted, ethanol-precipitated and finally resuspended in 15 µL of double-distilled (dd)H$_2$O. We added 9 µL of ddH$_2$O, 3 µL of 10× buffer for calf intestinal alkaline phosphatase (CIAP) and 3 µL of 0.012 U/µL CIAP (Life Technologies, Gaithersburg, MD, USA) to the above solution, and dephosphorylation was performed at 37°C for 30–60 min. After dephosphorylation, 2 µL of the reaction mixture were mixed with 2 µL of 250 µM Na$_3$VO$_4$ (Sigma Chemical, St. Louis, MO, USA) and incubated at room temperature for 2–3 min. The entire 4 µL of this solution were then used directly for ligation in a total volume of 10 µL, containing 1 µL of 10× ligation buffer [500 mM Tris-HCl (pH 7.8), 100 mM MgCl$_2$, 100 µM dithiothreitol (DTT), 10 mM ATP and 250 µg/mL bovine serum albumin (BSA)], 8 U of T4 DNA Ligase (New England Biolabs, Beverly, MA, USA) and a significant molar excess of insert digest. The subsequent transformation was done according to a standard calcium chloride transformation method (2).

Table 1. Cloning Efficiency with or without Using Na$_3$VO$_4$

<table>
<thead>
<tr>
<th>Treatment of pUC19 Before Transformation</th>
<th>No. of Colonies</th>
<th>Recombinants$^a$ (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dark Blue</td>
<td>Light Blue or White</td>
<td></td>
</tr>
<tr>
<td>EcoRI/Sphl-digested, no ligation</td>
<td>3</td>
<td>-</td>
</tr>
<tr>
<td>No CIAP, ligate with EcoRI/Sphl insert</td>
<td>354</td>
<td>44</td>
</tr>
<tr>
<td>CIAP-treated, purified by a general method$^b$, ligate with EcoRI/Sphl insert</td>
<td>2</td>
<td>24</td>
</tr>
<tr>
<td>CIAP-treated, inactivated by Na$_3$VO$_4$, ligate with EcoRI/Sphl insert</td>
<td>1</td>
<td>28</td>
</tr>
<tr>
<td>Sall-digested, no ligation</td>
<td>27</td>
<td>6</td>
</tr>
<tr>
<td>Sall-digested, no CIAP-Na$_3$VO$_4$ treatment, ligate with Sall insert</td>
<td>ca. 2100</td>
<td>34</td>
</tr>
<tr>
<td>CIAP-treated, purified by a general method$^b$, ligate with Sall insert</td>
<td>10</td>
<td>9</td>
</tr>
<tr>
<td>CIAP-treated, inactivated by Na$_3$VO$_4$, ligate with Sall insert</td>
<td>18</td>
<td>8</td>
</tr>
</tbody>
</table>

$^a$Determined by blue/white selection on the 5-bromo-4-chloro-3-indolyl-β-d-galactopyranoside (X-gal)-containing plate and a restriction analysis using PvuII.

$^b$Heated to 65°C for 1 h in the presence of 5 mM of EDTA, extracted twice with phenol chloroform and precipitated with ethanol.

To demonstrate the efficiency of this protocol, we separately inserted a 860-bp, EcoRI/Sphl-digested DNA fragment and a 750-bp, Sall-digested DNA fragment, derived from the coding region of the 69-kDa outer membrane protein pertactin of Bordetella pertussis (1), into pUC19 vector (Life Technologies). Using roughly equal amounts of restriction-digested pUC19 (ca. 50 ng, based on 1 µg of starting plasmid for ligation and transformation), we evaluated the cloning efficiencies of the various procedures including that in which Na$_3$VO$_4$ was used as an inhibitor (Table 1). As observed by other researchers, CIAP treatment effectively eliminates nonrecombinant background and substantially increases the recombination frequency. However, the most significant finding was that the percentage of recombinants generated from the procedure using a very brief treatment with Na$_3$VO$_4$ following CIAP cleavage (e.g., 90% and 27%) was basically equivalent to that obtained using the more time-consuming purification steps instead (e.g., 82% and 31%). This indicates that the presence of the Na$_3$VO$_4$ (50 µM in the ligation solution) and of vanadate-inactivated CIAP do not interfere with ligation and transformation. Consequently, a comparable efficiency of
cloning could be achieved by using this quick, alternative protocol.

Not only does this Na$_3$VO$_4$-inhibition procedure save time, but also note that in this protocol the adjustment of vector-to-insert ratio in the ligation reaction is not necessary. To ligate with the CIAP/Na$_3$VO$_4$-treated vector (ca. 50 ng), we recommend using as much excess insert as is practical. Eliminating the quantitation step for both vector and insert further simplifies the process and reduces the hands-on time of the cloning procedure.

In summary, the procedure we describe in this report should make the subcloning method using alkaline phosphatase not only a less costly but also a time-effective procedure. A high percentage of recombinants could be quickly achieved without using any special cloning vector or bacterial strain. We feel that this protocol is especially useful for cloning procedures having no appropriate screening method (e.g., blue/white colony selection) or no background reduction strategy [e.g., digesting the ligation product before transformation with a selection enzyme that cuts the vector between the two cloning sites (3,4)].

REFERENCES


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Division of Biologics Quality Control, National Institute of Preventive Medicine, Department of Health, Administrative Yuan, Taipei, Taiwan, R.O.C.

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Yu-Yuan Peter Wo, Der-Shyan Sheu and Cheng-Hsiung Lu National Institute of Preventive Medicine Taipei, Taiwan, R.O.C.

Photographic Recording of Fluorescent DNA Bands on Agarose Gels


Electrophoretic separation of DNA fragments by size on agarose gels is one of the most important techniques in molecular biology, and Polaroid® (instant) photography is frequently used to record the fluorescent DNA banding patterns from this analysis. Polaroid photography allows a quick assessment of photographic quality and permits a repeat of the photography if needed. Small-format gels are often used to conserve agarose, to reduce run time and to conserve the DNA sample for other analyses. With less DNA per band, it becomes more important to have a sensitive and accurate method for recording the reduced fluorescence. With less distance between each band, lateral diffusion of DNA over time—longer than approximately 2 h between the end of electrophoresis and photography—reduces resolution, therefore further justifying the need for a quick and accurate record of low-intensity bands.

Here, we present a method to obtain a quick, sensitive and more accurate image of the fluorescent DNA banding patterns on agarose gels using Polaroid film (Polaroid, Cambridge, MA, USA). The principles and techniques presented are applicable to all photographic films for reduced light recordings (3) and have been applied to X-ray films used for autoradiographic records from weak β emitters (1,2).

Photographic exposure in normal light easily initiates silver grain formation on film and exhibits an exact reciprocal relationship for exposure time and aperture opening (f-stop). For example, the same silver grain density can be obtained with a 1/30-s exposure at f = 8 or a 1/15-s exposure at f = 11. Thus, in normal light, for the same amount of silver grain development, a doubling of exposure time is required with each 50% reduction in aperture opening.

Photography in low-light environments, such as ethidium bromide fluorescence (DNA staining), requires progressively greater exposure times to...