Puc18 plasmid engineering

Abstract

The objective of the experiment was to engineer a pUC18 plasmid so that it contained a kanomycin resistance gene in its multiple cloning site and to transform it into cells. The kanomycin resistance gene was obtained from a pKAN plasmid. The desired plasmid was constructed by digesting pUC18 and pKAN with the same restriction enzymes,(BamHI and HindIII) and religating the products to give the engineered pUC18. The created plasmid was then transformed into E. coli strains DH5α. The strains that contained the engineered plasmid were selected using two methods of selection. According to the indirect method of selection the percentage of competent cells transformed with the plasmids was 0. 063% which is a low number. According to the direct method of selection on the other hand no cells were transformed. In conclusion even though some colonies with the engineered plasmids were obtained the percentage of cells transformed was very low. Also, the indirect method of selection gives better results for selection of desired strains.

Introduction

Bacteria can carry antibiotic resistance genes either in their chromosomes or extrachromosomally in phage or a plasmid(Hausner and de Jong 2010).

B-galactosidase is an enzyme involved into the cleavage of lactose into glucose and galactose and is encoded by the lac Z gene of the lac operon. (Glick et al 2010) The lac operon is prevented from being transcribed through repression of the lac promoter. Activation of this promoter can be done by the addition of lactose or isopropyl-β-D-thiogalactopyranoside(IPTG)

to the medium. Lactose and IPTG simply prevent binding of the lac repressor(the product of the Lac I gene) to the promoter. (Glick et al 2010)

In the following experiment plasmids pUC18 and pKAN are used to provide the genes to be transformed into bacteria. pUC18 is 2686 base pairs(bp) long and contains a bacterial origin of replication, an ampicillin resistance gene, a lacI gene, a segment of the lac Z' gene encoding part of B-galactosidase(which breaks down X-gal) and a multiple cloning sequence(MCS) that is within the lac Z' gene.(Glick et al 2010) The lac Z' gene encoded by the plasmid is part of the B-galactosidase protein which complements a gene carried by the Escheria. coli chromosomally thus forming a functional B-galactosidase.(Glick et al 2010) If a DNA segment is cloned in the MCS then the lacZ' gene will be interrupted and will not give rise to a functional protein. If that occurs then the Bacteria transformed with the plasmid will not break down5-bromo-4-chloro-3-indolyl-β-D-β-galactosidase(X-gal) present in the plates. When X-gal is broken down by β-galactosidase it turns blue whereas when it is not broken down it stays white. This color differentiation is a way to tell if there has been any DNA incorporated in the MCS of pUC18. Finally in order for the β-galactosidase in pUC18 to be transcribed, IPTG has to be present in the medium so that the lac operon can be induced.(Glick et al 2010)

pKAN plasmids can serve as sources for the kanomycin resistance gene. In the following experiment the kanaomycin resistance gene will be inserted in the MCS of pUC18. pKAN contains an origin of replication, a kanomycin resistance gene and multiple restriction sites.(Hausner and de Jong 2010) More importantly it contains only one BamHI and HindIII recognition sites in

the whole plasmid which flank the kanomycin resistance gene.(Hausner and de Jong) This allows researchers to cut out the antibiotic resistance gene by simply using BamHI and HindIII producing only two fragments of DNA: the gene and the rest of the plasmid.

Once experimenters have inserted the pKAN gene into the MCS of pUC18 and transformed the E. coli strains they need a way to select for the desired plasmid. There are two methods to select for the desired those colonies: the direct method and the indirect method. The direct selection method involves spread plating transformed strains into plates containing both the antibiotic ampicillin and kanomycin. (Hausner and de Jong 2010) Since the pUC18 plasmid confers amplicillin resistance(Glick et al 2010) and the kan gene confers kanomycin resistance (Hausner and de Jong 2010) then only the cells that contain Puc18 with the kanomycin resistance gene should be able to grow in these plates. The indirect method on the other hand is a two step selection process. In the first step the transformed strains are plated onto LB plates containing ampicillin and X-gal.(Hausner and de Jong 2010) Only the cells that have up-taken pUC18 will grow since they will be resistant to ampicillin. Furthermore cells that contain pUC18 with inserted DNA in the MCS will produce white colonies since they can’t produce a functional β-galactosidase. Cells that give rise to blue colonies will have up-taken pUC18 without any DNA inserted in their MCS since they are able to break down X-Gal. (Glick et al 2010) To select the cells with pUC18 containing the kanomycin resistance gene the white colonies are plated in plates containing kanomycin. Only the cells that have the kanomycin resistance gene in their pUC18 will grow.(Hausner and de Jong 2010)

The objectives of the following experiment include the construction of a pUC18 plasmid containing the kanomycin resistance gene in the MCS, the transformation of that plasmid into the E. coli DH5α cells and the selection of the cells containing the engineered plasmid. If both pUC18 and pKAN plasmids are digested with BamHI and HindIII and the digests are ligated then a plasmid which contains both kanomycin and ampicillin resistance genes should be produced; consequently cells transformed with the engineered plasmid should be resistant to both antibiotics.

Materials and Methods

Plasmid extraction and plasmid engineering

pUC18 and pKAN plasmids were extracted from the DH5α and MM294 E. coli strains respectively using a DNA isolation kit as described by (Hausner and de Jong 2010). Confirmation for proper extraction was done through agarose gel electrophoresis by running the extracted DNA in a 0. 7% gel at 100V for 1 hour. The gene containing kanomycin resistance from pKAN was cloned into pUC18. The restriction digests to do the cloning were prepared as described in Table 2 in (Hausner and de Jong 2010). After plasmid digestion the kanomycin resistance gene was inserted into the multiple cloning sequence of pUC18 in a ligation reaction using the enzyme ligase and the reaction was allowed to go to completion for 24 hours at room temperature. The ligation reactions were set up according to table 3 in (Hausner and de Jong 2010)

E. coli transformation and strain selection

E. coli strain DH5α was sub-cultured for 1 hour at 37°C. The cells were then made competent by washing them in 10mM CaCl. Next cells were transformed with three different combinations of plasmids. The set of cells in

tube 1 was transformed with uncut pUC18 DNA. The set of cells in tube 2 was transformed with cut pUC18. Cells in tube 3 were transformed with pUC18 containing the cloned pKAN resistance and finally cells in tube 4 were transformed with just water as a negative control. The transformation procedure has been described in (Hausner and de Jong 2010). Transformed cells from all tubes were spread plated onto LB+carb+X-gal plates for indirect selection. Furthermore cells from tube 3 were plated onto LB+carb+ kan plates for direct selection of cells containing pUC18 with the insert from pKAN.

To determine the density of competent cells cells dilutions of , and were prepared. The two highest dilutions were plated onto LB plates. All the plates were incubated at 37°C and they were allowed to grow for ~24 hours.

After the colonies had grown on plates plate they were counted and their numbers were recorded. White and blue colonies from the LB+carb+X-gal plates were then streaked onto LB + kan plates to obtain the colonies that had the kanomycin resistance gene incorporated in the MCS.

For more information on the procedure refer to Experiments in Biotechnology Laboratory Manual (Hausner and de Jong 2010)

Results

Extraction of plasmids from E. coli strains

Figure 1 contains the image of the 0. 7% agarose gel in which the isolated plasmids Puc18 and pKAN were run to check for product. As it can be seen in lane 1 a lot of Puc18 was extracted from the DH5α strain. Less plasmid DNA was collected for pKAN from the MM294 strain since the band in lane 2 is of

much weaker intensity. There is more than one band in lane two. The additional bands represent additional plasmids isolated from the bacteria.

Calculation of Competent cell density

Table 1 shows the dilutions performed on the competent cells in order to calculate their cell density. It also shows the number of colonies on the plates that were spread plated with dilution 2 and dilution 3. The results for the dilution were not used for cell density calculation since less than 30 colonies grew on the plate. Dilution was used to calculate the cell density because the number of colonies was between 30 and 300.

Indirect method of selection

Cells plated from tubes 2 and 3 were used to calculate the % of transformed cells. Every colony represents a single transformed cell since it can be assumed the every colony has arisen from a single cell. Furthermore for tube 3 since five plates were spread plated the percentage of the transformed cells was obtained by using the average amount of colonies for all five plates.

Calculation the percentage of transformed cells in tube 2:

%of transformed cells= x 100

= 0. 0045% of cells transformed

Calculation of transformed cells in tube 3

Average for blue colonies:

= 58. 6 ≈ 59 blue colonies

Average for white colonies

= 11. 4 ≈ 11colonies

Total number of colonies = 59 blue colonies + 11 blue colonies

= 70 colonies in total

Both blue and white colonies from tube 3 represent transformed cells since they both up-took plasmid DNA whether it was just pUC18 or pUC18+kanomycin resistance gene. Therefore since every colony came from a single cell there were 70 cells in total that were transformed from 100µl of media spread plated in each plate.

% of transformed cells in tube 3:

%of transformed cells= x 100

= 0. 063% of cells transformed

Direct selection of clones containing the kanomycin gene:

No colonies grew on LB + carb + kan plates. That means that there were no cells that were transformed with the engineered plasmid. Furthermore an accurate number for % of transformed cell could not have been calculated even if cells had grown in these plates. That is because this selection method takes into account only the cells that were trasformend with pUC18 which contained the kanomycin resistance gene and not the cells that were transformed with only pUC18.

Discussion

Isolation of plasmids from cells

The optimal results for the gel would have been to see one strong band at ~2. 7 kb representing pUC18 and one strong band at 4. 2 kb which represents pKAN. For the pKAN lane there is more than one band seen. Those bands represent different sized plasmids that were also isolated from the cell. Since there was no DNA ladder on the gel it cannot be concluded what plasmid the lanes represent but the only thing that can be concluded is that there was plasmid DNA isolated from both the DH5α and the MM294 strains which most likely was pUC18 and pKAN. In order to conclude whether pUC18 and pKAN plasmids were isolated from the bacteria the students should be provided next time with a DNA ladder in order to determine the sizes of the lanes.

Indirect selection method

The cells from tube 1 were transformed with un-digested pUC18. The cells from this tube represented a positive control for transformation. The colonies in the plates were all blue and they were too many to count. The reason for the high number of colonies was that these cells were transformed with undigested plasmids which are all stable and all allow bacteria to carry information extrachromosomally, making the transformation percentage of competent cells very high. All the cells from tube 1 produce blue colonies. That is because they all had a functional B-galactisidase since no genes were cloned into the multiple cloning site located within the lacZ’ gene.

The cells from tube 2 were transformed with digested pUC18 plasmid. The cells from this tube represented a negative control for kanomycin resistance

gene cloning. Tube 2 gave rise to very few colonies in comparison to tube 1 because the cells in tube 2 were transformed with unstable DNA. pUC18 had been previously digested with HinDIII and BamHI and a lot of plasmid did not re-ligate and for that reason the DNA was unstable. Since the DNA was unstable it was not able to maintain the ampicillin resistance gene in bacteria and consequently the strains were not able to grow in carbonicillin plates. As a result the number of percent transformed cells was as low as 0. 0045%.

The cells from tube 4 were transformed with sterile water i. e no DNA. These cells represented the negative control for transformation. Because no DNA was inserted in them none of the cells contained the ampicillin resistance gene and as expected none grew in the plates containing carbomicillin.

The cells from tube 3 were transformed using pUC18 that contained insertion on the MCS as well as pUC18 that didn’t. All five plates that were spread plated with E. coli from tube 3 contained blue colonies as well as white ones. The reason for the color difference is that the blue colonies contained a functional β-galactosidase whereas the white ones didn’t. The functional β-galactosidase in the blue colonies was due to the fact that no DNA was inserted in the MCS to interrupt the lacZ’ gene. The white colonies on the other hand did not contain a functional β-galactosidase since they had a DNA insertion in their multiple cloning site, which interrupted the lacZ’ gene. Consequently they could not break down X-gal. However just because they had a DNA insertion in their MCS it did not mean that they contained the kanomycin resistance gene. They might have contained the rest of the pKAN plasmid. As a result the white colonies needed to be streaked into plates that

selected for kanomycin resistance. If the cells then grew on LB + Kan plates and they also originated from white colonies on LB + Carb + X-gal plates then they contained a Puc19 plasmid with a kanomycin resistance gene inserted in the MCS. The percentage of transformed cells was also not very high: 0. 063%. A way to improve this would be to maybe increase the molarity of the CaCl solution to make the cells more competent.

Direct selection method

According to the direct method of selection there were no cells that were transformed. This is contradictory to the results obtained from the indirect method of selection. This error could have been produced because of either improper spread plating of plates or because of improper transformation procedure. Also the conditions in the LB + carb + kan plates could have been too harsh(two antibiotics) for the bacteria to pick up growth even if they were resistant to both antibiotics. In following experiments it is better to use the indirect selection method since it seems more successful in selecting desired strains.

Comparison of direct VS indirect selection methods

The direct and indirect selection methods have both advantages as well as disadvantages. The main disadvantage of indirect selection is that it takes longer since it contains two steps and each step takes at least a day for completion. The main advantage is that if done correctly, the indirect selection methods gives very accurate selection for the desired cells. The reason for that is that first it selects for colonies that just have an insertion in the MCS and this tells the researcher that some type of cloning has occurred in plasmids. The second step then selects for the colonies that contain

pUC18 with the kanomycin resistance gene inserted in the MCS. Thus the criterion of indirect selection is that cells have both pUC18 with an inserted DNA in MCS and also have kanomycin resistance. The colonies that grow in the second step fulfill both the criteria.

The main advantage of the direct method is that it takes a shorter time to complete and it also uses up less equipment which can also save researchers some money. The main disadvantage with this selection is that it has a higher chance of giving false positives. Direct selection does not select for strains that have DNA inserted in the MCS of Puc18 but only selects for strains that have ampicillin and kanomycin resistance. Therefore the strains that grow in LB + carb + kan plates might have both pUC18 and pKAN plasmids but not the kanomycin resistance gene inserted in the pUC18 MCS. Those strains would still be able to grow since they still have both ampicillin and kanomycin resistance. However the genes would on different plasmids and not on the engineered one. Therefore even though the indirect selection method is longer it is more accurate in selecting the desired strains for this experiment.

In conclusion, according to the indirect selection the desired plasmid was engineered by digesting both pUC18 and pKAN with HindIII and BamHI. Also when selecting for cells transformed with pUC18 it is better to employ the indirect method of selection because it gives more accurate results.

Question 1: Although both lanes contain plasmid DNA, why doesn’t the DNA appear to be in the same location in both lanes?

The DNA does not appear in the same location in both lanes because pUC18 and pKAN are of different sizes. pUC18 is 2686 base pais long whereas pKAN is 4194 base pairs long.(Hausner and de Jong 2010) Because pUC18 is of smaller size it will travel farther from the wells than pKAN.

Question 2: How would you verify that the transformed cells actually contain the carb/kan plasmid that was used for transformation?

One accurate way would be to isolate the plasmid DNA from the transformad cells and run it on an agarose gel. If the kanomycin resistance gene was inserted into pUC18 then on the gel one will be able to see a band of the size 4548 base pairs which is different from both the pUC18 and the pKAN plasmids. The size of the created plasmid was calculated the following way by obtaining the information from (Hausner and de Jong 2010):

To find the size of kanomycin resistance gene inserted in pUC18, the number of base pairs from the origin or replication of HindIII was subtracted to the number of base pairs from the origin of replication of BamHI. This was done because pKAN was digested with HindIII and BamHI to obtain the kanomycin resistance gene:

2095 – 233 = 1862 base pairs

The size of the insert was then added to the size of Puc18:

2686 + 1862 = 4548 base pairs