Production of Biotin-5HT$_{3A}$-His$_6$

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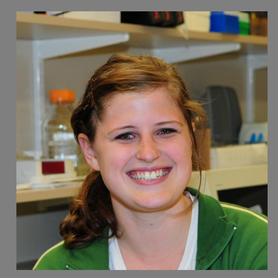

Introduction

Transfection is a process in which bacterial DNA is incorporated into human tissue and results in the production of protein. This protein can then be extracted, purified, characterized, and used for further experimental purposes. In this study, DNA from *E. coli* that has a gene carrying a double mutant of the human serotonin type three receptor (biotin-5HT$_{3A}$-His$_6$), is being prepared for the purpose of transfection with HEK-293 (human embryonic kidney) cells. This process will allow for the expression of a biotin-5HT$_{3A}$-his$_6$ membrane protein that can be used in further studies of serotonin receptors.

Experimental Strategy

- Growth of DH5α *E. coli*: incubation of double mutant bacteria in LB broth for 12 hours, as determined through the growth curve.
- DNA Extraction: Use a kit involving syringes and a series of buffers ("miniprep"). DNA is in plasmid form.
- DNA Digestion: A mixture of Bgl II enzyme, buffer, water and the DNA are heated in a 37°C water bath for about 2 hours. This cuts the DNA to a linear shape.
- DNA Purification: Ultimately performed via dialysis during which the digested DNA is placed in a 50000 MWCO (molecular weight cut-off) dialysis tube with the membrane facing downward floating in stirring 1X TE (Tris·EDTA) buffer for about 3 hours.
- Measuring DNA concentration: used a spectrophotometer to find the absorbance of the DNA at 260 nm, then used the Beer-Lambert law to solve for concentration of the DNA (ε = 0.020 mL/ug·cm for double stranded DNA).

$$A = \varepsilon \cdot c \cdot l$$

$$c = \frac{A_{260}}{\varepsilon \cdot l} = \frac{A_{260}}{0.020 \frac{mL}{\mu g \cdot cm}(0.1cm)\left(1000 \frac{\mu L}{mL}\right)}$$

$$c_{gel} = \frac{0.0083}{0.020 \frac{mL}{\mu g \cdot cm}(.1cm)\left(1000 \frac{\mu L}{mL}\right)} = 0.0042 \frac{\mu g}{\mu L}$$

$$c_{dialysis} = \frac{0.173}{0.020 \frac{mL}{\mu g \cdot cm}(.1cm)\left(1000 \frac{\mu L}{mL}\right)} = 0.0865 \frac{\mu g}{\mu L}$$

Shown above is the Beer-Lambert law and the calculation for concentration of the DNA by using the absorbance at 260 nm read with a spectrophotometer. The first written equation is the Beer-Lambert law. The second line shows a rearranged form of the equation as well values for the path length and extinction coefficient. The third line that yields a 0.0042 ug/uL concentration corresponds to figure 1 and was found after purification with a kit. The fourth equation, expressing a significantly higher concentration of 0.0865 ug/uL, corresponds to figure 2 in which DNA was purified much more successfully via dialysis.

- Concentrator: Concentration required for transfection was 0.1 ug/uL, and after purification the concentration was slightly below 0.1, so concentrator was used to remove excess water from the DNA.
- HEK Cell Culture: Cells were laid down in a 25 cm^2 flask with 10 mL of media and first changed after 6 hours time in the incubator. Once changed, cells continued to grow and the media was changed once every 48 hours or when a color change occurred.

Figure 1

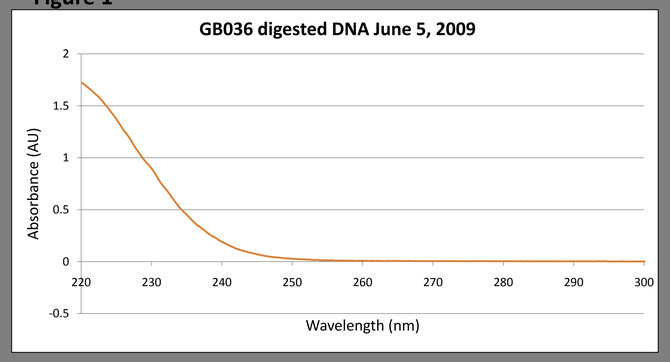

Figure 1: Using a spectrophotometer as seen above, the curve of the DNA after the first purification process, using the kit appeared to have no DNA. The overall concentration of DNA from this curve is about 0.004 ug/uL, a fraction of what is needed.

Figure 2

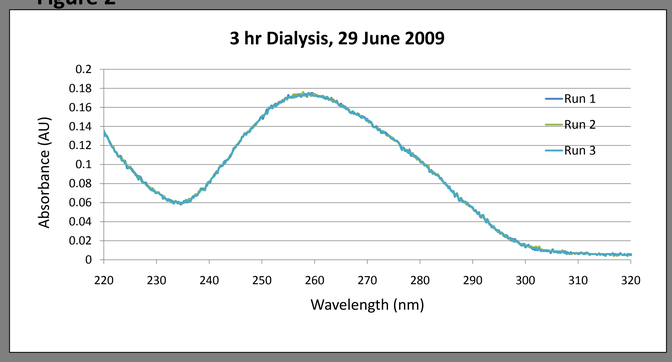

Figure 2: The above curve is what a DNA curve should appear to look like with the large peak at 260 nm, about twice the absorbance units of 230 nm. This is the curve measured after a 3 hour dialysis. The concentration of this DNA came out to be 0.0865 ug/uL, then a concentrator was used to pull it up to 0.1 ug/uL.

Figure 3

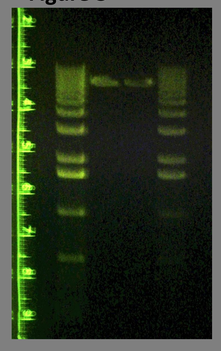

Figure 4

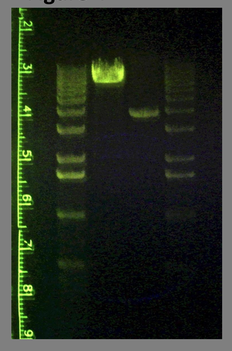

Figure 5

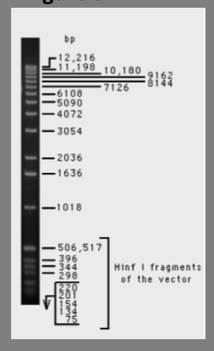

Figure 3: This gel image shows that the DNA is intact following digestion. The agarose gel was made at 0.7% with an 8 well comb. In this image, lanes 2 through 5 are in use, with lanes 2 and 5 acting as markers, and 3 and 4 have DNA.

Figure 4: This gel image is also an agarose gel of 0.7% with an 8 well comb, as well as lanes 2 through 5 loaded. Both lanes 2 and 5 once again contain a marker, and lane 3 contains digested DNA whereas lane 4 contains undigested DNA. Once again, it shows the digestion is complete and the DNA is intact after dialysis and concentration.

Figure 5: Lined up with figures 3 and 4, this figure shows the entire 1 KB DNA ladder and provides a reference for each marker band in the other two gel images.

Discussion

For the DNA purification, dialysis was not the original approach. The first procedure involved running the digested DNA through an agarose gel, cutting out the 7189 bp band, and then using a kit (QIAquick Gel Extraction kit) for purification. However, following this protocol the measured concentration of the DNA was only a fraction of what was necessary for use in transfection. Concentration outcomes from this procedure appeared to be 0.004 ug/uL rather than being relatively close to 0.1 ug/uL (figure 1). Following multiple test trials with a DNA ladder and a few more alterations each time the purification took place, it was determined that the necessary concentration was not going to be achieved using the kit. Another approach for purification involved dialysis. In this protocol, the digested DNA was transferred to a 50000 MWCO dialysis tube, and then set in stirring 1X TE buffer for 12 hours. Following dialysis, DNA concentration was significantly higher, but still only 0.026 ug/uL. Following this, the dialysis was repeated for only 3 hours, in hopes that less DNA would fall through the membrane of the dialysis tube. As hoped, the concentration of the DNA after this second dialysis yielded 0.0865 ug/uL (figure 2). Since the concentration was still below 0.1 ug/uL, though incredibly close, a concentrator was used to pull the concentration to the appropriate setting. With the concentrator, excess water was pulled off the DNA to raise its concentration, and thus, achieve about 0.1 ug/uL. The concentration of the TE buffer is slightly higher as well, but only about 1.2X, not enough to adversely affect the DNA.

Conclusion

To reach the process of transfection, a minimum DNA concentration of 0.1 ug/uL must be acquired following the digestion and purification of the double mutant DNA to be sure it is in a pure linear shape. Once this concentration of DNA has been achieved, HEK cells can be prepared. As of now, HEK-293 cells are in the growing process and will be used with the DNA for transfection. After the cells incorporate the DNA, the protein will be expressed during replication when the start codon of the inserted DNA is reached. This protein is the serotonin receptor (5-HT$_{3A}$) with additional labels of N-biotin and C-his$_6$. Once the HEK cells express the protein, it will be extracted and purified through a Ni-NTA column. Next, the purified protein will be characterized using SDS-PAGE and an avidin assay, a functional Ca^{2+} assay and a Bradford assay. The purpose of the protein will be to further study the orientation and kinetic properties of serotonin receptors.

Acknowledgements

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