Isolation of the Vimentin Protein from Breast Cancer Epithelial Cells

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Abstract

Vimentin is one of the proteins that make up the intermediate filaments of regular mesenchymal cells. However, overexpression of the Vimentin protein has also been observed in different epithelial cancers (Satelli et al., 2011) such as breast cancer, where its increased expression has often been correlated with the aggressiveness of the cancer. The aim of this thesis project is to isolate the Vimentin protein from the mRNA of breast cancer epithelial cells (MDA-MB-231) which is of interest in order to be able to investigate its function further as well as finding out what its actual connection to cancer is.

In order to start the experimental procedure, the mRNA had to be converted into cDNA to isolate the Vimentin gene through PCR. The Vimentin gene would then have to be purified before it could be ligated into the pGEM®-T Easy maintenance vector. During transformation into XL1 blue competent cells, the pGEM®-T Easy vector with the ligated Vimentin gene would enter the cells and as the cells grew the vector with its insert would be cloned. In order to afterwards screen for which colonies had taken up the pGEM®-T Easy vector with the ligated Vimentin gene, a colony PCR was carried out. Lastly, the plasmids were extracted and in order to express the final Vimentin protein its coding sequence had to be transferred from the pGEM-T Easy vector into a new pET-22b(+) cloning/expression vector.

The Vimentin protein was isolated well from the mRNA of breast cancer epithelial cells, and even though the last part was not performed in the laboratory due to time constraints but was designed instead, the fact that the Vimentin gene was shown able to transfer onto the pET-22b(+) cloning/expression vector could be of wide use. The Vimentin protein could be overexpressed in different cells, such as cancer cells and from there its function could be investigated. It is hoped that the Vimentin protein can become a future therapeutic target, and the use of therapeutic agents in combination with other anti-cancer agents directed at Vimentin should be encouraged as well (Satelli et al., 2011).
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Abbreviation list

°C: Celsius degree
µg: Micrograms
µl: Microliters
µM: Micromolar
A: Adenosine
Amp and Ap: Ampicillin
C: Cytosine
cDNA: Complementary DNA
Conc: Concentration
dATP: Adenosine deoxyribo-nucleotides
dCTP: Cytosine deoxyribo-nucleotides
dGTP: Guanine deoxyribo-nucleotides
DNA: Deoxyribonucleic acid
dNTPs: Deoxyribo-nucleotides
dTTP: Tyrosine deoxyribo-nucleotides
EMT: Epithelial-mesenchymal transition
F primer: Forward primer
g (for centrifugation): Gravitational force
g: Grams
G: Guanine
His Tag: Histidine Tag
IPTG: Isopropyl-β-D-thiogalactopyranoside
Kb: Kilobases
kcal: Kilocalorie
LA: Luria Agar
lacZ: Lactose operon of Escherichia Coli
LB: Lysogeny broth
M: Molarity
MDA-MB-231: Human Breast Carcinoma cell line
mg: Micrograms
ml: Milliliters
mM: Millimolar
mol: Mole
mRNA: Messenger RNA
MCS: Multiple cloning site
MW: Molecular weight
NaCl: Sodium chloride
NCBI: National Center for Biotechnology Information
ng: Nanograms
NT and nt: Nucleotide
OD: Optical density
Oligo(dt): Tyrosine deoxyribo-oligonucleotide primer
ORF: Open reading frame
PCR: Polymerase chain reaction
R primer: Reverse primer
RE: Restriction enzyme
RE site: Restriction enzyme site
RNA: Ribonucleic acid
RT: Reverse transcription.
T: Tyrosine
UV-light: Ultraviolet light
X-Gal: 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside
XL1 blue: Competent cells
1 Introduction

Vimentin is a protein found in the intermediate filaments of regular mesenchymal cells, where it is of a big importance (Satelli et al., 2011). Together with the microfilaments and the microtubules, the intermediate filaments make up the cytoskeleton of cells. Therefore, one of the functions of Vimentin is that it helps to maintain a stable and strong structure of the mesenchymal cells where it is being expressed.

It has also been observed that the Vimentin protein is overexpressed in different kinds of epithelial cancers (Satelli et al., 2011). Therefore, it is of interest to isolate it in order to investigate its real function and its actual connection with cancer, which to a large extent remains unsure today. In previous studies, it has been seen how overexpression of the Vimentin protein was correlated with faster tumor growth as well as an increased migration of the cancer cells (Satelli et al., 2011). It is hoped that the overexpression of the protein in various cancers could serve as a possible therapeutic target in the future (Satelli et al., 2011).

In the context of breast cancer Vimentin’s increased expression has often been related to the aggressiveness of the cancer, as the cells become more invasive when there is more of the protein present. It has also been observed how Vimentin is of great importance during the process of epithelial-mesenchymal transition (EMT) in breast cancer (Satelli et al., 2011). EMT is a cellular process where epithelial cells acquire the same constitution as mesenchymal cells, resulting in their shape being altered as well as they experience an increase in motility (Satelli et al., 2011).

With this thesis project the aim is to isolate the Vimentin protein from the mRNA of breast cancer epithelial cells (MDA-MB-231). In order to do so, the Vimentin gene sequence will first have to be isolated from the mRNA through PCR. The ligation of the Vimentin gene into the pGEM®-T Easy maintenance vector (Promega) will then be followed by the transformation of pGEM®-T Easy into XL1 blue competent cells in order for it to be cloned. Finally, the Vimentin gene will be isolated from pGEM®-T Easy in order to be transferred to the pET-22b(+) cloning/expression vector (Novagen) for the final expression of the Vimentin protein.
2 Materials and methods

The experimental procedure for the isolation of the Vimentin protein started from the mRNA of the breast cancer epithelial cells (MDA-MB-231). The mRNA had already been received from another laboratory and the reason as to why it was preferred, rather than the DNA was because both contained the exons that code for the Vimentin protein, but the mRNA is free of introns. This makes the mRNA shorter and easier to work with.

2.1 cDNA conversion

To start with, the mRNA from breast cancer epithelial cells was converted into cDNA through reverse transcription (RT). This was done since a PCR would be carried out afterwards in order to isolate the Vimentin gene from its cDNA. In general, mRNA is much less stable than cDNA since it is single stranded compared to cDNA which is double stranded. The fact that mRNA is unstable makes it more vulnerable to degradation, which would not bring the best results after running the PCR.

In order to proceed with the cDNA conversion, the Promega protocol for Standard First-Strand Synthesis of cDNA was followed. For the mRNA mix, 2 µl of mRNA, 1 µl of Oligo(dt) and 11 µl of nuclease-free water (to reach the final volume of 14 µl) were added together into a sterile eppendorf tube. The Oligo(dt) is a primer which is often used in RT reactions and would also be used this time. Before use, all materials would also have to be centrifuged before added together, so that all the contents in each tube would gather at the bottom.

The eppendorf tube containing the mRNA mix was afterwards placed on a heating block for incubation at 70 °C degrees for 5 minutes, which was done in order to denature the secondary structure of the mRNA. Once the tube was taken out it was left to cool on ice for an additional 5 minutes, so that the Oligo(dt) primer would successfully anneal to the mRNA template.

During the 5 minutes of heating and the 5 minutes of cooling of the mRNA mix, the second mix for the transcription reaction was prepared in a new, sterile eppendorf tube. The mix would contain 5 µl of M-MLV RT 5x reaction buffer, 1.25 µl dATP (10 mM), 1.25 µl dCTP (10 mM), 1.25 µl dGTP (10 mM), 1.25 µl dTTP (10 mM), 1 µl M-MLV (H-) point mutant and 1 µl of RNasin® (Promega) which prevents the action of RNases that would otherwise catalyze the hydrolysis of mRNA.

Once both mixes were ready, the second mix was added to the mRNA mix. The final mix was then placed on a heating block for incubation at 40 °C degrees for the initial 10 minutes, then the temperature was increased to 55 °C degrees for an additional 50 minutes, in order to allow reverse transcription to occur. Once 50 minutes had passed, the mix was placed on a heating
block at 70 °C for 15 minutes in order to inactivate the reverse transcriptase and stop the cDNA conversion.

2.2 PCR

2.2.1 Primer design

In order to design the primers for the PCR, the access number of Vimentin in Homo Sapiens was looked up on the NCBI website and was found to be NM_003380.3. The coding sequence (see Figure 1) was then used to design the forward and reverse primers, which would have to allow a Vimentin PCR product of 1.4 kb to be isolated, with the help of DNA Man (Sequence alignment program, version 8 for Microsoft Windows).

| 361 | ggagccagtc cgccgaccg ccgccgccca ggccatcgca gccatgttca |
| 421 | ccagcatcgt gttctgttc ccctaccgca ggatgttgg gggcccgggc accggagccc |
| 481 | ggccgagct cagccgagac tagtgcacta cgccccaccg cacctacagc tggggacgcg |
| 541 | gtcgctgcc ccagccacag ccgcccccct ccgctctgct ccggggccgc gttatgtgca |
| 601 | cgccctctcc tcgcgtgctc ctgcggagca ggtggcgtct ctcagccagct |
| 661 | cggttgactt ggtctggtgc gaccccatca acacccatgt cggaaacacc cgacccacca |
| 721 | agagagttgg aatgcggagg catgatgcc cttgctgccca ctacatcagc aaggtgccc |
| 781 | tccttgagca gcaataaag atctctgctg cgcagctcag gcagcataag gcacccaggc |
| 841 | atgcctgccct ctcgggacgc ctgcggtctc gctgttgagc gcgtgtgctc |
| 901 | acgtacacca ccacacagcg cgctctgga gttgagcagc cccgccggcc agggccagcc |
| 961 | tcgcctgccg ggagaaattt cctggagca gcagaataag atcctgctgg cggagctcga |
| 1021 | tgcaatcttt ccagcagagt ggtgcaaatg gttctctgct gcgtctgctc cctggacgc |
| 1081 | aagttggaact ttgcaagaa gagattgctt ttttgaaagl acttcacagc gagaaatcc |
| 1141 | agagctgctg caagctgagtc ccacagccag tgggtcgctg caggttgagc gtttctttga |
| 1201 | ctcctgcatttgtgagc gttgatccag gagatggcct tggcctcagc |
| 1261 | accctgagga ggccagagat tggatcaat ccagttttgc ttgatcactc agggctgctc |
| 1321 | acgggcaacaa tgcacccctg ccgccgcagc gggaggtgagc acgagccacag |
| 1381 | tgccacccct ccacccgagc cttgattgcc ctttcgacta cagagccagt ccacatgcagc |
| 1441 | agattggctga aatggagag gacctttgct ggaatgctct ttaacttact ccgctgctcag |
| 1501 | cggcgctcgc gatgtgatg caaatatat cagcttctgc gcctggtggc ctgtgccagt |
| 1561 | ccaccacact ccctcagatt aagatggctt tggctgtctc cgcacatcag ccgccggagc |
| 1621 | tgctgggaga cggagagag cggagttcct tgcctcttgc aacgttactc ccctctcccag |
| 1681 | tggagagcaac tatactgctt ccctctctct ccctctgtat ccacactcag gcacccagcc |
| 1741 | tcattacacg gttggaacta tggagaggtg acggcttctg tcgcagcttc cccttacagc |
| 1801 | atgacccctga ataaatttg cacacactca gttccagcaat atattaccag ccagaaaat |

Figure 1: Coding sequence (in shaded colour) for Vimentin in Homo Sapiens, taken from the NCBI website.

There are some important points to take into account during primer design, in order for them to give an amplification with high yield. Some of the most important ones that were considered were the melting temperature for both primers, the self-complementarity for each one as well as the complementarity between the two. When it came to the melting temperature, i.e. the
temperature at which a primer would anneal to their complementary cDNA sequence, it was important that the primers did not differ too much. The PCR would also have to be set to the lowest annealing temperature (one primer would have a lower one than the other primer). If set to a higher temperature, the primer with the lowest melting temperature would no longer be able to bind to its complementary cDNA sequence (which is fine for the primer with the highest melting temperature, as it would still be able to bind at a lower temperature).

2.2.2 Running of PCR

In order to prepare for the PCR the primers had to be diluted in nuclease-free water first, which was done to obtain the same amount of each primer for the best results i.e. an outcome of as much PCR product as possible. The PCR mixes had to be prepared as well in small PCR tubes, which was done following the Promega protocol for Go Taq® PCR Core Systems (with some slight changes). The PCR was done in order to isolate the gene of interest coding for Vimentin from the cDNA and three reactions were carried out in total. Two of them contained the cDNA (made two in order to compare them) while the third one was used as a negative control and therefore only contained nuclease-free water.

Both the forward and reverse primers were each diluted in an eppendorf tube in order to attain the amount of 10 µM for each primer for the PCR. However, some calculations had to be carried out first in order to be able to do so,

\[
\text{MW: } 330 \text{ g mol}^{-1} \text{ nt}^{-1} \times \text{NT} \\
\text{Conc.}^n: 37 \mu \text{g ml}^{-1} \times \text{OD} \\
\text{M: } \frac{\text{Conc.}^n}{\text{MW}} \\
\]

Where MW, Conc.\(^n\) and M where given on each primer bottle, for both the forward and reverse primers.

*For the forward primer:*

NT: 21
OD: 445.4

\[
\text{Molarity} = \frac{\text{OD} \times \text{Conc.}^n \times \text{Unit of Conversion}}{\text{NT} \times \text{MW}} = \frac{445.4 \times 37 \times 1000}{21 \times 330} = 2378.04 \mu \text{M}
\]
In order to reach the final volume of 500 µl (which had been chosen, both for usage and storage) the final amount of primers (10 µM) was divided with the initial amount of primers (2378.04 µM) times 500,

Needed primer volume: \( \frac{10}{2378.04} \times 500 = 2.1 \) µl

Needed nuclease-free water volume (to reach the final volume of 500 µl) : 500-2.1 = 497.9 µl

*For the reverse primer:*

NT: 24  
OD: 746.7

Molarity \( = \frac{746.7 \times 37 \times 1000}{24 \times 330} = 3488.4 \) µM

Needed primer volume: \( \frac{10}{3488.4} \times 500 = 1.4 \) µl

Needed nuclease-free water volume: 500-1.4 = 498.6 µl

In continuation, the two cDNA reactions and the negative control were prepared. Master mix for four PCR tubes was made in order to not run out of it and each PCR tube would hold a total volume of 25 µl (see *Table 1*).

*Table 1*: Components of the PCR reactions, where the cDNA would be substituted by nuclease-free water for the negative control.

<table>
<thead>
<tr>
<th>Initial concentration</th>
<th>Final concentration</th>
<th>For 1x PCR tube (µl)</th>
<th>For 4x PCR tubes (µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>cDNA</td>
<td></td>
<td>2</td>
<td>8</td>
</tr>
<tr>
<td>10 mM dNTPs</td>
<td>0.2 mM</td>
<td>0.5</td>
<td>2</td>
</tr>
<tr>
<td>10 µM F primer</td>
<td>1 µM</td>
<td>1.25</td>
<td>5</td>
</tr>
<tr>
<td>10 µM R primer</td>
<td>1 µM</td>
<td>1.25</td>
<td>5</td>
</tr>
<tr>
<td>Go Taq®</td>
<td>1 unit</td>
<td>0.1</td>
<td>0.4</td>
</tr>
<tr>
<td>5x Go Taq® Buffer</td>
<td>1x</td>
<td>5</td>
<td>20</td>
</tr>
<tr>
<td>Nuclease-free water</td>
<td>14.9</td>
<td>25</td>
<td>100</td>
</tr>
<tr>
<td><strong>Total volume</strong></td>
<td></td>
<td><strong>25</strong></td>
<td><strong>100</strong></td>
</tr>
</tbody>
</table>

Where the Go Taq® (Promega) is a thermostable DNA polymerase (also one of the reasons why it was chosen, as it can withstand high temperatures without degrading) which binds to the 3’ end of each primer, in order to build a new DNA strand from the cDNA template by addition of dNTPs (i.e. small DNA units). During the course of the PCR this new DNA strand, which in this case would be the Vimentin gene, would get amplified and isolated. The Go Taq® buffer (Promega) was important in order to activate the Go Taq® and maintain a stable pH for the PCR reactions to take place.
In order to calculate the volume of each component that was needed the following equation was used for all components,

\[
\text{Ex. For 10 mM dNTPs: } \frac{\text{Final concentration}}{\text{Initial concentration}} \times \text{Total volume} = \frac{0.2}{10} \times 25 = 0.5 \mu l
\]

Once the primers had been diluted and the PCR mixes were ready it was time to run the PCR.

The tubes were left in the thermocycler machine for about two hours in order to let the reactions take place. The thermocycler machine was set to 30 cycles and according to the Promega protocol for Go Taq® PCR Core Systems (with some slight changes, see Table 2).

**Table 2:** The temperatures and times chosen for each step for all 30 cycles in the thermocycler machine.

<table>
<thead>
<tr>
<th>Steps</th>
<th>Temperature (°C)</th>
<th>Time (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial denaturation of DNA</td>
<td>94</td>
<td>5</td>
</tr>
<tr>
<td>Denaturation of DNA</td>
<td>94</td>
<td>0.5</td>
</tr>
<tr>
<td>Annealing of primers</td>
<td>54</td>
<td>1</td>
</tr>
<tr>
<td>Elongation of primers</td>
<td>72</td>
<td>1.5</td>
</tr>
<tr>
<td>Complete elongation of primers</td>
<td>72</td>
<td>7</td>
</tr>
</tbody>
</table>

Then the PCR tubes were taken out and stored in the fridge at 4 °C. The choice of the annealing temperature was made from looking at the melting temperatures of the primers, which showed to be 57.6 °C for the forward primer and 52 °C for the reverse primer. In reality, the PCR should have been run at 52 °C so that the reverse primer could bind efficiently to its complementary cDNA sequence, but 54 °C was close enough and would still be an optimal temperature for the designed primers.

**2.3 Gel electrophoresis**

The gel electrophoresis was carried out according to standard procedures with 0.8% agarose gel after the PCR, in order to see if the Vimentin gene had been successfully isolated from the cDNA.

To start with, 0.8 grams of agarose were weighted and added into a beaker with 100 ml of nuclease-free water. The beaker with its contents was heated up in the microwave oven until the agarose was dissolved and the solution looked transparent. It was important to avoid the contents from boiling in the microwave oven, as some water could then evaporate and the amount of agarose would then become more than 0.8%.

The solution was allowed to cool down, then 3.5 μl of 10 mg ml⁻¹ ethidium bromide was added under the fume hood, as ethidium bromide is cancinogenic. Ethidium bromide was added because it is a fluorescent tag that binds to the DNA. That way, when exposed to UV-light, it
would fluoresce and the DNA could be detected. The gel was poured onto a gel chamber afterwards and was then left for about 20-30 minutes to solidify.

Once the gel was solid 12 µl of each PCR product was mixed together with 3 µl of blue loading dye, which would allow the tracking of the DNA as the gel electrophoresis proceeded, in order to reach a total volume of 15 µl. The Fermentas 1 kb ladder was used and 5 µl was loaded onto the first lane. Then the negative control was loaded onto the second lane, followed by the two cDNA reactions on lane three and four.

The gel electrophoresis ran for about one hour and a half before any clear bands for the Vimentin gene could be seen from the results on the gel.

2.4 Purification of PCR products

Once the gel electrophoresis had been carried out, either one could dissolve the gel slices or process the PCR products in order to get rid of excess primers, excess Go Taq enzyme etc. and be left with the purified Vimentin gene solely. The second option, i.e. the processing of the PCR products was chosen, which was further divided into three steps according to the Promega protocol for Wizard® SV Gel and PCR Clean-Up System, namely binding, washing and elution of the gene DNA.

Firstly, an equal volume of membrane binding solution, used to activate the gene DNA in the PCR product so it would later bind to the membrane of the minicolumnn, was added to the two PCR products. Then they were transferred from the small PCR tubes onto two SV minicolumn assemblies (with collection tubes attached below) and were after incubated for 5 minutes at room temperature. The PCR products were then centrifuged at 16 000 x g for 1 minute, followed by the removal of the flowthrough from both collection tubes.

During the washing step 700 µl of membrane wash solution i.e. ethanol, used to wash out all PCR product excess components but the gene DNA from the membrane, was added. The SV minicolumnn assemblies were centrifuged at 16 000 x g for 1 minute and the flowthrough was again removed. This same procedure was repeated one more time, but with 400 µl of membrane wash solution instead and 5 minutes (rather than 1 minute) of centrifugation at 16 000 x g. To finish off the washing of the gene DNA, only the minicolumns were centrifuged for about 1 minute with the centrifuge lid open. This was done in order to allow evaporation of any remaining ethanol.

In continuation, following the elution step the minicolumns were put into two new, sterilized 1.5 ml eppendorf tubes, followed by the addition of 50 µl of nuclease-free water (to store the gene DNA with it) to each tube and a 1 minute long incubation at room temperature. The minicolumns were then centrifuged one more time at 16 000 x g for 1 minute, followed by the quantification
of the purified Vimentin gene which was carried out in the Nanodrop spectrophotometer. Lastly, the purified PCR product was stored in a fridge at -20 °C.

2.5 Ligation

In order to later be able to clone the purified Vimentin gene in XL1-blue cells, i.e. competent cells for further research, its sequence had to be ligated into a maintenance vector first. The one chosen was the pGEM®-T Easy vector for a number of reasons (see Figure 2),

- It is a stable vector, which makes it good to maintain and save the gene sequence (hence called a maintenance vector).
- It has many different restriction sites, which makes it easier to move the gene sequence into another vector later for cloning and expression of the protein it codes for, as an example.
- It contains the ampicillin resistance gene, which makes it possible to select the colony that contains the pGEM®-T Easy vector, as it will allow the cells that have taken up the vector to survive in an environment where ampicillin is present.
- It contains the lacZ gene, which allows detection of the cells that have taken up the pGEM®-T Easy vector containing the gene sequence or not in the presence of the substrate X-Gal in blue-white colony screening.

Three ligation reactions were carried out, one standard reaction containing the PCR product, one positive control containing the control insert DNA (which was supposed to fit well in the vector) as well as one negative control, which did not contain any DNA but only nuclease-free water. The Promega protocol for Ligations using the pGEM®-T and pGEM®-T Easy Vectors and the 2X Rapid Ligation Buffer was used.

Figure 2: Map and sequence reference points for the pGEM®-T Easy vector (Promega).
Before each reaction was mixed, both the tubes containing the pGEM®-T Easy vector and the control insert DNA were centrifuged, in order to collect the contents at the bottom of the tubes. The ligation buffer had to be vortexed as well before each use, to make sure its contents (ex. salts which could have formed crystals while stored at -20 °C) were well mixed. The components for each reaction were pipetted into three eppendorf tubes (one tube for each reaction). Both the components (see Table 3) and the eppendorf tubes containing the reaction mixes were on ice at all times, as the DNA ligase is temperature sensitive.

**Table 3:** Overview of the reaction components in the standard reaction as well as in the positive and negative controls.

<table>
<thead>
<tr>
<th>Reaction components</th>
<th>Standard reaction</th>
<th>Positive control</th>
<th>Negative control</th>
</tr>
</thead>
<tbody>
<tr>
<td>pGEM®-T Easy vector</td>
<td>1 µl</td>
<td>1 µl</td>
<td>1 µl</td>
</tr>
<tr>
<td>Purified PCR product</td>
<td>1.2 µl*</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Control insert DNA</td>
<td>-</td>
<td>2 µl</td>
<td>-</td>
</tr>
<tr>
<td>DNA ligase</td>
<td>1 µl</td>
<td>1 µl</td>
<td>1 µl</td>
</tr>
<tr>
<td>Ligation buffer</td>
<td>5 µl</td>
<td>5 µl</td>
<td>5 µl</td>
</tr>
<tr>
<td>Nuclease-free water</td>
<td>1.8 µl</td>
<td>1 µl</td>
<td>3 µl</td>
</tr>
<tr>
<td><strong>Final volume</strong></td>
<td><strong>10 µl</strong></td>
<td><strong>10 µl</strong></td>
<td><strong>10 µl</strong></td>
</tr>
</tbody>
</table>

*It was meant to be only 1 µl but some more was added in order to be sure enough had been pipetted into the eppendorf tube.

Where DNA ligase binds the Vimentin gene sequence into the pGEM®-T Easy vector and also closes the gaps between the purified PCR product and the vector. It is also important that the water is nuclease-free as it could otherwise degrade the purified PCR product and the vector. The ligation buffer was added so that the DNA ligase was activated and to maintain a stable pH.

In order to make the calculations for what amount of PCR product was needed, the following equation was used,

\[
\text{ng of vector} \times \frac{\text{kb size of insert}}{\text{kb size of vector}} \times \text{insert: vector molar ratio} = \text{ng of the insert}
\]

Where the ng of the vector as well as its kb size were stated in the protocol to be 50 ng µl⁻¹ and 3 kb respectively.

The ratio of 3:1 was used (suggested in the protocol) in order to optimize the insert i.e. since there would be three times more of the insert than of the vector it would increase the possibility of the insert to ligate successfully into pGEM®-T Easy.

So, \[
\frac{50 \times 1.4}{3} \times \frac{3}{1} = 70 \text{ ng of the insert was needed}
\]
In order to calculate the volume of the PCR product needed, its mass was divided with its concentration according to the following equation,

\[
\frac{m}{c} = \frac{70}{71.7} = 1 \mu l \text{ of the insert was needed}
\]

Where 71.7 ng µl\(^{-1}\) was the concentration of the purified Vimentin gene that had previously been quantified from the Nanodrop spectrophotometer.

Once the reactions were mixed, they were placed for incubation in a fridge at 4 °C overnight, in order to get the maximum number of transformants.

### 2.6 Transformation

In order to proceed with the transformation, the Promega protocol for Transformation using the pGEM®-T and the pGEM®-T Easy Vector Ligation Reactions (with some slight changes) was followed. The transformation into XL1 blue, i.e. competent cells was carried out in order to clone the Vimentin gene.

To start with, the Luria agar (LA) plates with ampicillin/IPTG/X-Gal were prepared. In order to blend for 500 ml of LA (which was the amount made) 2.5 grams of yeast extract, 5 grams of tryptore, 2.5 grams of NaCl as well as 7.5 grams of bacteriological agar were added to 500 ml of nuclease-free water. The same method for the transformation was used for all three ligation reactions (i.e. for the standard reaction, the positive control and the negative control).

Firstly, 100 µl of XL1-blue were added to the whole 10 µl ligation reaction. The reaction mix was placed on ice for 10 minutes, then heated at 37 °C for 4 ½ minutes. Heating up the cells during this step would allow the pGEM®-T Easy vectors to be able to enter them. Since the cells were said to be permeable, this step was used to heat-shock them. The reaction mix was then placed on ice for another 2 minutes before 900 µl of LB medium (per 100 ml of LB: 1 gram tryptone, 0.5 gram yeast extract, 2 grams NaCl and 95 ml of nuclease-free water) was added, in order to let the cells grow. In continuation, the reaction mix was put in a water bath at 37 °C for about one hour, to allow the expression of all proteins (including the lacZ protein).

Once an hour had passed, the LA plates were taken out from the fridge were they had been stored at 4 °C. Four plates were used for the standard reaction, three plates were used for the positive control and another three plates were used for the negative control (so 10 plates in total). Different amounts of each reaction mix were pipetted onto their respective plates.

For the standard reaction there were three plates with ampicillin and one which was left without. For the three plates with ampicillin the amount of reaction mix that was pipetted onto them was of 50 µl, 100 µl and onto the third plate, the remaining amount of reaction mix was poured (the reaction was centrifuged, almost all the supernatant was absorbed and the pellet was then
resuspended with the rest of the supernatant before pipetted onto the plate). Before that though, 50 µl of the reaction mix were also pipetted onto the plate with no ampicillin.

The same was done for the positive and negative controls, although no plate with 100 µl was used for these. It was only used in the standard reaction in order to be able to compare it with the plate with 50 µl (should get twice as many colonies on the one with 100 µl of reaction).

Between each addition of reaction mix onto the plates, the plate spreader was dipped in ethanol and held under the flame from a bunsen burner in order to sterilize it. The plates where afterwards put to shake in an incubator at 37 °C overnight so that the colonies could grow.

### 2.7 Colony PCR

The same procedure was carried out as for the PCR earlier, i.e. the same Promega protocol for Go Taq® PCR Core Systems (with some slight changes) was used. However, 16.9 µl of nuclease-free water was to be used here rather than 14.9 µl in each PCR tube. This was because the colony that was going to undergo the Colony PCR was so small, and would not reach 2 µl. Otherwise all the materials were the same. The Colony PCR was carried out in order to isolate the Vimentin gene from the pGEM®-T Easy vector in 10 different colonies.

Since it was not for certain that all pGEM®-T Easy vectors, which had been taken up by all the white colonies, had ligated the Vimentin gene (some of the pGEM®-T Easy vectors could have ligated something else to them that closed them, given that some sort of contamination had occurred) 10 PCR tubes, i.e. 10 PCR reactions were carried out in total. 12x mastermix was made in order to not run out of it, and each PCR tube would hold a total volume of 25 µl (see *Table 4*).

**Table 4**: Components of the Colony PCR reactions.

<table>
<thead>
<tr>
<th>Initial concentration</th>
<th>Final concentration</th>
<th>For 1x PCR tube (µl)</th>
<th>For 12x PCR tubes (µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Colony</td>
<td>One colony</td>
<td>0.5</td>
<td>6</td>
</tr>
<tr>
<td>10 mM dNTPs</td>
<td>0.2 mM</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10 µM F primer</td>
<td>1 µM</td>
<td>1.25</td>
<td>15</td>
</tr>
<tr>
<td>10 µM R primer</td>
<td>1 µM</td>
<td>1.25</td>
<td>15</td>
</tr>
<tr>
<td>Go Taq</td>
<td>1 unit</td>
<td>0.1</td>
<td>1.2</td>
</tr>
<tr>
<td>5x Go Taq Buffer</td>
<td>1x</td>
<td>5</td>
<td>60</td>
</tr>
<tr>
<td>Nuclease-free water</td>
<td>16.9</td>
<td></td>
<td>202.8</td>
</tr>
<tr>
<td><strong>Total volume</strong></td>
<td><strong>25</strong></td>
<td></td>
<td><strong>300</strong></td>
</tr>
</tbody>
</table>
For each PCR reaction, a different colony from the LA Amp, pGEM, Vimentin, 100 µl plate (from the previous standard reaction, chose this plate as it had most colonies) was picked with a small tip. The individual colonies were then transferred onto a LB plate by rapidly inserting the tip according to the colony plate map shown in Figure 3 below,

![Colony plate map](image)

**Figure 3:** Colony plate map, with a circle around the space that was used for the 10 colonies.

The reason as to why this was done was in order to save the colonies. Since it was not known whether all (white) colonies would contain the Vimentin gene or not it would be of use to be able to go back to the right colony which did contain the Vimentin gene for further use, or in case the experiment needed to be repeated. The plate was put away to shake in an incubator at 37 °C overnight (so that the colonies could grow) and then stored in a fridge at 4 °C.

Thereupon, the tip which still contained some cells was inserted into its respective PCR tube. The tip was moved and shaken around inside each tube in order to make sure all the cells mixed well. The same procedure was carried out for all 10 PCR reactions, and all the tubes were also labeled 1-10.

Once the PCR reactions were ready it was time to turn on the thermocycler machine, which was set to 30 cycles according to the Promega protocol for Go Taq® PCR Core Systems (with some slight changes, see Table 5). The PCR tubes were left in the machine for about two hours, so that the reactions could take place. Once the reactions were completed, the tubes were stored in the fridge at 4 °C.
Table 5: The temperatures and times chosen for each step for all 30 cycles in the thermocycler machine.

<table>
<thead>
<tr>
<th>Steps</th>
<th>Temperature (°C)</th>
<th>Time (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial denaturation of DNA</td>
<td>94</td>
<td>7*</td>
</tr>
<tr>
<td>Denaturation of DNA</td>
<td>94</td>
<td>1</td>
</tr>
<tr>
<td>Annealing of primers</td>
<td>54</td>
<td>1</td>
</tr>
<tr>
<td>Elongation of primers</td>
<td>72</td>
<td>1.5</td>
</tr>
<tr>
<td>Complete elongation of primers</td>
<td>72</td>
<td>7</td>
</tr>
</tbody>
</table>

*Compared to the previous PCR (see Table 2) the initial denaturation of DNA was set to last for a longer amount of time here in order to make sure the cells died and opened.

2.8 Colony screening

Once the Colony PCR had been completed, a gel electrophoresis was done in order to see in which colonies the pGEM®-T Easy vector, containing the ligated Vimentin gene, had been taken up successfully.

The procedure was practically the same as for the gel electrophoresis that had been carried out before, 0.8% agarose gel was also used here (i.e. 0.8 grams agarose were weighted and mixed in a beaker that contained 100 ml of nuclease-free water, according to standard procedures). The gel mixture was heated up in the microwave oven until it appeared transparent, but at the same time being careful that it would not boil.

The gel mixture was then allowed to somewhat cool. Only 80 ml of the gel mixture were used for the gel electrophoresis, so 2.8 µl of 10 mg ml\(^{-1}\) ethidium bromide was added to it under the fume hood. The gel was then poured onto a gel chamber and left for a bit more than 20 minutes, until it became solid.

In continuation, the gel was loaded with 5 µl of the Fermentas 1 kb ladder onto the first lane. 12 µl of each one of the 10 PCR products were mixed together with 3 µl of blue loading dye. The total volume of the PCR products, loaded in order onto lanes 2-11, would then be of 15 µl.

2.9 Plasmid extraction

In order to prepare for the plasmid extraction for the colonies 1, 3, 5 and 6, which had previously been seen to contain the pGEM®-T Easy vector with the ligated Vimentin gene, the LB plate containing all 10 colonies was used. The colonies (1, 3, 5 and 6) were picked up with a tip and put into four tubes, one for each that contained 8 ml of LB and 8 µl of ampicillin (to kill off any newborn cells which did not contain the plasmid). The tip was then left inside each tube. In order to be able to extract the plasmids, the cells had to be left to grow in their respective tubes at 37 °C overnight, shaking in an incubator.
The Plasmid extraction was divided into three main steps according to the Promega protocol for Wizard® Plus SV Minipreps DNA Purification System (Centrifugation protocol), namely production of lysate as well as binding, washing and elution of the p-GEM®-T Easy vector DNA. For the plasmid extraction, 5 ml from each overnight culture were used (3 other ml for glycerol stock, important for long-term storage at -20 °C) and the same procedure was done for all four tubes.

To start with, the 5 ml of overnight culture were pelleted for 5 minutes (i.e. the LB was removed) in a sterile eppendorf tube. Then the pellet was resuspended with 250 µl of cell resuspension solution in an eppendorf tube, followed by the addition of another 250 µl of cell lysis solution, used to degrade the cells completely. To the mix 10 µl of alkaline protease solution, used to degrade the remaining proteins (i.e. to clean up around the vector) was added and the tube was incubated for about 5 minutes in room temperature. In continuation, 350 µl of neutralization solution were added, used to stop all reactions (from lysis and alkaline protease solutions) followed by centrifugation of the tube at maximum speed for 10 minutes.

It was important to note that all the solutions had to be mixed well after they were added, so the tube would also be inverted four times between each addition.

In order to start the binding step of the extraction, a spin column was inserted into the collection tube and the lysate was poured into the spin column. The assembly was then centrifuged at maximum speed for 1 minute as well as the flowthrough was removed afterwards.

During the washing step 750 µl of wash solution, i.e. ethanol was added. The assembly was centrifuged at maximum speed for 1 minute and the flowthrough was removed. This same procedure was repeated, but this time 250 µl of wash solution was added. To finalize this step, the assembly was centrifuged again at maximum speed for another 2 minutes.

Lastly, and in order to elute the plasmid DNA, the spin column was transferred to a 1.5 ml new, sterile eppendorf tube. 100 µl of nuclease-free water was added (which was going to be stored together with the vector DNA) followed by centrifugation at maximum speed for 1 minutes and removal of the spin column. The DNA was then stored in a fridge at -20 °C before further use.

2.10 Cloning and expression design

The final cloning and expression of the Vimentin protein was, due to time constraints, designed but not carried out in the laboratory. The aim of this step was to move the coding sequence of Vimentin from the pGEM®-T Easy maintenance vector into the pET-22b(+) cloning/expression vector.

In order to do this, two common restriction enzyme (RE) sites had to be found on both the Vimentin coding sequence and the pET-22b(+) vector sequence. With the purpose of isolating
the largest Vimentin ORF from the pGEM®-T-Easy vector, PCR had to be carried out. For that, a set of primers had to be designed which also contained the RE sequences. The chosen REs would then also be used to cut the pET-22b(+) vector open so that the isolated Vimentin sequence could fit in it and ligate to it, in order to later clone and express the final Vimentin protein. Both the Vimentin coding sequence and the pET-22b(+) vector sequence had to be cut with two REs in order for the protein sequence to be in frame with the promoter of the vector.

### 2.10.1 RE site digestion analysis of Vimentin

The restriction site digestion analysis for the Vimentin coding sequence was done using DNA Man (Sequence alignment program, version 8 for Microsoft Windows) and from that, the following RE sites were found. The first list (see Figure 4) showed the REs which would cut the Vimentin gene (should not use, since those REs would not cut out the largest Vimentin ORF but they would cut it somewhere in the middle).

<table>
<thead>
<tr>
<th>Site</th>
<th>RE Name</th>
<th>Start</th>
<th>Length</th>
<th>RE Name</th>
<th>Start</th>
<th>Length</th>
<th>RE Name</th>
<th>Start</th>
<th>Length</th>
</tr>
</thead>
<tbody>
<tr>
<td>33</td>
<td>EcoNI</td>
<td>125</td>
<td>405</td>
<td>SciI</td>
<td>853</td>
<td>EcoNI</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>51</td>
<td>XmaI</td>
<td>168</td>
<td>448</td>
<td>EcoNI</td>
<td>854</td>
<td>PstI</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>53</td>
<td>SmaI</td>
<td>170</td>
<td>468</td>
<td>BspMI</td>
<td>857</td>
<td>BspMI</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>53</td>
<td>SrfI</td>
<td>223</td>
<td>473</td>
<td>Eco56I</td>
<td>895</td>
<td>EcoNI</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>65</td>
<td>Eco56I</td>
<td>225</td>
<td>475</td>
<td>NaeI</td>
<td>928</td>
<td>EcoNI</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>67</td>
<td>NaeI</td>
<td>242</td>
<td>535</td>
<td>EcoNI</td>
<td>1014</td>
<td>XcmI</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>67</td>
<td>EagI</td>
<td>309</td>
<td>565</td>
<td>Eco57I</td>
<td>1081</td>
<td>XcmI</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>67</td>
<td>XmaIII</td>
<td>323</td>
<td>636</td>
<td>Eam1105I</td>
<td>1097</td>
<td>PstI</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>67</td>
<td>Eco52I</td>
<td>327</td>
<td>732</td>
<td>AlwNI</td>
<td>1163</td>
<td>BspMI</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>74</td>
<td>EcoICRI</td>
<td>402</td>
<td>737</td>
<td>PstI</td>
<td>1267</td>
<td>SauI</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>74</td>
<td>Ecl136II</td>
<td>402</td>
<td>767</td>
<td>ClaI</td>
<td>1267</td>
<td>Bsu36I</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>76</td>
<td>SacI</td>
<td>403</td>
<td>813</td>
<td>SplI</td>
<td>1267</td>
<td>CvnI</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>76</td>
<td>SstI</td>
<td>404</td>
<td>813</td>
<td>SunI</td>
<td>1267</td>
<td>MstII</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>96</td>
<td>Tth111I</td>
<td>404</td>
<td>SacI</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Figure 4:** List of RE sites indicating which REs that would cut the Vimentin gene.
The second list (see Figure 5) showed the ones that would not cut the Vimentin gene (should choose from those).

Non Cut Enzymes
AatII       Acc65I       AccIII       AclI       AflII       AgeI
AhaIII       Alw44I       ApaBI       ApaI       ApaLI       AscI
Asp718I       AsuII       AvrII       BalI       BamHI       BbeI
BbvII       BclI       BglI       BglII       Bpu1102I       Bsc91I
BsiI       BamI       Bsp1407I       BspHI       BspMII       BstD102I
BstEII       BstXI       Csp45I       CspI       DraI       DraIII
DrdI       Eco31I       Eco47III       Eco72I       EcoRI       EcoRV
EheI       EspI       FseI       HindIII       HpaI       I-PpoI
KpnI       MfeI       Mlu113I       MluI       MscI       MstI
NarI       NcoI       NdeI       NheI       NotI       NruI
NsiI       PacI       PflMI       PinAI       PmaCI       PmeI
PvuI       PvuII       RleAI       SacII       SalI       SapI
ScaI       SfiI       SgrAI       SnaBI       SpeI       SphI
SpoI       SspI       SstII       StuI       SwaI       VspI
XbaI       XmnI       XorII

Figure 5: List of RE sites indicating which REs that would not cut the Vimentin gene, with four possible RE sites, which were also found in the MCS of the pET-22b(+) vector, highlighted in red.

2.10.2 The pET-22b(+) cloning/expression vector

The pET-22b(+) cloning/expression vector was chosen for the cloning and expression of the Vimentin protein because of a number of reasons (see Figure 6),

- It is a stable vector, which makes it good to clone and express the Vimentin protein (hence called a cloning/expression vector).
- It has a high expression level because of its high expression promoter, i.e. the T7 promoter.
- It has a His Tag, which is good for isolation of the Vimentin protein expressed by the vector.
- It has a pelB leader, which is good for localization of the vector in bacterial cells.
- It has the Ap gene, i.e. it is has the ampicillin resistance gene.
Figure 6: Map and sequence reference points for the pET-22b(+) cloning/expression vector.

The sequence and in particular the MCS of the pET-22b(+) vector was needed in order to see which RE sites coincided with the ones on the Vimentin coding sequence (i.e. with the ones that did not cut the Vimentin gene) in order to choose the two most suitable REs (see common RE sites highlighted in Figure 5 above as well as circled in Figure 7 below).

Figure 7: Sequence and MSC for the pET-22b(+) cloning/expression vector, with four possible RE sites, which are also found in the Vimentin coding sequence, circled in red.
2.10.3 Choice of adequate REs

Four common REs were found to be adequate (which did not cut the Vimentin gene and were common for both the Vimentin coding sequence and the pET-22b(+) vector sequence, as mentioned earlier) namely BamHI, EcoRI, SalI and HindIII.

Different combinations of the REs were attempted on the New Englad Biolabs website (Double digest finder). The best combination turned out to be BamHI and SalI, since they both shared a common reaction buffer (NEBuffer 3.1) to avoid any loss of product as well as a common temperature (37 °C) at which both REs were active.

2.10.4 BamHI and SalI RE sequences and cut sites

Both the sequences and the cut sites for the REs were also found on the New England Biolabs website (Enzyme finder). So that each RE would have space to land (on the Vimentin coding sequence and the pET-22b(+) vector sequence in order to be able to cut them) three more base pairs would have to be added to the primers,

BamHI:

5' - G^G A T C C - 3'
3' - C C T A G^G - 5'

Bp required: 3 for landing

SalI:

5' - G^T C G A C - 3'
3' - C A G C T^G - 5'

Bp required: 3 for landing

2.10.5 Primer design with RE sites

The two most important factors during the primer design were to achieve a good complementarity with the Vimentin coding sequence as well as finding matching melting temperatures (i.e. annealing temperatures) for both primers. The melting temperatures turned out to be 62.2 °C for the forward primer and 61.4 °C for the reverse primer, which were somewhat high but still good since the primers did not differ much.
Forwards

5'  NNNGGATCCATGTCCACCAGGTCCGTG  3'
5'  TCCGGATCCATGTCCACCAGG  3'

For the final forward primer, the first three nucleotides (from the left to the right) were for landing of the RE BamHI, the next six nucleotides made up the the BamHI site (where the RE would cut the Vimentin sequence, highlighted in yellow), the one C nucleotide (highlighted in red) was in order for the protein sequence to be in frame with the promoter of the vector and the last 12 nucleotides were to bind onto the Vimentin coding sequence (in order to isolate it during PCR).

The fact that the nucleotides TCC (for the landing of BamHI) and the nucleotide C (for the protein sequence to be in frame) as well as the amount of 12 binding nucleotides were chosen, was in order to find a matching melting temperature, since the factors that increase it are the primer’s GC-content as well as the amount of nucleotides the primer is made up of. The same applied when designing the reverse primer.

Reverses

5'  GATGACCTTGAATAAGTCGACNNN  3'
3'  NNNCAGCTGAATAAGTTCCAGTAG  5'
5'  NNNGTGACTTATTCAAGGTTCATC  3'
5'  CCCGTCGACTTATTCAAGGTTCATC  3'

For the final reverse primer, the first three nucleotides (from the left to the right) were for landing of the RE SalI, the next six nucleotides made up the SalI site (where the RE would cut the Vimentin sequence, highlighted in yellow) and the last 16 nucleotides were to bind onto the Vimentin coding sequence.
Once the primers had been designed, all would be set to start running the PCR. The primers would then bind to the Vimentin coding sequence (see Figure 8) in order to isolate it from the pGEM®-T-Easy maintenance vector.

Figure 8: The new Vimentin sequence with BamHI and SalI RE sites (highlighted in yellow) which have been added to it with the already designed primers (highlighted in grey) after PCR.

The pET-22b(+) vector sequence was then digested (see Figure 9) with BamHI and SalI with DNA Man (Sequence alignment program, version 8 for Microsoft Windows) in order to be able to ligate the isolated Vimentin sequence.

Figure 9: Digestion of pET-22b(+) with BamHI and SalI (RE sites highlighted in yellow, as well as the vector piece that is cut out highlighted in grey).
Once the Vimentin coding sequence had been isolated from the pGEM-T-Easy maintenance vector and the RE sites had been added to it after the PCR, it was time to ligate it into the pET-22b(+) cloning/expression vector for final expression of the Vimentin protein. Before this, the pET-22b(+) vector sequence had also been digested (with the help DNA Man, Sequence alignment program, version 8 for Microsoft Windows) with BamHI and SalI in order to cut the vector open and make sure the protein sequence was in frame with the promoter of the vector (see Figure 10).

Figure 10: Ligation of the pET-22b(+) vector (highlighted in grey) and Vimentin (highlighted in black).
3 Results

3.1 Primer design

The forward and reverse primers were designed with the help of the Vimentin coding sequence taken from NCBI as well as with the help of DNA Man (Sequence alignment program, version 8 for Microsoft Windows). Taking into account the melting temperature for both primers, the self-complementarity for each one as well as the complementarity between the two the following primers were designed,

*Forward primer:*

5'-ATGTCCACCAGGTCGTCC-3' (21 base pairs)

Melting temperature: 56.7 °C
Comments: Good, as an optimal melting temperature would be considered to be somewhere between 52 and 58 °C (PREMIER Biosoft).

Self-complementarity: -0.57 kcal mol\(^{-1}\)
Comments: Good, as the G-value is not too low. If the G-value had been much lower it would have increased the risk for self-complementation, i.e. when the primer binds to itself and creates secondary structures. This would lead to the Taq (DNA) polymerase not being able to bind well to the primer in order to carry out the elongation step during the PCR.

*Reverse primer:*

5'-TTATTCAAGGTCATCGTGATGC-3' (24 base pairs)

Melting temperature: 52 °C
Comments: Quite a low melting temperature. The maximum difference should also be ±5 °C between the primers, so this primer would be at the limit. However, it would have to do as the amount of base pairs had already been increased (which in turn increases the primer's melting temperature) and only these primers could be used (as the coding sequence for Vimentin was already known and the primers had to bind to it).

Self-complementarity: -0.25 kcal mol\(^{-1}\)
Comments: Good, as the G-value is not too low.

Once the designed primers were ready, they would be used for PCR. The purpose of the PCR was to isolate the Vimentin gene from the cDNA (that had been previously converted from the mRNA of breast cancer epithelial cells, MDA-MB-231).
3.2 Gel electrophoresis

When the gel electrophoresis was completed, in order to see if the Vimentin gene had been isolated well from the cDNA during the PCR, two clear bands could be seen on the gel as a result (see Figure 11). The bands showed to be 1.4 kb in size, which meant the Vimentin gene had been isolated successfully.

![Gel Electrophoresis Image]

Figure 11: Results from the gel electrophoresis, with two clear bands showing the successfully isolated Vimentin gene of size 1.4 kb.

3.3 Purification of PCR products

Since the PCR was successful based on the gel electrophoresis (see Figure 11), the PCR products were purified (in order to be left with the pure Vimentin gene solely). Before storing the two purified PCR products in a fridge at -20 °C, a quantification of the pure gene DNA was done from the Nanodrop spectrophotometer (see Table 6).

The purified PCR products were labeled Vimentin 1 and Vimentin 2. Good results were obtained from Vimentin 1, as the absorbance ratio at 260 and 280 nm should be around 1.8 and at 260 and 230 it should not be less than 1.8. Vimentin 2 was not used further.
Table 6: Quantification of the purified Vimentin gene from the Nanodrop spectrophotometer.

<table>
<thead>
<tr>
<th></th>
<th>Conc.\textsuperscript{n} (ng µl\textsuperscript{-1})</th>
<th>260/280</th>
<th>260/230</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vimentin 1</td>
<td>71.7</td>
<td>1.91</td>
<td>1.51</td>
</tr>
<tr>
<td>Vimentin 2</td>
<td>3.4</td>
<td>1.75</td>
<td>0.46</td>
</tr>
</tbody>
</table>

3.4 Transformation

In order to be able to clone the purified Vimentin gene in XL1-blue cells (competent cells) its sequence had to be ligated into the maintenance vector pGEM®-T Easy to start with. For the transformation 10 plates with ampicillin/IPTG/X-Gal were used, four for the standard reaction, three for the positive control and another three for the negative control. In order to see what results had been obtained, the plates were taken taken out from shaking in the incubator at 37 °C after the colonies had grown overnight.

There were some white colonies (containing vectors with inserts) and some blue colonies (containing vectors without inserts) observed on all plates, for all reactions. The white colour of the colonies was because once the insert ligated with the vector, the lacZ protein could no longer be expressed. When the insert does not manage to ligate into the vector, expression of the lacZ protein is induced by the IPTG in the presence of the substrate of X-gal, which gives the colonies their blue colour. The observations that were made from the 10 LA plates could be summarized in Table 7 below.

Table 7: Observations made from blue-white colony screening of the LA plates for the standard reaction as well as for the positive and negative controls.

<table>
<thead>
<tr>
<th></th>
<th>Standard reaction</th>
<th>Positive control</th>
<th>Negative control</th>
</tr>
</thead>
<tbody>
<tr>
<td>LA Amp, 50 µl</td>
<td>Few colonies</td>
<td>Few colonies</td>
<td>Very few colonies</td>
</tr>
<tr>
<td>LA Amp, 100 µl</td>
<td>Twice as many colonies as for 50 µl</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>LA Amp, pelleted</td>
<td>Many colonies, but can still see the individual colonies</td>
<td>Many colonies, but can still see the individual colonies</td>
<td>Few colonies</td>
</tr>
<tr>
<td>LA, 50 µl</td>
<td>So many colonies one can no longer see the individual ones. No amp to kill them, whether they take up the plasmid or not</td>
<td>So many colonies one can no longer see the individual ones. No amp to kill them, whether they take up the plasmid or not</td>
<td>So many colonies one can no longer see the individual ones. No amp to kill them, whether they take up the plasmid or not</td>
</tr>
</tbody>
</table>

Some differences were observed between the plates for all three reactions. The three first plates for the standard reaction got somewhat more colonies compared to the plates for the positive
control. Both reactions should have gotten more or less the same amount of colonies, so the results turned out good for those plates. However, some colonies were observed on the plates for the negative control as well, even though there should be none since there was no DNA that could have been inserted into the vectors. The vectors would need something to ligate to them and close them in order to be able to enter the cells and help them survive against the ampicillin on the plates, which means something else rather than DNA could have ligated to them. On the LA, 50 µl plate there was a lawn of cells for all reactions.

### 3.5 Colony screening

Following the transformation a Colony PCR was carried out, in order to isolate the Vimentin gene from the pGEM-T Easy vector in 10 different colonies, which had in turn been picked from the LA Amp, 100 µl plate. In order to see in which colonies the Vimentin gene had been transformed successfully, a gel electrophoresis was carried out.

It could be seen that the Vimentin gene had been transformed successfully into the 1\textsuperscript{st}, 3\textsuperscript{rd}, 5\textsuperscript{th} and 6\textsuperscript{th} colonies (see Figure 12) from the gel electrophoresis results.

**Figure 12:** Results from the gel electrophoresis, with four clear bands showing the transformed Vimentin gene of size 1.4 kb into the 1\textsuperscript{st}, 3\textsuperscript{rd}, 5\textsuperscript{th}, and 6\textsuperscript{th} colonies.

These four colonies would then undergo plasmid extraction in order to separate the plasmid DNA from the bacterial cells, as it was necessary to purify the plasmid DNA before it could be used further.
3.6 Plasmid extraction

Once the plasmid DNA had been extracted from the 1\textsuperscript{st}, 3\textsuperscript{rd}, 5\textsuperscript{th} and 6\textsuperscript{th} colonies, and before it was stored at -20 °C, it was taken to the Nanodrop spectrophotometer in order to see how much of the plasmid DNA the colonies contained, i.e. the concentration of the Vimentin gene sequence. The results were 60.4 ng µl\textsuperscript{-1} for the 1\textsuperscript{st} colony, 233.1 ng µl\textsuperscript{-1} for the 3\textsuperscript{rd} colony, 139.4 ng µl\textsuperscript{-1} for the 5\textsuperscript{th} colony and 72.8 ng µl\textsuperscript{-1} for the 6\textsuperscript{th} colony. Since colonies 3 and 5 showed to have the highest concentration of the gene sequence, they were sent off to another laboratory for sequencing (in order to see that the sequence obtained agreed with the Vimentin gene sequence from the NCBI website, which was later confirmed).
4 Discussion

The results from all parts of the experimental procedure showed to be good and therefore the Vimentin protein was isolated successfully from the mRNA of breast cancer epithelial cells (MDA-MB.231). Even if there was not enough time to do the last part in the laboratory but it was designed instead, the cloning and the expression design showed how it is possible to transfer the Vimentin gene onto a high expression vector such as pET-22b(+). The pET-22b(+) vector would make it possible for the Vimentin protein to be overexpressed in different kinds of cells, and from there it would in turn be feasible to investigate its function in both normal conditions as well as in disease conditions and compare them. This would be of interest for further studies, as it is still quite uncertain which is the real function of the Vimentin protein.

In some previous studies it had been suggested how the overexpression of the Vimentin protein was correlated with the progression of aggressive cancer cell lines as well as an increased migration of such cells (Satelli et al., 2011). In the future, it is hoped that the Vimentin protein can become a therapeutic target where research of new therapeutic agents, together with other anti-cancer agents, should be done. The Vimentin protein could also serve as a useful biomarker, i.e. its expression could be of help when looking at what stage a given cancer is at (Satelli et al., 2011).
Acknowledgements

I would like to express my sincerest thanks to Catherine Kaschula and David van der Spoel for letting me take part in this research project. I would also like to thank Suhail Rafudeen for all his support and also to the two best supervisors one could have, Tamaryn Ellick and Ali Kiyaei for their great guidance and constructive advice. Special thanks goes to Chiara Testini who was with me from the beginning to the end, from helping me find the project to helping me with my thesis during the last week before it had to be handed in. Last but not least I would also like to thank all the members of the Plant Biotechnology laboratory at the University of Cape Town for making me feel so welcome and for an excellent team spirit, which made it into an awesome experience both from a professional and social perspective.
5 References

A. Satelli, S. Li; "Vimentin in cancer and its potential as a molecular target for cancer therapy"; *Cellular and Molecular Life Sciences*, 2011

pGEM®-T Easy maintenance vector (Promega)

pET-22b(+) cloning/expression vector (Novagen)

Promega protocol for Standard First-Strand Synthesis of cDNA

RNasin® (Promega)


Promega protocol for Go Taq® PCR Core Systems

Go Taq® (Promega)

Go Taq® buffer (Promega)

Promega protocol for Wizard® SV Gel and PCR Clean-Up System

Promega protocol for Ligations using the pGEM®-T and pGEM®-T Easy Vectors

Promega protocol for Transformation using the pGEM®-T and the pGEM®-T Easy Vector Ligation Reactions

Promega protocol for Wizard® Plus SV Minipreps DNA Purification System (Centrifugation protocol)


DNA Man, Sequence alignment program, version 8 for Microsoft Windows