Cloning of *Polo* into the selection vector pBluescript.

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Introduction

Molecular cloning is a common technique of molecular biology that allows copying, manipulation or isolation of a specific DNA sequence. Often the DNA sequence of interest is a complete gene, which is inserted into another DNA molecule called a vector to form a circular plasmid, and then introduced into a host. Usually, the host cells (commonly *Escherichia coli*) are grown in culture, where they replicate the cloned plasmid as they divide. The high quantity of replicated plasmid can then be purified from the culture and used in downstream applications.

A fundamental concept in cloning is the use of restriction enzymes to introduce double stranded breaks in a DNA strand at a predictable location. Restriction enzymes are isolated from bacteria and recognize specific, palindromic DNA sequences that are 2-8 base pairs long. Digestion of the desired insert gene and the vector with the same restriction enzymes produces cuts at matching sequences between the two strands. This enables insertion of the insert into the vector at a specific location using a ligation reaction. After ligation, the vector and insert are now circularized into a plasmid, which can be transformed into bacterial cells. Circularization is important as bacterial cells recognize linear DNA as foreign and degrade it. The vector usually contains a gene coding for antibiotic resistance that allow selection for transformed cells only. The vector can also contain sequence elements or genes that allow controlled expression of the insert gene, or selection for presence of the insert.

In this experiment, the vector being used is a plasmid called pBluescript (pBS; Figure 1), which encodes two genes used for selection. pBS encodes an ampicillin resistance gene, allowing the transformed bacteria to be grown on ampicillin containing media to select for cells with plasmid. It also contains the gene LacZa, coding for the alpha subunit of β-galactosidase. This polypeptide combines with other β-galactosidase subunits which are endogenously expressed and forms an enzyme that can break down a substrate present in the media to a blue precipitate. This is used to select for plasmid containing the insert: because the restriction enzyme recognition sites used for the insertion are within the LacZa coding sequence, the colour of a colony reveals if the LacZa gene is intact or disrupted. Blue colonies mean the LacZa sequence is intact, whereas a white colony means it was disrupted and there is likely a successful insertion into the pBS vector.

The restriction enzymes used in this experiment are XBaI and BamHI. Both of these enzymes produce double stranded breaks that are staggered, meaning there is a few nucleotide stretch of single stranded DNA on each end of the double stranded break. These stretches are complementary with other sites of the same restriction enzyme due to the palindromic nature of the recognition sequences. These 'sticky ends' are excellent to promote directional hybridization by base pairing and then ligation of two different DNA fragments digested with the same enzymes. The restriction sites are each present exactly once within the LacZa coding sequence of pBS (Figure 2) with a twelve base pair region between them.

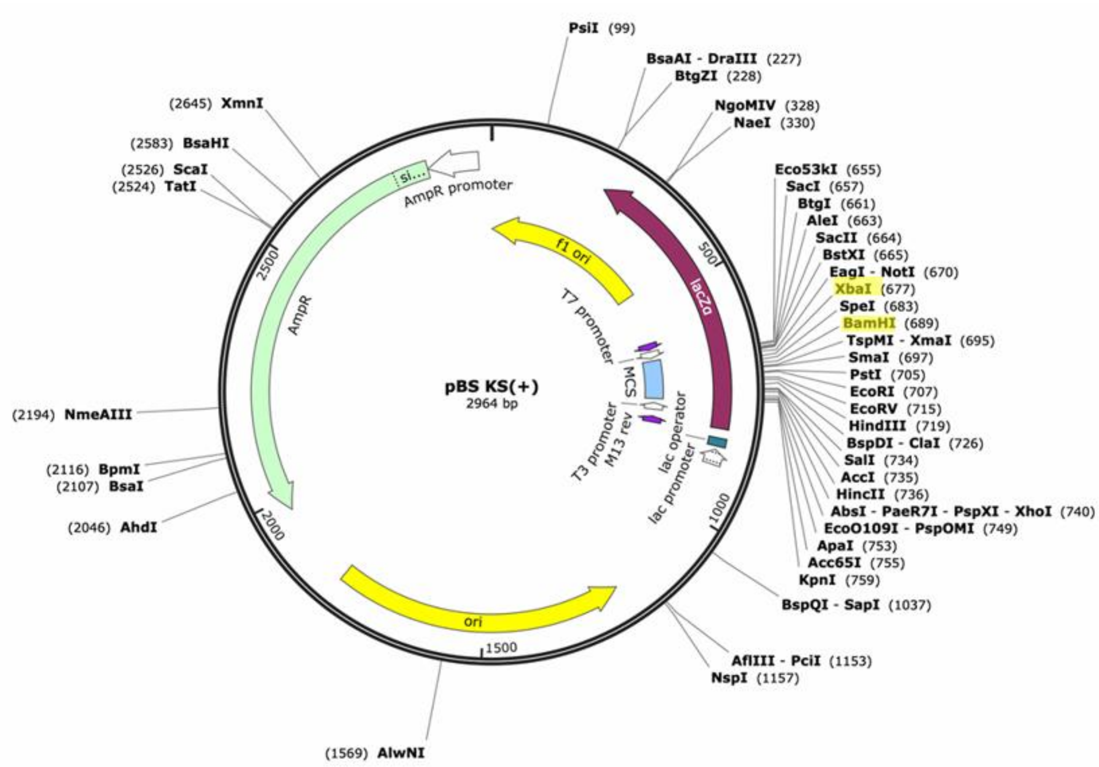

Figure 1. Map of the plasmid pBluescript used in this experiment. XbaI and BamHI restriction sites are present in the LacZa coding sequence. (Image from Bourouh 2026)

In this experiment the gene *Polo*, which codes for a mitosis-regulating kinase in *Drosophila melanogaster* will be amplified by PCR and then cloned into pBS. *Polo* is an essential gene in fruit flies, but in this case its function is not relevant, and other genes could have been used in its place. The amplification of *Polo* will be done from genomic DNA extracted from *Drosophila melanogaster* adults and purified using phenol-chloroform. After ligations and transformation, the success of the cloning will be evaluated by redigesting the propagated plasmid from a white colony and visualization by gel electrophoresis. If *Polo* was successfully inserted, the digestion would excise the *Polo* insert back out of the plasmid, and the corresponding bands would appear on the gel.

Results

Genomic DNA extraction and amplification of *Polo*

The coding sequence for the kinase *Polo* was first amplified by PCR for cloning into pBluescript (pBS). Genomic DNA was extracted from ten *Drosophila melanogaster* adults using an SDS based lysis buffer and phenol-cholorform purification. A yield of 92.6 ng/µL in 50 µL Tris-HCl was obtained (purity ratios: 260/230 = 2.05, 260/280 = 1.09). During the DNA extraction, samples of extract before (Tube A) and after (Tube B) the RNase digestion but before the phenol chloroform step were taken for diagnostic purposes. The extracted gDNA was used as the template for a PCR amplification using a forward primer with a 5' XbaI recognition sequence and linker, along with a reverse primer with a 5' BamH1 recognition sequence and linker:

XbaI-*Polo*-FOR 5'-GATC-TCTAGA-AAAGGAGAGCAAGATGGCCGC-3'
BamH1-*Polo*-REV 5'-GATC-GGATCC-TGAACATCTTCTCCAGCATTTTCC-3'

To evaluate the results of the gDNA extraction and the PCR, all samples were run on 1% agarose gels in TAE buffer (Figure 1). The PCR reaction mixture had a reduced volume after thermal cycling, suggesting the tube or lid was not sealed properly and the water evaporated off. Since the volume was unusable downstream, two new PCR reactions were prepared using 50 ng of another gDNA extract and validated on a separate agarose gel (Figure 1B).

The RNase digestion step was successful as cellular RNA is visible only in the sample from before the digestion (Tube A), and not after (Tube B; see Figure 1A). Notably, the high molecular weight band labelled as gDNA was present in both tubes A and B, but not observed in the final extract after the phenol-chloroform step (Figure 1A). The PCR product showed a strong band that matched the expected molecular weight of the *Polo* amplicon (~3200 bp), with no off-target bands visible, suggesting it is suitable for downstream use. The PCR products were then combined and purified by isopropanol precipitation to yield 873.8 ng/µL in 10 µL water.

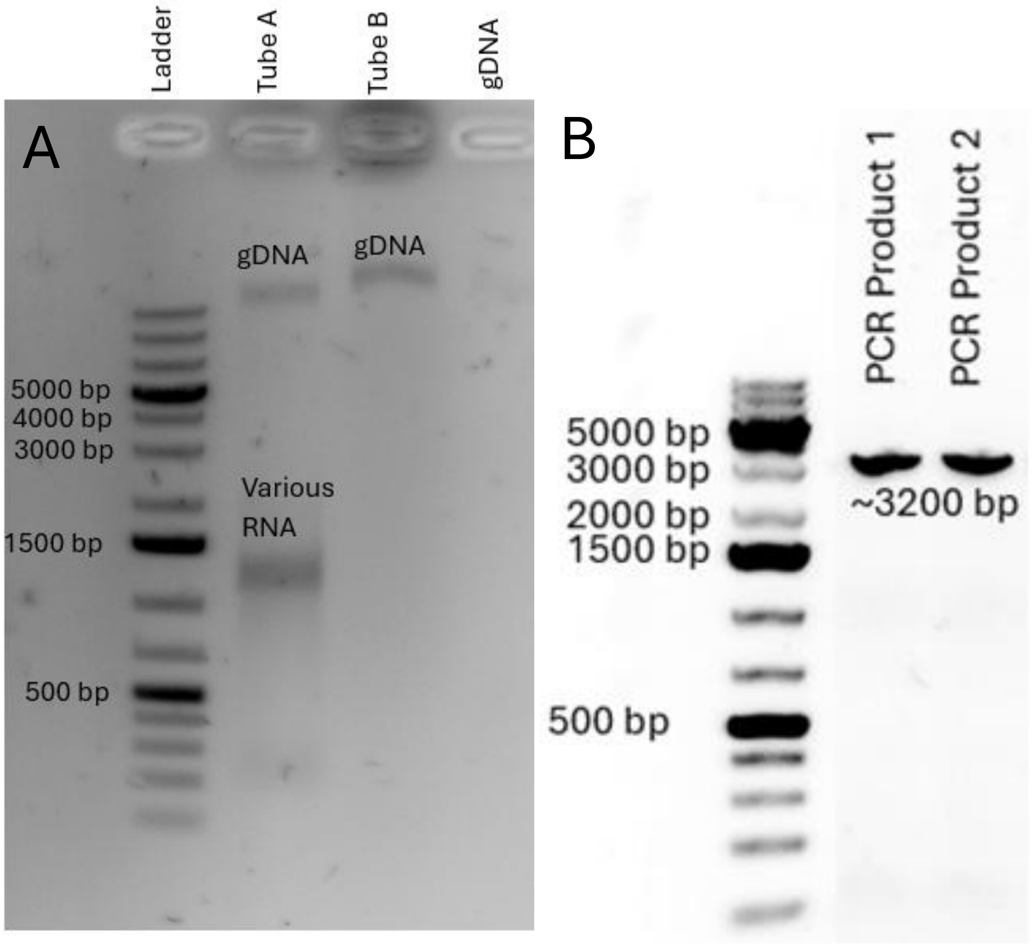

Figure 2. A) Gel electrophoresis of *Drosophila* genomic DNA extraction samples, and (B) a PCR product of the *Polo* gene.

Restriction enzyme digestion

To continue the procedure of cloning the *Polo* gene into pBS, both the PCR product and the plasmid were digested with two restriction enzymes. The restriction enzymes used, XbaI and BamH1, both have exactly one restriction site on the PCR product and on pBS (within the LacZa coding sequence) where overhanging ends will be produced. For diagnostic purposes, pBS was also digested with each enzyme individually, and with no enzyme as a negative control. The negative control, single digests, and PCR product all had 1 µg of DNA digested, while 5 µg of pBS was double digested. Extra pBS was used for the double digestion as the efficiency of the subsequent gel extraction was expected to be low. Each of the pBS digestions were run on a 1% agarose gel in TAE buffer to evaluate the results (Figure 3, left). Importantly, the linearized, single digested pBS samples are distinguishable on the gel from the circular, undigested pBS even though they are the same molecular weight because of supercoiling. This is observed in Figure 3 with the band at ~2700 bp, along with the band at ~4100 bp which could be the non-supercoiled circular form of pBS, which would run slower down than gel than the linearized version. The gel results also indicate that both restriction enzymes worked independently of one another and linearized pBS by producing one double stranded break. The double digested pBS produced a much stronger band and also appears to be a linear molecule with a slightly smaller molecular weight than the single digested controls. Furthermore, the bands of each treatment match when compared to an example gel (Figure 3, right).

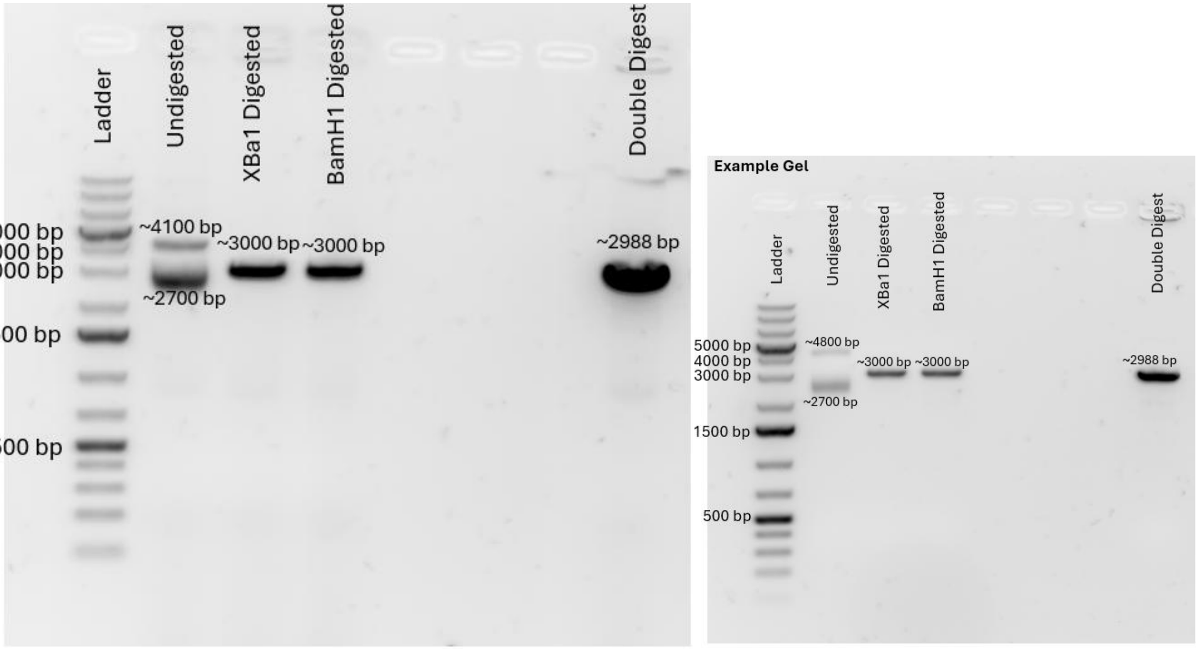

Figure 3. Restriction digest reactions of pBluescript visualized by gel electrophoresis. Left: Experimental gel completed in this experiment. Right: Example gel from a known successful experiment for comparison.

Gel Extraction and Ligation Reactions

Next, a gel extraction was completed to purify the double digested pBS sample. The double digested band was excised and incubated at -80 °C for five minutes before being spun down through a filter for five minutes at max speed. About 11 µL of 32.1 ng/µL eluate was obtained (purity ratios: 260/280 = 1.73, 260/230 = 0.13). Before ligations, the digested PCR product and gel extraction eluate were both purified by isopropanol precipitation to yield 223.9 ng/µL PCR product (purity ratios: 260/280 = 1.78, 260/230 = 1.88) and 15.6 ng/µL plasmid digest (purity ratios: 260/280 = 1.91, 260/230 = 1.92) each in 10 µL of water. The efficiency of the gel extraction was 3.1%, with 156 ng of pure plasmid obtained (see Calculation 1).

Calculation 1. Gel extraction efficiency.

$$15.6\frac{ng}{\mu L} \times 10\ \mu L \times \frac{1}{5\ \mu g\ into\ gel} \times 100\% = 3.1\%$$

Both the PCR product and plasmid digestion had good purity ratios, and the precipitation effectively removed the large amount of salt and organic impurity from the gel extraction eluate. Ligation reactions containing vector-to-insert molar ratios of 1:3 and 1:5 were prepared, along with a negative control containing no insert. All three reactions contained 50 ng of digested pBS. The 1:3 reaction contained 163 ng of digested PCR product and the 1:5 contained 272 ng (see Calculation 2). The purified digested pBS from the gel extraction was too low yield and was only able to be used for the 1:5 ligation reaction, with the other two reactions containing digested pBS prepared by the instructor. Ligation reactions were incubated at 18 °C overnight.

Calculation 2. Molar ratios of ligation reactions.

Size of insert = 3220 bp
Size of vector = 2964 bp

$$\frac{5\ insert}{1\ vector} \times 50\ ng\ vector \times \frac{3220\ bp}{2964\ bp} = 272\ ng$$

$$\frac{3\ insert}{1\ vector} \times 50\ ng\ vector \times \frac{3220\ bp}{2964\ bp} = 163\ ng$$

Bacterial Transformation and Cloning Validation

The ligated plasmids were transformed into competent *Escherichia coli*. A volume of 5 µL of the three 20 µL ligation reactions (1:3 and 1:5 vector-to-insert ratio, negative control) was added to three 50 µL aliquots of homemade chemically competent *E. coli* on ice. A positive control of 50 ng undigested pBS was also added to an aliquot of cells. Transformation was completed by 30 second heat shock at 42 °C followed by 1 hour incubation in SOC media at 37 °C. Cultures were then spun down and resuspended in 100 µL before being spread-plated onto X-Gal/IPTG/Amp agar and incubated overnight for subsequent blue/white selection (Figure 4). Both controls showed the expected results with no colonies on the negative control (no insert ligation; Figure 4C), and a good number of blue colonies (but no white colonies) on the positive control plate (Figure 4D). The number of colonies on the positive control plate suggests the transformation procedure was successful. Interestingly, only the 1:3 ligation plate (Figure 4A), but not the 1:5 ligation plate (Figure 4B), had colony growth, with at least 4 white colonies.

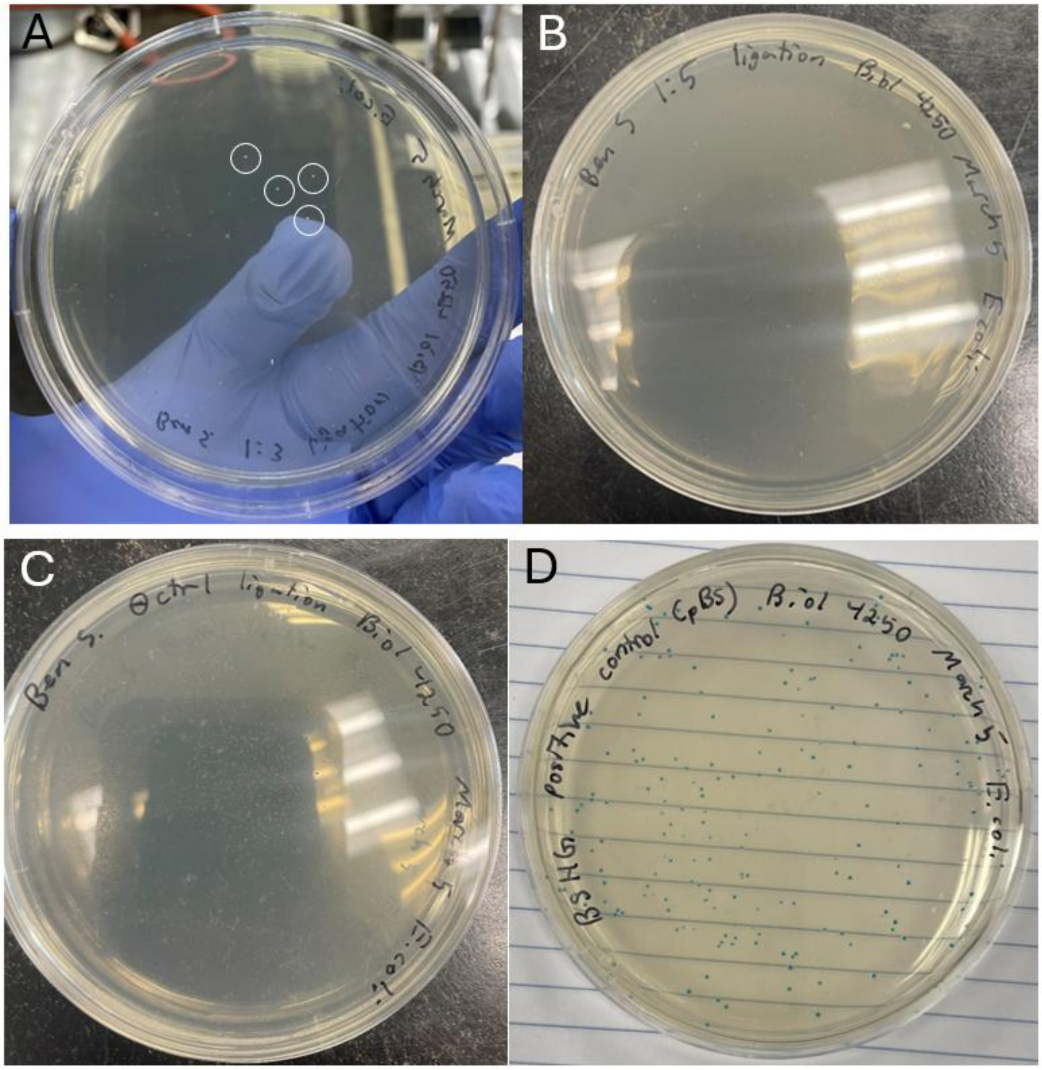

Figure 4. Plated transformations of A) 1:3 ligation ratio, B) 1:5 ligation ratio, C) no insert ligation negative control, D) transformation positive control. White colonies are outlined on the 1:3 ligation plate.

To validate that both the blue and white colonies contained the expected plasmid, they were first inoculated into 5 mL, ampicillin containing broth cultures and incubated overnight. Plasmid was isolated from 3 mL of the overnight culture using an NaOH containing lysis buffer to select against genomic DNA. The purified DNA was resuspended in 50 µL of TE buffer and a yield of 56.8 ng/µL was obtained from the blue colony culture (purity ratios: 260/280 = 1.90; 260/230 = 1.77) and 155.6 ng/µL was obtained from the white colony culture (purity ratios: 260/280 = 1.73; 260/230 = 0.86). Each plasmid was then digested with XbaI and BamH1. The digestions were visualized with gel electrophoresis (see Figure 5). Plasmid isolated from the cultured blue colony should be intact, circular pBluescript, and the gel suggests this was the case. The single digested bands appear at ~3 kb, the expected molecular weight of linearized pBS, while the undigested, circular plasmid runs faster due to supercoiling and appears at ~2.5 kb. These along with the double digest, agree with what was seen on the previous gel with restriction digests of pBS (see Figure 3). The digestions of plasmid isolated from the cultured white colony did not produce any bands, suggesting the colony did not contain any plasmid and the cloning was unsuccessful.

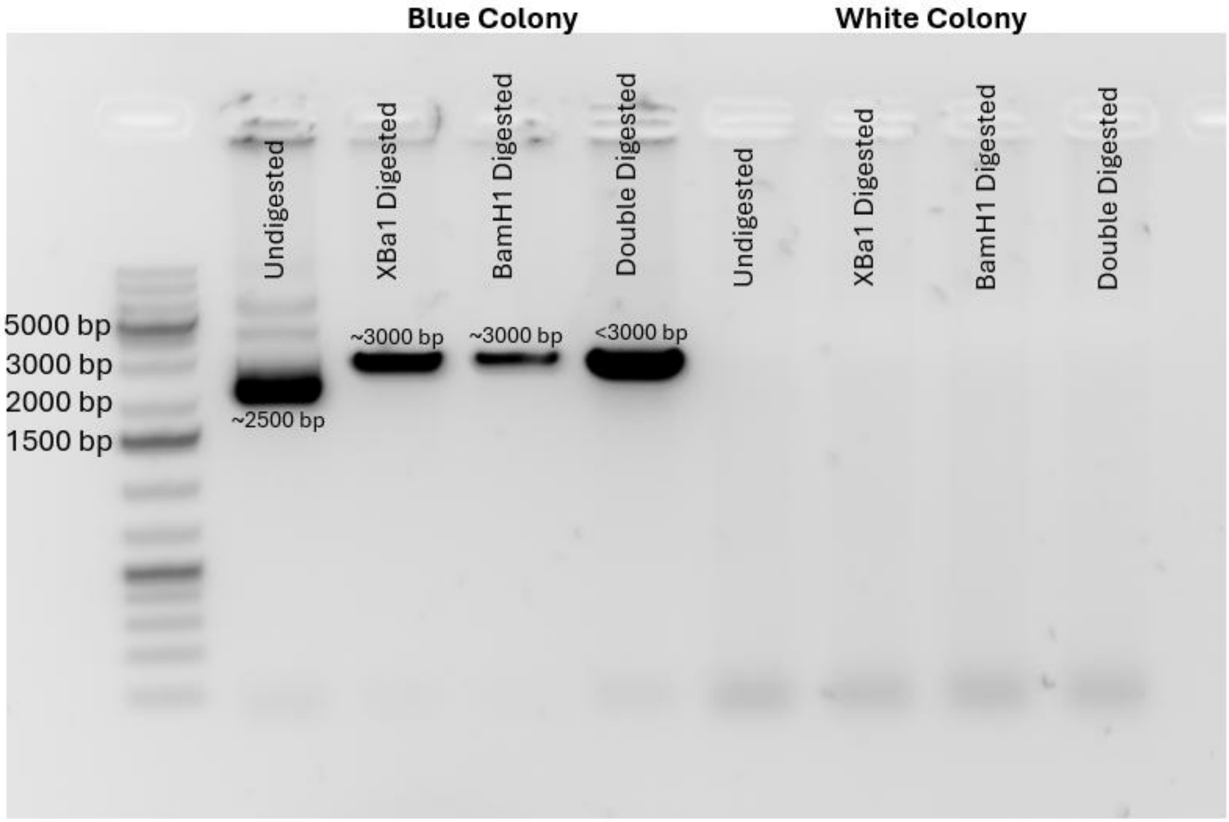

Figure 5. Restriction digests of plasmid purified from transformant colonies visualized by gel electrophoresis.

Discussion

An experiment was completed to clone the gene *Polo* into pBluescript (pBS) by restriction digest and ligation, followed by bacterial transformation. The methodology of this experiment is standard in molecular biology but some steps have notable considerations. The use of phenol-chloroform in genomic DNA purification is not strictly necessary, but it is exceptional at removing protein impurities from the aqueous extraction layer. The hydrophobic core of proteins have a high affinity for the phenol and they are readily denatured and dissolved into the organic phase of the mixture. A similar methodology consideration is the inclusion of alkaline phosphatase in the pBS double digestion. This enzyme dephosphorylates the terminal nucleotides of the double stranded breaks produced by the restriction enzymes. Since the phosphate groups are necessary for ligation enzymes to fuse the DNA backbone, this prevents re-circulization of the empty vector in later steps. This inclusion is also not strictly necessary because the two sticky ends of the empty vector are not complementary and are unlikely to hybridize regardless.

At the end of the procedure, a validation digest of plasmid purified from a white transformed colony (see Figure 4A) indicated that the cloning was not successful, and no plasmid was present in the colony. This result is highly unexpected as the tested colony successfully grew on the ampicillin plate, but no non-genomic DNA of any size was observed on the validation gel (see Figure 5). Therefore, the coding sequence Amp^R present on pBS, which acts as the selection marker for successful transformants, must have been incorporated into the genome of the *E. coli*. This phenomenon would likely have occurred following transformation of a linearized plasmid molecule, which would then have been degraded following incorporation and expression of the Amp^R coding region.

Another unexpected observation is the apparently high DNA concentration of the white colony extract (155.6 ng/µL) despite bands visible at all after restriction digest. One possible explanation is that despite the RNase digestion step in the plasmid purification protocol, the extract had a large amount of RNA. However, large amounts of cellular RNA would appear as a smear on the gel which is not observed (see Figure 5).

Plasmid purified from a positive control blue transformant colony (see Figure 4D) was also digested and ran on the validation gel, matching the expected band pattern. This, along with the large number of blue colonies on that plate, indicates that the transformation procedure was successful. However, it should be considered that the insert is slightly larger than pBS, and so the ligation product is over double the size of the pBS transformed into cells on the positive control plate. The transformation protocol, specifically the use of homemade chemically competent cells instead of commercial grade or technique focused steps like mixing the cells and DNA solution carefully, may have not been good enough to facilitate transformation of that size of plasmid (~6 kb).

To account for the uptake of the Amp^R coding sequence, there likely was successful transformation of a large amount of linear, empty, pBS which made it through the ligation. Based on the positive control plate, this molecule would be readily transformed, giving many opportunities for the Amp^R sequence to be integrated into the genome before degradation of the linear vector. This unlikely event would account for the small number of colonies on the 1:3 ligation plate compared to the positive control. Furthermore, the lack of colonies on 1:5 ligation plate also agrees with this hypothesis as the higher insert:vector ratio would decrease the amount of empty vector that made it through. That implies that transformation of the 1:5 ligation product would likely produce correct colonies if done with an optimized protocol or commercial grade competent *E. coli* cells.

That hypothesis aside, the ligation with a 1:3 vector-to-insert ratio should ideally leave little empty vector molecules that make it through, and there is not certain evidence that the 1:5 ligation had high efficiency. However, a clear reason for poor ligation efficiency is not apparent. The restriction digestion of pBS used in the 1:5 ligation had a comparable band pattern to a reference digestion (see Figure 3), and the 1:3 ligation reaction used digested pBS provided by the instructor. Interestingly on the restriction digest gel (Figure 3), the 12 base pair size difference between single and double digested pBS is slightly visible, which further indicates the double digest was correct. This suggests that the DNA molecules used for ligations are correct, and another factor led to the low efficiency. There is the possibility of chemical contaminants like EDTA making it through the gel extraction into the ligation, but the 260/230 ratio of 1.92 suggests minimal impurity.

In the future, the double digested plasmid could be purified further by isopropanol precipitation and used in another set of ligation reactions before another attempt at transformation. Another possibility is to test another colony from this experiment's transformation by plasmid purification and test digestion. The improbability of Amp^R genome integration may suggest that another of the colonies does contain the correct plasmid.

Literature Cited

Bourouh, M. (2026). BIOL 4250: Biochemical Techniques II, *Molecular Cloning Lab Module*. Thompson Rivers University Department of Biological Sciences.