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Exploring the Availability of *NdeI* Cut Ends For Additional Molecular Reactions

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***NdeI* digested fragments have a low ligation efficiency, and the cause is unknown. One possible explanation is the unavailability of *NdeI* digested ends to the ligase enzyme. Using PCR, a DNA fragment was created, which was the same sequence and size of pUC19 digested with *NdeI* and *HindIII*. The *NdeI/HindIII* digested fragment had slower mobility and appeared to be larger than its PCR counterpart when run on a polyacrylamide gel. To assess the accessibility of the 3' ends of *NdeI* cuts, the 3' recess was filled with a biotinylated dATP using Klenow enzyme. Only one 3' end appeared to be available to the Klenow enzyme. However due to unequal retaining efficiency of the fragments to nitrocellulose, the labelling efficiency of each end of *NdeI* and *HindIII* remain unknown.**

Cloning of fragments into vectors is an important technique in molecular biology. This involves the use of restriction enzymes to cut the fragment and vector, and ligase to join them together. From previous studies, *NdeI* appears to have lower ligation efficiency than *HindIII* in both λ DNA and pUC19 (1, 2).

Different studies have been conducted to determine the reason for the difference in ligation efficiency. Some studies looked at the possibility of sequence preference by ligase. Larger fragments appeared to ligate better than smaller fragments (4). GC rich overhangs demonstrate a better ligation efficiency then AT rich overhangs and longer overhangs ligate better than shorter ones (4). However, *SspI,* which is a blunt end restriction enzyme flanked by AT, had better ligation efficiency than *NdeI*, which had a two nucleotide AT overhang (4).

Other studies have examined the activity of *NdeI* enzyme itself. *NdeI* once heat inactivated, no longer had enzymatic activity even when returned to non-denaturing temperature (5). Therefore, it seems that the lower ligation efficiency was not due to reactivation of the restriction enzymes. The possibility that *NdeI* remained on the fragment preventing ligase from interacting with the fragment was also examined. However, the results were inconclusive due to inconsistency in the mobility in the electric field and the buffer used to run the agarose gel (5).

Based on previous studies, the possibility that *NdeI* cut ends are distorted and therefore unavailable is feasible. Figure 1 shows the different ends that would be involved in different molecular reaction and the designations that will be used in this paper. If *NdeI* remains bound to the DNA, it would retard the mobility of the DNA through an agarose or polyacrylamide gel. Filling in the 3' recess ends with biotinylated-14-dATP was attempted to determine if enzymes, other than ligase, are affected by *NdeI* digestion.

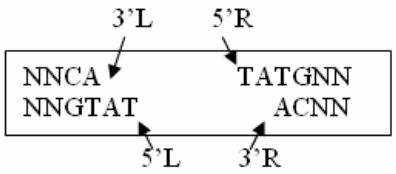

FIG. 1 The cut site by *NdeI* restriction enzyme. The separated ends are arbitrarily designated left and right to distinguish between the four different positions that may be involved in molecular reactions.

MATERIALS AND METHODS

pUC19 extraction. *Escherichia coli* DH5α containing the pUC19 plasmid was grown overnight in 100mL of LB broth, which contains 10g/L of tryptone, 10g/L NaCl, and 5g/L of yeast extract, with 100μg/mL ampicillin. The plasmid in the culture was isolated in a Maxiprep (Invitrogen K2100-07) according to manufacturer's instruction. The DNA pellet was dissolved in TE buffer, which contains 10mM TrisHCl and 1mM EDTA at pH7.5, and the plasmid concentration was determined using Pharmacia Biotech Ultraspec 3000.

Creating Fragments from pUC19. Half a microgram of pUC 19 was digested with 5 units of *HindIII* (Fermentas ER0501) and *ApaII* (Fermentas ER0041) each in 1X Tango buffer in a reaction volume of 30μl. Sample was incubated at 37°C for 2hrs and heat inactivated by heating to 65°C for 20min. This was repeated with pUC19 digested with *HindIII* and *NdeI* (Fermentas ER0582) in 1X buffer R. Products were run on a 1.5% agarose gel to ensure that complete digestion occurred. pUC 19 was also used in a PCR reaction to amplify a fragment similar to the *HindIII/NdeI* digested fragment. Primers used were catR- 5' CTT GCA TGC CTG CAG GTC 3', and catF- 5' ATG CGG TGT GAA ATA CCG C 3'. The reaction contained 0.2mM dNTP, 1X Taq buffer, 0.5u Taq (Fermentas EP0402), 1μM catR, 1μM catF, 10ng pUC19, and 2mM of $MgCl_2$ in a 20μl volume. The PCR conditions included an initial denaturation of 95°C for 3 min, then 32 cycles of 95°C for 1 min, 68°C for 30sec, and 72°C for 2 min, followed by a final extension of 72°C for 1 min. The product was run on a 1.5% agarose gel to ensure specific amplification.

Polyacrylamide gel. An acrylamide gel was used to resolve the various fragments. The resolving gel contained 8% 29:1 acrylamide in 1X TBE at a final volume of 6mL with 100uL of 10% APS and 5ul of TEMED for polymerization. The stacking gel contained 3.5% acrylamide. A 1kb plus ladder (Invitrogen 12308-011) was used.

The gel was run at 23V at 4°C in an ice bucket overnight, approximately 15hrs. The gel was then stained in a solution of 0.2µg/mL of ethidium bromide and visualize under a transilluminator.

Labelling *NdeI/HindIII* fragment. Two microgram of pUC19 was digested with *NdeI* and *HindIII* as described above. It was then labelled with biotin-14-dATP (Invitrogen 19524-016). The reaction used 20µM of each biotin-14-dATP, dGTP, dTTP, and dCTP, 1µg of digested pUC19, 0.5u of Klenow (Invitrogen 18012-021), in 1X REact 2 Buffer in 30µl volume. Samples were left at room temperature for 15min. Reaction was then stopped with phenol extraction, heat inactivation, or left untreated. For phenol extraction, 100ul of water and 130ul of phenol was added to 30ul of biotinylated sample. The sample was mixed, and spun at 9000rpm for 1 min. Eighty microliter of the aqueous layer was extracted for ethanol precipitation. Then 8uL of 3M sodium acetate and 150ul of cold 100% ethanol was added to the sample, mixed, and left at -80°C for 1hr. The sample was then spun at 13000rpm for 15 min, air dried, and resuspended in 20ul of water. For heat inactivation, the sample was heated to 65°C for 15min. Half a microgram of the labelled products were digested with 2u of *PvuII* (Fermentas ER0631) in 1X buffer R in a 20uL volume. The reaction was incubated at 37°C for 2hrs. Some of the sample that used phenol extraction was then digested with 2u of *AatII* (Fermentas ER0991) in 1X tango, *Cfr10I* (Fermentas ER0181) in 1X cfr buffer, or *AflIII* (New England BioLabs R0541S) in 1X NEBuffer 3 by incubating at 37°C for 2hrs. This was done to create different sizes from the large *PvuII/NdeI* fragment. Figure 2 shows the cut sites of restriction enzymes on pUC19 that were used in this paper.

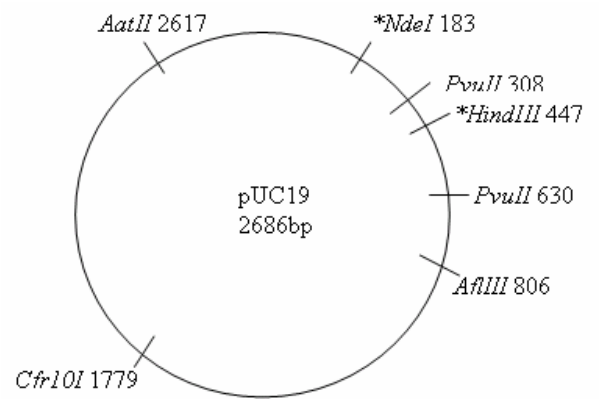

FIG. 2 Cut sites and position number on pUC19 of restriction enzymes that were used in these experiments. (*) refers to the biotin labelled sites.

Blotting. All the samples and a 1kb plus standard were then used in a 2% agarose gel (Amresco agarose 3:1, 9012-36-6). The gel was stained by soaking for 20min in 0.2ug/mL of ethidium bromide and visualized. Then it was soaked in a denaturing solution of 1.5M NaCl and 0.5M NaOH for 30 min at room temperature, and then soaked in neutralizing solution of 1.5M NaCl and 0.5M TrisHCl (pH 7.5) for 30min. It was then blotted overnight to transfer the fragments from the gel to a nitrocellulose membrane (BioRad 1620114) in 20X SSC. The membrane was allowed to air dry at room temperature for 5min. Then 300ng of the positive control (biotinylated primer: Eub338B) was spotted onto the membrane. The membrane was then baked at 80°C for 2hrs.

Detection. The membrane was blocked in 1X blocking solution (Roche 11921673001) diluted in 1X TBS-T, which contains 7.88g of TrisHCl, 8.76g NaCl and 0.05% tween in 1L total volume at pH7.5 for 1hr. Then the membrane was rinsed with 0.5X blocking solution and incubated at room temperature with Streptavidin-POD (Roche 11089153001) at 1:2000 dilution in 0.5X blocking solution. The membrane was then washed with 1X TBS-T 4 times at 5min intervals. The lumilight plus substrate (Roche 12015200001) was added to the membrane and incubated for 5 min and then exposed to film for 30sec.

RESULTS

Polyacrylamide gel. Table 1 lists the fragments that can be seen in the acrylamide gel in figure 3. The fragments generated by *NdeI* and *ApalI* appears to be larger than their expected size, even though the PCR fragment migrated at the expected size.

Table 1. Summary of the DNA fragment sequences and calculated lengths. (*) indicates the cut sites. Lengths from sequences were calculated from the start of one cut site to the next. Calculated lengths have an uncertainty of 7bp.

Fragment	Observed ends for different combination of digests		Length from Sequence (bp)	Calculated Length from Gel (bp)
ApaII/HindIII	G*TGCA C	A*AGCT T	270	282
	C ACGT*G	T TCGA*A		
NdeI/hindII	A*TA T	A*AGCT T	263	275
	T AT*A	T TCGA*A		
PCR amplified	AT*AT	AG*CT	264	264
	TA*TA	TC*GA		

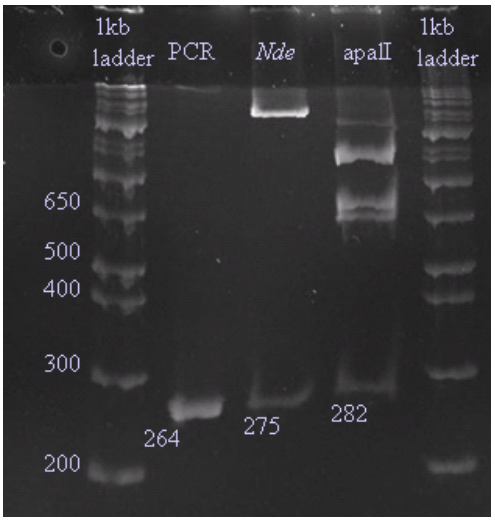

FIG. 3 Size distribution of pUC19 fragment generated by PCR, *NdeI/HindIII* double digest, and *ApaII/HindIII* double digest on an 8% polyacrylamide gel.

Labelling Reaction. The *NdeI/HindIII* digested samples were labelled with biotinylated ATP then digested with *PvuII* to give fragments that were separated on an agarose gel and blotted onto a nitrocellulose membrane. The gel was loaded according to the order in Table II, which also shows the size of the fragments that have been labelled. Figure 4

showed the developed film and its corresponding agarose gel. As can be seen, the strongest band is the L end of *NdeI.* The 139bp and 124bp bands did not separate as can be seen clearly in lane six and seven in figure 4b. The first four lanes showed that the same L end of *NdeI* cut to different lengths by restriction enzymes have different intensities. The intensity of each band was quantified using a grey scale with white at 225 and black at 0, so the brighter samples have a smaller number, using Corel Photo-Paint 9 and the box area method. The larger fragment appeared to have stronger intensities even though each band had the same number of labelled sites. The corresponding band intensity can be seen in table II. The correlation of length to intensity can be seen in figure 5. There appeared to be no difference between phenol extracted, heat inactivated, and untreated sample.

Table 2 Summary of the predicted contents of each lane in figure 4 and the corresponding fragment lengths (bp) in each lane. Bands that can be seen on the film in fig. 4a also show the quantify intensity in greyscale units for lanes one to four. Intensities for lane 6-8 were not included, since their intensity values were not required for interpretation. (n/a) indicates that no bands can be seen on the film. The more intense bands have a lower greyscale value.

Lane	Sample	Predicted Labelled Fragments								Predicted Unlabelled Fragments
		(L) *NdeI*		(R) *HindIII*		(L) *HindIII*		(R) *NdeI*		
		length	Intensity	length	Intensity	length	Intensity	length	Intensity	
1	*NdeI*, *HindIII*, PvuII digest (phenol extracted)	2241	82	183	107	139	n/a	124	n/a	
2	AflIII digested	2065	87	183	108	139	n/a	124	n/a	176
3	Cfr101 digested	1091	94	183	111	139	n/a	124	n/a	1149
4	AatII digested	249	110	183	113	139	n/a	124	n/a	1992
5	1kb plus ladder									
6	*NdeI*, *HindIII*, PvuII digest (heat inactivated)	2241		183		139		124		
7	*NdeI*, *HindIII*, PvuII digest (untreated)	2241		183		139		124		
8	positive control									

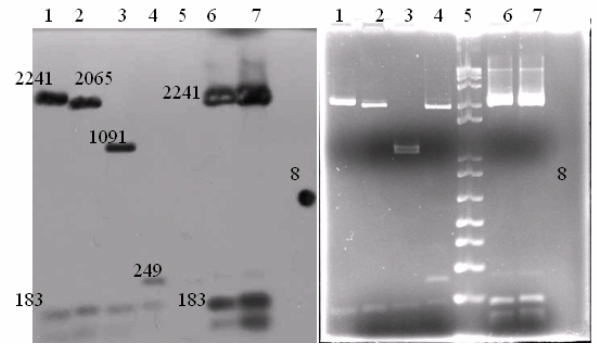

FIG. 4 Assessment of biotin labelled pUC19 fragments predicted in the summary in Table II. a) Luminescent pattern when autoradiograph film is exposed to the blot developed in streptavidin-POD to make a luminescent product and b) band pattern on the ethidium bromide stained agarose gel that was used to prepare the nitrocellulose blot.

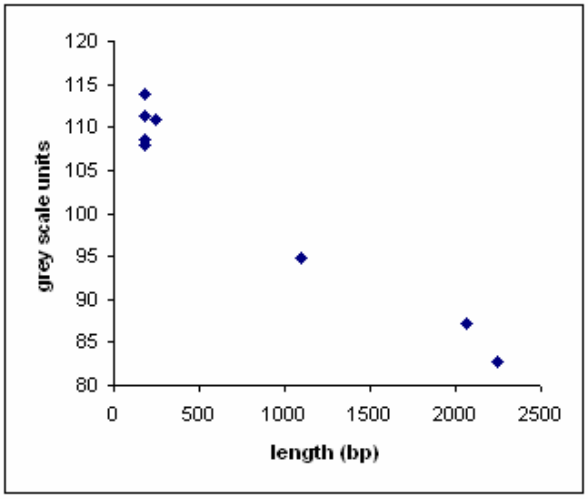

FIG. 5 Relationship between intensity of band brightness and the corresponding observed fragment length.

DISCUSSION

The *NdeI* fragment, R end, appears to be larger than its PCR counterpart (table I). This suggests that *NdeI* remained bound on the fragment or that the ends are convoluted, therefore not linear, and retards movement. The *ApaII* digested fragment is also bigger than predicted. However, since a ligation efficiency test was not performed, it is difficult to speculate the cause. Since the samples were run on the gel are flank by DNA ladders, the results are unlikely to be due to artefacts in gel electrophoresis.

The 3'L end from *NdeI* has the strongest luminescent signal after labelling with biotinylated ATP and the largest fragment at 2241bp even though all fragments should be equally labelled. There were several potential explanations. One possible explanation is that the larger fragments are retained on the membrane better than smaller fragments. According to the manufacturer, 0.2um pore size nitrocellulose membranes are recommended for fragments smaller than 500bp (Bio-Rad bulletin 1939, Bio-Rad Co., Hercules, Ca). The smaller fragments may have passed through the 0.45um membrane that was used. This may also explain the strong intensity from the positive control, since the biotinylated primer was spotted on instead of transferred through capillary action of blotting. However, since the positive control contains many more molecules of biotin, it is difficult to speculate whether smaller fragments passed through the membrane during the blot or the fragments were washed off the membrane during one of the steps. Another explanation is that nick translation could have occurred in the larger fragment causing more than one biotin to bind per fragment. Klenow enzyme does have the ability to displace one of the strands and extend

from a nick site (6). A third explanation is that smaller fragments could have been lost in the phenol extraction, or the 3'L end has strong affinity to Klenow. Since there was no difference between the phenol extracted, heat inactivated, and untreated sample, it is unlikely that the strong signal is due to significant loss during the phenol extraction. To determine which one of the explanations was most likely, the labelled samples were digested with *AflIII*, *Cfr101*, or *AatII*, each of which cuts once somewhere within the labelled 2241bp fragment. From these digests, it was determined that no nick translation occurred, because nick translation would have labelled the whole fragment but only the 3'L *NdeI* fragment had a signal. Since all the fragments contained the 3'L end and the same amount was loaded into each lane, the differences in intensity would be due to retention of the fragments to the membrane. As can be seen in figure 5, the larger fragments have better the retention to the nitrocellulose. Without knowing the exact relationship between the fragment retention and size, rather linear or exponential, it is impossible to compare the labelling efficiency of each end in this experiment. It is also difficult to extrapolate an accurate relationship with the few data points that were available. What can be concluded is that 3'L of *NdeI* is open for labelling, as are both 3' *HindIII* ends. Although a 2% agarose gel can resolve the smallest fragment, including the R end of *NdeI* and *HindIII*, this particular gel did not (figure 4b). It is unclear if the 3'R of *NdeI* is free to be labelled.

FUTURE EXPERIMENTS

In order to compare labelling efficiency of the *NdeI* ends and *HindIII* ends, the fragments need to be of similar size. It would also be advantageous to discover the relationship between fragment size and nitrocellulose retention. This would show if there is a range of fragment size with similar retention but will still be resolved on a gel. Another consideration is to use a restriction enzyme with the same overhang. The longer *HindIII* overhang may allow Klenow enzyme to attach better. However, the restriction enzyme must first be tested for ligation efficiency.

Another question to be considered is the availability of other ends. Using Klenow fragment, we explored 3' ends of the *NdeI* cut site. However, there is still the 5' end to be considered. One possibility is to use T4 polynucleotide kinase (T4 PNK) to transfer the γ-phosphate from an ATP to the 5'-OH group.

Another option to study the ends is to do PCR using a series of primers, each starting a nucleotide away from 3' or 5' end. If the 3' ends are blocked by *NdeI* or convulated, the primer will be unable to bind, and we will be able to determine how far along the sequence the end is blocked. If the 5' ends are unavailable, the PCR should be unable to extend to the end, so that the reverse primer has nothing to anneal with. However, due to the nature of PCR, as long as the 3' end of the primer anneals, it is possible for extension to occur. There will most likely be less amplification, since the primer is only partially annealed and fewer extensions will occur per cycle. Possibly instead of a traditional PCR with amplification to endpoint, a quantitative PCR should be used with fewer cycles to compare the number of product present after PCR using whole pUC19 or digested pUC19 as template.

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