A dehydrogenase linked assay of DNA ligase activity
by Jerold Randall Morgan

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in Chemistry
Montana State University
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Abstract:
A novel dehydrogenase linked assay for prokaryotic DNA ligase activity is presented. This assay is based on the quantification of NAD+ utilization by the DNA ligase. The ligase enzyme splits NAD+ into NMN and AMP during the joining of DNA pieces. This assay is simple to use, inexpensive, and the reagents are stable for six months if stored in the dark. The assay avoids the use of radiolabels and mutagenic chemicals that are found in many DNA ligase assays. Finally, data is presented that indicates that the assay can be used to measure DNA ligase activity in crude extracts from prokaryotes.
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DNA LIGASE ACTIVITY

by

Jerold Randall Morgan

A thesis submitted in partial fulfillment
of the requirements for the degree
of
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in
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APPROVAL

of a thesis submitted by

Jerold Randall Morgan

This thesis has been read by each member of the thesis committee and has been found to be satisfactory regarding content, English usage, format, citations, bibliographic style, and consistency, and is ready for submission to the College of Graduate Studies.

27 January 1987
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27 January 1987
Date

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Head, Major Department

Approved for the College of Graduate Studies

29 January 1987
Date

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Graduate Dean
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Date
To my Grandfather

Rev. Jerry Sheldon Nelson

who taught me that life without knowledge and wisdom is a veritable desert
ACKNOWLEDGMENTS

I would like to acknowledge the following persons for their assistance in the course of this Master's project.

I extend my gratitude to Dr. Pete Bartels for allowing me to use his equipment in Seattle. The use of this equipment permitted me to finish the necessary experiments proving the usefulness of the assay. I wish to also thank Dr. Gordon Julian, my advisor, for helping me through difficult phases of the project and teaching me how to handle problems. Without his extensive knowledge and creative ideas, I would have been unable to create the workable solutions necessary to bring the project to a successful conclusion.

I especially wish to thank my wife and family who believed in the values of effort and persistence.
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A novel dehydrogenase linked assay for prokaryotic DNA ligase activity is presented. This assay is based on the quantification of NAD$^+$ utilization by the DNA ligase. The ligase enzyme splits NAD$^+$ into NMN and AMP during the joining of DNA pieces. This assay is simple to use, inexpensive, and the reagents are stable for six months if stored in the dark. The assay avoids the use of radiolabels and mutagenic chemicals that are found in many DNA ligase assays. Finally, data is presented that indicates that the assay can be used to measure DNA ligase activity in crude extracts from prokaryotes.
EVOLUTION OF COLORIMETRIC ASSAY

Originally, the objective of the project was to purify a DNA ligase from the thermophilic bacteria T-2. However, the problems encountered with recommended assays for DNA ligase activity prompted a search for an alternative assay. The developed assay utilizes an enzymatic cycling of NAD⁺, which in turn converts a dye from one oxidation state to another. This assay is sensitive and easy to use, and does not depend on the use of radiolabels. The following section of the thesis presents the problems encountered in the early stages of the project, and reveals the evolution of the new assay.

The first stage of the project involved familiarization with the ³²P labeled d(A-T)n ligase assay (1,2). Within the first three months, the procedure for the assay could be used routinely. The assay was complicated by the short half-life of the substrate (14 days) (3). Once the substrate was formed, it had to be used as quickly as possible to minimize the reduction in decay CPM (radioactive counts per minute). Also, the substrate had to be handled with special care to prevent exposing people to radiation. Additionally, all materials that came into contact with the labeled substrate had to be isolated until the radioactivity was sufficiently low to dispose of the
The main reason that the $^{32}\text{P}$ labeled d(A-T)n assay was not found to be suitable for the purification of the enzyme was not the hazardous nature of the radiolabel, nor the short half-life, but rather the costliness of the $^{32}\text{P}$ labeled ATP that was used to produce the substrate. The $^{32}\text{P}$-ATP that was first utilized was kindly donated to Dr. Julian's lab by another research group. We used the donated ATP to produce the first lot of substrate. When it was time to order more of the labeled ATP, the full price became apparent to us. It was determined that the price of the substrate was too high to allow the use of the assay for the purification of the ligase. The purification stage of an enzymatic study requires a large number of activity assays to be run at each step. This decision not to use the $^{32}\text{P}$-ATP assay was reinforced by the finding that there was endogenous phosphatase activity in the crude extract that would have to be removed or destroyed before the assay could measure any ligase activity. The phosphatase enzyme removes the radiolabel from the DNA before the ligase is able to join the fragments which lowers the measured ligase activity and gives false negative results. If there is a large excess of phosphatase activity, the activity of the ligase can be completely hidden. Thus, another assay was sought that would be useful during the purification of the ligase. Once the
enzyme had been purified, the $^{32}$P labeled d(A-T)n assay was to be used in the kinetic studies of the enzyme if the other assay could not be used for kinetic studies.

The assay that was substituted for the $^{32}$P labeled d(A-T)n assay was based on the electrophoresis of restriction fragments from lambda phage genomic DNA. The assay procedure involved digesting the lambda DNA with EcoRI restriction endonuclease, exposing the fragments to the ligase sample, and then separating the resulting mixture by electrophoresis through agarose gels. Ligase activity was interpreted by witnessing an alteration in the band pattern in the gel. The monitored change was an increase in the size of all the fragments, which would be expressed as a slower rate of electrophoretic movement. Several catalogs showing activity of commercial ligase preparations showed gels that contained an increase in the size of restriction fragments after being exposed to the ligase at 16°C. Many of these electrophoretic assays were run during the first year of the project. Several times the band pattern showed results that were interpreted as ligase activity.

However, a reinterpretation of the assay results became necessary late in the first year of research. A new culture of the thermophilic bacteria was received from ATCC, and an attempt was made to purify active material from several large growths of the bacteria.
Problems first appeared when the electrophoretic assay failed to reveal any bands in the gel at all. The assay was modified to include a digestion of protein with proteinase K before electrophoresis. The results from the assays showed no activity. A trial of material that had earlier shown activity was run as a control, and it did not show the change in band pattern that had been taken as characteristic of ligase activity.

At first the absence of the expected alteration in band pattern gave rise to the suspicion that the reagents used in the assay had gone bad. However, a check of the reagents as used in the old electrophoretic assay configuration showed that they were not the cause of the difficulty. When the samples were exposed to proteinase K, the fragments of DNA possessed the same mobilities as DNA that had not been incubated with ligase sample. If the proteinase K treatment was omitted, the samples showed activity. Also, samples of crude extract that lacked NAD$^+$ showed similar results as samples that had NAD$^+$ added prior to incubation with ligase. During the early stages of the project this result was justified by the suggestion that residual NAD$^+$ in the crude extract was responsible for the ligation activity. This explanation was no longer sufficient when more highly purified fractions that had been subjected to steps such as dialysis that should have removed any NAD$^+$. 
A search of relevant literature was completed in order to find a possible explanation for the observed failure of the assay. It was found that the problem with the assay resulted from a misinterpretation of the data. At 37°C the cohesive ends that were present on EcoRI generated fragments did not possess enough hydrogen bonding attraction to remain joined together (4). Thus, the probability of ligating the fragments was low. However, the ends of the lambda genome possess a twelve base pair match that is joined during the replication stage of the phage. The two fragments that contain the matching end sequences are the only fragments to undergo an alteration in electrophoretic mobility (4). These two fragments are the largest and the smallest fragments that are formed by EcoRI digestion. Thus, the expected result of the electrophoresis of ligated fragments should have been the disappearance of the smallest and the largest band, and the appearance of a band with an even larger size. In practice, the mobility of the new band was not sufficiently different from the mobility of the larger of the two unligated fragments to be separated from it. Thus, a disappearance of the smallest band would have to be interpreted as ligase activity. Since the recovery of DNA after the digestion with proteinase K was not quantitative, there was an inherent variation in the amount of the smallest fragment present during electrophoresis. This variation
rendered the assay unsuitable for determining ligase activity in crude samples where proteinase K digestion was necessary.

The artifact that had been interpreted as ligase activity was suspected to be caused by the binding of proteins to the DNA restriction fragments. The protein-DNA complex would have a greater molecular weight than the DNA alone. An increase in the protein concentration could give rise to larger complexes and thus increase the observed molecular weight. This is also the expected result from a sample containing ligase activity that would be ligating fragments into larger fragments.

Even before the mistaken interpretation of data from the electrophoresis assay was discovered, another assay had been explored. It had been anticipated that the other assay would prove to be more rapid than the electrophoresis assay. This assay involved the healing of nicks introduced into plasmids by S1 nuclease (5,6). The assay proved to be more difficult than was assumed. The production of large amounts of plasmid without random nicks was not successful. The assay was abandoned finally because of the presence of endogenous endonuclease activity in crude samples. The endonuclease activity presented the same problems that were present with the phosphatase activity when the $^{32}P$ labeled d(A-T)n was attempted on crude samples. A very small amount
of endonuclease activity would cover up any ligase activity present in a sample. This suppression of observed ligase activity would be caused by the greater concentration of endonuclease substrate relative to ligase substrate. This greater relative concentration results from the fact that the endonuclease is able to use many different base pair sequences on the same plasmid as a substrate, whereas the ligase must find those sequences that possess a break in the phosphodiester backbone.

After a full year of experimental work, no usable assay had been found. At this point, a decision was made to develop an assay that could work during the purification of the ligase. A review of the literature revealed that many assays could not detect ligase activity in crude samples (4, 7, 8). The project was then altered to find an assay that could circumvent some of the problems encountered with the other assays that had been previously attempted.

In examining the ligase reaction in prokaryotes, (Figure 1), it is apparent that there are two major alterations in the molecular structures of the reactants. The first, and biologically most important, is that a phosphodiester bond is formed between two pieces of DNA. However, NAD$^+$ is also cleaved to form AMP and NMN (Figure 2). This cleavage is similar to the reaction in eukaryotes where ATP is cleaved to form PPI
Figure 1. Mechanism of prokaryotic DNA ligase reaction.
Figure 2. Structures of NAD$^+$ (top), NMN (middle), and AMP (bottom).
and AMP (9). The Wiess unit has been used for some time to measure eukaryotic DNA ligase activity (10), and the Wiess unit follows the conversion of ATP. The assays used to measure prokaryotic ligase activity have largely ignored the NAD$^+$ cofactor in favor of the DNA.

A method for measuring the use of NAD$^+$ by prokaryotic ligases was developed from other work that was accomplished during the project. When it was discovered that the culture of T-2 that had been used as a stock culture had become contaminated, a method for rapid identification of T-2 was developed. Dr. Julian had previously used a test that takes advantage of the lack of a pentose shunt in T-2. One of the enzymes in the pentose shunt is glucose-6-phosphate dehydrogenase. The test developed by Dr. Julian used the shift in UV absorbance that occurs when NADP$^+$ is converted to NADPH by glucose-6-phosphate dehydrogenase, in order to monitor activity of the enzyme. Since the enzyme should be absent in T-2, there should be no increase in the OD$_{340}$ when glucose-6-phosphate and NADP$^+$ is mixed with a sample of disrupted cells.

The new test for T-2 culture purity was a colorimetric test for glucose-6-phosphate dehydrogenase activity developed from a scheme for measuring lactose dehydrogenase activity that had been found in an article (11). The test involves a dye and electron carrier. The NADP$^+$ is reduced by the enzyme, and, in
turn, the NADPH reduces an electron carrier, phenazine methylsulfate. The reduced form of PMS then reduces a dye, which alters the dye's absorbance spectrum. This colorimetric test is much more sensitive than the other test that involves UV absorbance.

The idea occurred that if the dye reaction was as sensitive to NAD$^+$ as it was to dehydrogenase, and the concentration of NAD$^+$ was made the rate limiting component, an assay for concentration of NAD$^+$ could be established. This test of NAD$^+$ concentration could then be incorporated into an assay for ligase activity. The assay would measure the decrease in the amount of NAD$^+$ present after incubation with a ligase sample. The more ligase activity present in a sample, the greater the decrease in NAD$^+$ concentration. A representation of the ligase activity assay is given in Figure 3.

The main aspects of such an assay that would need to be tested were sensitivity, linearity, and reproducibility. Any useful assay for DNA ligase would need to be sensitive enough to detect the small changes in NAD$^+$ concentration occurring in a reasonable sample size. The assay would also need to be linear, i.e. two units of DNA ligase should give double the measured NAD$^+$ usage that one unit of DNA ligase would use. Lastly, an useful assay needs to be reproducible over a wide range of samples, or have an internal standard built into the assay to calibrate the measurements.
Phase I
Nicked DNA + NAD$^+$ $\xrightarrow{\text{Ligase}}$ Healed DNA + AMP + NMN

Phase II
Ethanol + NAD$^+$ $\xrightarrow{\text{ALD}}$ NADH + H$^+$ + Acetaldehyde

PMS or PES reduced

PMS or PES oxidized

INT or MTT oxidized

INT or MTT reduced

Figure 3. Representation of ligase assay. Phase I is the incubation of the DNA substrate with the ligase sample. This is followed by Phase II, which measures the amount of NAD$^+$ not utilized by the ligase.

The first step in forming a new assay was to determine if the dye conversion was sufficiently sensitive to detect the small amount of NAD$^+$ that would be present in a ligase reaction. By using a combination of a dehydrogenase and PMS to cycle the NAD$^+$ between its oxidized and reduced forms, a large amplification of the sensitivity of the assay for NAD$^+$ could be achieved.

The concentration of NAD$^+$ to be monitored must lie in the range that would be used by a ligase sample in a reasonable length of time. It was determined that 50 to 500 pmoles of NAD$^+$ would be a usable range. This range was chosen because it was able to detect a significant change in the amount of NAD$^+$ present, after incubation
of the NAD\(^+\) and one unit of DNA ligase at 37°C for two hours. One unit of ligase activity is defined as the amount of enzyme required to give 50% ligation of Hind III digested lambda DNA in 30 minutes at 16°C in a final volume of 5 \(\mu\)l and a 5'termini concentration of 0.12 \(\mu\)M (12). One unit would use 4.8 pmoles of NAD\(^+\) in two hours under the assay conditions listed above. If a 5 pmoles alteration in the amount of NAD\(^+\) present could be assayed consistently, then the assay would be usable for reasonable sample sizes.

The first trial of the colorimetric assay used ethanol as the dehydrogenase substrate and alcohol dehydrogenase as the enzyme responsible for cycling the NAD\(^+\) to NADH. A range of NAD\(^+\) concentrations that began at 500 pmoles, and decreased to 100 pmoles was used to test the linearity of the relationship between the dye conversion and the NAD\(^+\) concentration. The correlation coefficient for the plotted data was calculated by Hewlett Packard linear regression as 0.977. The low value can be attributed to the inaccuracy of the delivery of alcohol dehydrogenase by the micropipetter. The replacement of the pipetter with a more accurate one resulted in an increase in the correlation coefficient to 0.988 (Figure 4).
Figure 4. Test of absorption at 503 nm versus pmoles of NAD+. Color development time was 1800 sec. Dye and electron carrier were INT and PMS respectfully.

The higher value of the correlation coefficient encouraged the trial of the colorimetric assay of NAD⁺ concentration in a ligase activity assay measuring the decrease in NAD⁺ that results from the cleavage of NAD⁺ into NMN and AMP by the ligase. The DNA substrate used in the assay was prepared by digesting salmon sperm DNA 10ug/ml with Mlu-1 overnight at 37°C. This enzyme was
used due to the fact that the recognition and cleavage sequence is composed of G-C base pairs (13), which are more temperature stable (14). The ligase sample consisted of a commercial preparation of E. coli ligase purchased from New England Biochemicals. (The results are tabulated in Table I.) Also, a test was conducted to determine the optimal temperature at which to run the assay. (The results from this test are also presented in Table I.) The results from these two tests show that the assay for ligase activity is feasible. The optimal temperature was determined to be 37°C using Mlu I digested DNA.

<table>
<thead>
<tr>
<th>Tube #</th>
<th>Temperature/Time of ligation</th>
<th>OD&lt;sub&gt;503&lt;/sub&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0°C / 0 hours</td>
<td>0.326</td>
</tr>
<tr>
<td>2</td>
<td>20°C / 4 hours</td>
<td>0.169</td>
</tr>
<tr>
<td>3</td>
<td>25°C / 4 hours</td>
<td>0.170</td>
</tr>
<tr>
<td>4</td>
<td>30°C / 4 hours</td>
<td>0.163</td>
</tr>
<tr>
<td>5</td>
<td>37°C / 4 hours</td>
<td>0.153</td>
</tr>
</tbody>
</table>

Table 1. Test of optimal temperature for ligation reaction. Color development time was 1800 sec. Dye and electron carrier were INT and PMS respectfully. All tubes contained 10 units ligase and 500 pmoles NAD<sup>+</sup>. Substrate consisted of 1 mg digested DNA.

A test of the linearity of dye conversion versus time of alcohol dehydrogenase reaction was performed to determine the precision of the assay, which could be highly dependent of the accuracy in the timing of the colorimetric incubation period (Figure 5). The plot was
Figure 5. Test of linearity of dye conversion versus time. All samples contained 500 pmoles NAD$^+$. Dye and electron carrier were INT and PMS respectfully.

obviously nonlinear, which might indicate that the reaction rate of the alcohol dehydrogenase reaction is not limited by the concentration of NAD$^+$. The assay was modified to include the addition of 3% BSA to the alcohol substrate. The BSA stabilized the dye, and
prevented the precipitation of the dye, which dramatically increased the linearity of the dye conversion (Figure 6) (11). However, the BSA also increased the background considerably (Figure 7).

Figure 6. Test of linearity of dye conversion using 3% BSA to stabilize the dye. Dye and electron carrier were INT and PMS respectfully. All samples contained 500 pmoles NAD+.
To reduce the background, the BSA was removed from the assay. A reference suggested that an alternative dye and electron carrier might work better to assay NAD$^+$ (15). The dye was changed to thiozalyl blue (MTT), and the electron carrier was switched to phenazine ethyl sulfate (PES). The linearity of the assay was retained, but the background was reduced. (A typical plot of OD$_{570}$ versus NAD$^+$ concentration is given in Figure 8.) The sensitivity of the assay was also improved from 500
Figure 8. Plot of color development of dye versus NAD$^+$ concentration. Dye and electron carrier were MTT and PES respectively. Color development time was 3600 sec.

Additionally, the DNA substrate changed during the development of the assay. At first, EcoRI was used to produce ligatable breaks in salmon sperm DNA (10mg/ml), and then MluI was used to produce the DNA substrate because of the G-C content of its cleavage sequence. (A plot of a test assay is given in Figure 9.) As can be
observed, the amount of NAD⁺ used by the ligase levels off over time. The leveling off can be attributed to the exhausting of ligatable breaks in the DNA. To produce more ligase substrate per ml of DNA, the DNA digesting enzyme was switched to DNase (2). The amount of DNase

Figure 9. Plot of ligase test verses time of ligation. All tubes contained 500 pmoles of NAD⁺ at start of test. Dye and electron carrier were INT and PMS respectfully. Color development time was 1500 sec. All tubes contained 10 units of ligase.
digestion that would give the best results was determined empirically. DNA was digested by several different concentrations of DNase. The conditions that produced DNA substrate with the highest concentration of ligase substrate was used to produce 10 ml aliquots of DNA substrate. (The results from the DNase digestions are given in Table 2. The data of a ligase activity assay using the DNase substrate is given in Table 3, and are plotted in Figure 10.)

<table>
<thead>
<tr>
<th>Tube #</th>
<th>OD570</th>
<th>pmoles NAD⁺ used by ligase</th>
<th>DNase (mg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.415</td>
<td>22</td>
<td>0.1</td>
</tr>
<tr>
<td>2</td>
<td>0.422</td>
<td>22</td>
<td>0.5</td>
</tr>
<tr>
<td>3</td>
<td>0.695</td>
<td>5</td>
<td>1.0</td>
</tr>
<tr>
<td>4</td>
<td>0.211</td>
<td>35</td>
<td>1.5</td>
</tr>
<tr>
<td>5</td>
<td>0.214</td>
<td>35</td>
<td>2.0</td>
</tr>
<tr>
<td>6</td>
<td>0.147</td>
<td>39</td>
<td>2.5</td>
</tr>
<tr>
<td>7</td>
<td>0.226</td>
<td>34</td>
<td>3.0</td>
</tr>
</tbody>
</table>

Table 2. Test of DNase digested DNA as substrate for ligase assay. Ligation time was 4 hours. All tubes contained 50 pmoles NAD⁺ and 1 mg DNA at start of ligation time. Color development time was 3600 sec. The dye and electron carrier were MTT and PES respectfully.
Table 3. Data of ligase assay. All tubes contained 50 pmoles of NAD$^+$ at beginning of ligation time and 1 mg DNA substrate. The ligation time was set at 4 hours, and the temperature was 37°C. The dye and electron carrier were MTT and PES.

Many of the assays developed to measure ligase activity work well for pure enzyme preparations, but fail to produce meaningful results for crude extracts or partially purified samples. Other components in the samples can interfere with either the ligase reaction or with the measuring of the ligated substrate. Enzymes that modify DNA can override the measurable changes that other assays monitor. The class of enzymes that would interfere with this new assay are the NAD$^+$ modifying enzymes. The majority of the NAD$^+$ altering enzymes are dehydrogenases. These enzymes convert NAD$^+$ to NADH. This conversion would destroy the ability of the ligase to utilize the NAD$^+$, but would not interfere with the colorimetric assay of NAD$^+$ concentration. Other than the ligase enzyme itself, there are few enzymes that use NAD$^+$ in a nonredox reaction, thus there would be few enzymes that could degrade NAD$^+$, and give false positive
Figure 10. Plot of utilization of NAD$^+$ by DNA ligase. The conditions are the same as Table 3.
data in a ligase assay.

The main interference encountered with the new assay involved the colorimetric part of the assay. The conversion of the dye appeared to be inhibited by the presence of proteins in crude samples. The first test of the new ligase assay on a crude cell lysate produced no color development. A test was performed to observe what might be the interfering component. (The results from these two tests are tabulated in Table 4.) As can be seen from the results, the interfering substance was found not to be the buffers, but rather a protein was seen to inhibit. Since the proteins tested did not originate in the bacteria from which the crude sample came, it is impossible to prove that a particular protein is responsible for the inhibition. However, it is an indication that the interfering component is a protein.

<table>
<thead>
<tr>
<th>Tube #</th>
<th>Test Substance</th>
<th>Dye Conversion (+/-)</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>50 ul (1.4 mg/ml) lysozyme</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>50 ul (1.4 mg/ml) BSA</td>
<td>+</td>
</tr>
<tr>
<td>3</td>
<td>50 ul crude extract of T-2</td>
<td>-</td>
</tr>
<tr>
<td>4</td>
<td>50 ul 1 M (NH₄)₂SO₄</td>
<td>+</td>
</tr>
<tr>
<td>5</td>
<td>50 ul 1 mM DTT</td>
<td>+</td>
</tr>
</tbody>
</table>

Table 4. Test of interfering substances.

In order to remove the interference problem, the
assay was modified to include an ultrafiltration of the sample after the ligase reaction occurred, but before the colorimetric reaction began. The filtration is accomplished through a membrane that possesses a molecular cut-off of 10,000 daltons. This pore size is adequately large to allow the NAD\textsuperscript{+} to pass through the membrane, but yet the majority of proteins are retained. Also included is a control that is used to measure the nonspecific absorption of NAD\textsuperscript{+} onto proteins. This control does not possess any DNA substrate, and thus there should be scarcely any ligase reaction occurring on the sample. (The results from a ligase assay using the modified protocol is given in Table 5). The results indicate an absorption of NAD\textsuperscript{+} by the crude sample, but also show that there is ligase activity by the further reduction on the amount of NAD\textsuperscript{+} present after incubation with the crude sample and DNA. Thus, it is manifest that the assay can provide meaningful data when used to measure the activity of DNA ligase in a crude sample.
<table>
<thead>
<tr>
<th>Tube #</th>
<th>Conditions</th>
<th>pmoles (NAD⁺)</th>
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<tbody>
<tr>
<td>1</td>
<td>no sample</td>
<td>48</td>
</tr>
<tr>
<td></td>
<td>no centrifugation</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>no sample</td>
<td>43</td>
</tr>
<tr>
<td></td>
<td>ultracentrifuged</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>100 ul crude extract</td>
<td>---</td>
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<tr>
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Table 5. Data from assay of DNA ligase activity in crude extracts from E.coli. The extracts were prepared by sonic disruption of bacterial cells, precipitation of DNA and associated enzymes with polyamine P and elution of enzymes into 10mM tris pH8.0.
LIGASE ASSAY METHODOLOGY

Materials: PES (Sigma)
Thiozyl blue (Sigma)
Alcohol Dehydrogenase (Sigma)
NAD⁺ (Sigma)
Salmon sperm (DNA Sigma)
DNase (Sigma)
DNA ligase (E. coli) (New England Biochemicals)
Centricon (Amicon)

Preparation of Substrate:
1. Dissolve 100mg salmon sperm DNA in 9ml 10mM tris, (pH 8.0), 1mM Na₂EDTA.
2. Add 1.0ml 10× DNase buffer (900mM tris pH 8.0, 100mM MgCl₂,10mM Na₂EDTA). Add 10ul DNase 3 mg/ml in 1× DNase buffer.
3. Incubate overnight at 37°C.
4. Incubate at 75°C for thirty minutes to stop digestion of DNA.
5. Dispense DNA into 100ul aliquots to use for ligase assays, and freeze at -20°C.

Ligase Assay
1. Add 25ul 10× LAB (100mM tris pH 8.0, 30mM, MgSO₄, 100mM NH₄Cl), and 50ul NAD⁺ 1.0uM to each aliquot of DNase digested DNA.
2. Add ligase samples to tubes. Mix thoroughly. For negative control use 50ul 1% BSA.
3. Add DD H₂O to bring final volume to 250ul.
4. Incubate at 37°C or appropriate temperature for two hours.
5. Add 1.0ml 10% EtOH in 10mM HEPES (pH 8.0).
6. (Optimal) To remove interference in crude samples centrifuge sample through Centricon. Collect material that goes through membrane. Measure an
aliquot of 1.0 ml of sample for use in remaining phase of assay.

7. Add 50ul dye reagent, (40mM phenazine ethyl sulfate, 10mM thiozyl blue).

8. Add 50ul alcohol dehydrogenase (4mg/ml).

9. Incubate for 3600 seconds at 30°C.

10. Add 1.0ml 12mM iodoacetate.

11. Measure and record OD<sub>570</sub>.

**Interpretation**

A set of standard solutions with known concentrations of NAD<sup>+</sup> should be run with each assay. These standards can be used to define the concentration of NAD<sup>+</sup> in the ligase tests and the negative control. A positive ligase reaction is indicated when the negative control possesses a higher concentration of NAD<sup>+</sup> than the ligase sample. If an ultrafiltration of the sample is performed (step 6), then the quantity of NAD<sup>+</sup> calculated from the calibration standards must be corrected for the amount of sample lost in the centrificon.
DISCUSSION

The DNA ligase assay presented has several advantages over the assays that had been tried earlier in the project. Each of these advantages alone might not be sufficient to justify the development of the assay, but combined they represent a significant improvement over other assays. These advantages are: 1) quantitative results; 2) stability, expense and safety of reagents; 3) use of a primary standard for measuring ligase activity, and; 4) avoidance of interference by other DNA modifying enzymes.

The use of the electrophoretic assay throughout the first year of the project pointed out the weakness of a qualitative assay. Without quantitative results, comparisons between separate runs of the assay can be difficult. Also, interpretations of the results is influenced by the experience of the investigator. Thus, artifacts are more easily interpreted as positive results during the beginning stages of a study when the experience of the investigator is less. In a purification project, the beginning of the project is also the point at which artifacts are more likely to exist. When the experience of the investigator is more extensive, and the sample has a greater purity,
qualitative assays might work better. Additionally, the use of a quantitative assay allows the investigator to evaluate the progress of a purification procedure in terms of activity, recovery and degree of purification.

The assay presented avoids the use of radiolabels and/or strongly mutagenic reagents. Avoiding these hazardous substances is especially an advantage to labs in which large numbers of such assays are run, and in which an increased work load causes more frequent mistakes. An additional difficulty with radiolabels is also avoided. Most highly sensitive radioactive assays use $^{32}$P as the label. The short half-life of this isotope renders substrates unusable within a couple of months after preparation. The only reagent that was seen to suffer from instability over a period of eight months was dilute solutions (1uM) of NAD$^+$. However, a 1mM stock solution was workable after six months if stored at 4°C. Also NAD$^+$ is stable in dry powder for several years.

Another advantage of this assay is that the reagents are easily prepared and inexpensive. Many other ligase assays are substrates that involve multiple steps of preparation. The substrates for this new assay are more easily produced because most of the preparation involves only dissolving the reagents. The only enzymatic preparative step is the digestion of the DNA with DNase, a one-step procedure.
One of the great advantages of this new assay is that it uses a primary standard to measure ligase activity. This standard is NAD$^+$ which can be weighed out and dissolved to a known concentration. Other assays must rely on the use of a characterized ligase sample as a standard for calibration of the results, which demands the purchase or preparation of a ligase standard that can be both time consuming and expensive. NAD$^+$, on the other hand, is inexpensive and requires only to be accurately weighed. Also, NAD$^+$ concentration is directly related to ligase activity since each ligation uses one molecule of NAD$^+$.

In monitoring the decrease in NAD$^+$ concentration, this assay avoids the majority of interference by other DNA modifying enzymes. Other enzymes that use DNA as a substrate are often copurified with DNA ligase during the beginning steps of a purification scheme. This copurification can be problematic when these other DNA modifying enzymes can render the DNA substrate unsuitable for measuring ligase activity. In this new assay the DNA substrate is present in excess, and is thus not as sensitive to the action of other DNA modifying enzymes.
REFERENCES CITED


APPENDIX

TEST FOR T-2 CULTURE PURITY

Introduction

When growing large quantities of bacteria, it is important to periodically check a sample of the cells to verify that the culture has remained uncontaminated. Gram staining is one procedure used to identify strains of bacteria. However, it is difficult to rely solely upon staining patterns to identify cell type. Unless there is a significant visual difference in appearance, the identification process can be quite difficult.

This difficulty can be avoided if a strain of bacteria is chosen that possesses a rare metabolic difference. In this manner, an assay of that metabolic uniqueness can be used to verify purity. T-2 possesses a metabolic trait that can be used in a purity verification assay. This trait is the absence of a pentose shunt. More specifically, the lack of any activity for glucose-6-phosphate dehydrogenase, (the first enzyme in the pentose shunt pathway), can be used in this way. Bacillus Stearothermophilus does have glucose-6-phosphate dehydrogenase activity, and a clear distinction can formed between these two organisms.

An assay was developed previously to detect glucose-
to achieve consistent results. This assay used the absorbance of NADPH at 340nm.

The reaction catalyzed by glucose-6-phosphate dehydrogenase is:

\[
\begin{align*}
\text{HCOH} & \quad \text{NADP}^+ \\
\text{HOCH} & \quad \text{NADPH} \\
\text{HCOH} & \quad \text{HOCH} \\
\text{CH_2OPO_3}^--2 & \quad \text{HC} \\
\text{C=O} & \quad \text{HCOH} \\
\text{HOCH} & \quad \text{HCOH} \\
\text{HC} & \quad \text{HC} \\
\end{align*}
\]

glucose-6-phosphate \quad \rightarrow \quad \text{6-phosphogluconolactone}

Stearothermophilus can utilize either NAD$^+$ or NADP$^+$ as an electron acceptor in this reaction.

The developed assay measures the appearance of the reduced form of the cofactor. The production of NADPH (NADH) is coupled to another redox reaction that forms a dye with an absorbance at 503nm (11). This enables a visual assay to develop since the positive reaction results in a bright red solution. The reaction is two-fold. An electron carrier is reduced by the NADPH (NADH), and thus reduces a tetrazolium salt. The electron carrier is phenazine methosulfate, (PMS) and the tetrazolium salt is 2-p-iodophenyl-3-p-nitrophenyl-5-phenyl tetrazolium chloride (INT). Very small quantities of PMS and NADP are required for the assay, because both are recycled in the coupled reaction. The
absorbance of the reduced form of INT is three-fold greater than NADPH, and this also increases the sensitivity of the assay.

Solutions
I. Glucose-6-phosphate (.9g/100ml)
   Tris pH 8.2 (2.42g/100ml)
II. PMS (12.5mg/25ml)
   INT (50mg/25ml)
   NADP⁺ (10mg/25ml)
   NAD⁺ (100mg/25ml)

Procedure
1. Suspend 1-2 grams of T-2 cells from centrifuged pellet in 5ml 100mM Tris pH 8.0.
2. Pass cells through french pressure cell or other mechanical lysing procedure.
NOTE: Do not use lysozyme due to interference with assay reactions.
3. Place 1.0ml of solution I in a clean 3ml test tube.
4. Add 25ml of lysate to test tube and vortex.
5. Place in 65°C bath for five minutes.
6. Add 100ml of solution II and vortex.
7. Wait ten minutes. If red color appears, then test is positive, and glucose-6-phosphate is present.
   If color remains unchanged, then test is negative.
NOTE: A blank consisting of 10mg/ml bovine serum albumin should be run. Also, a blank of glucose-6-phosphate dehydrogenase should be checked.