# Erythropoietin Manufacturing and Purification with Recombinant DNA Plasmids to Treat Anemias

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**Abstract**

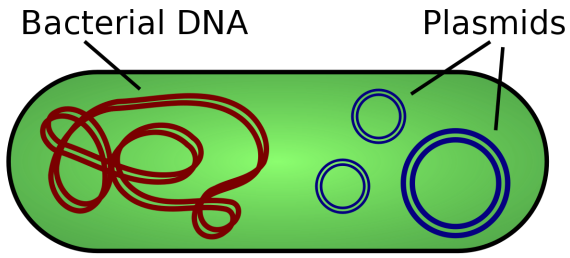
The human erythropoiesis stimulating agent, recombinant human erythropoietin (rHuEPO), was manufactured and purified using a pET-based expression system in *Escherichia coli (E. coli)*. The pET-28 backbone plasmid was used as a vector for the erythropoietin (EPO) gene to be expressed. The EPO gene was cloned into the pET-28 plasmid using the Gibson Assembly method after the plasmid was cut using restriction enzymes NotI and NcoI. The correctly assembled plasmids containing the EPO gene were verified using DNA gel electrophoresis and DNA sequencing, and then transformed into *E. coli* to manufacture the rHuEPO. The cell lysate of the transformants were purified using poly-histidine tag enabled purification by Ni2+ affinity chromatography which was possible due to the specialized construction of the purchased EPO gene which was designed to have homologous ends to the backbone plasmid and a poly-histidine tag. The purified rHuEPO extracted from the cell lysate was verified using an SDS PAGE gel which showed the correct banding pattern of the purified protein. The results of the study indicate that rHuEPO can be manufactured using recombinant DNA plasmids in *E. coli* and purified using affinity chromatography with Ni2+ beads. Future experiments include performing an assay with the purified protein on red blood cell precursors grown in culture.

**Introduction**

Protein manufacturing and purification with recombinant plasmids is a way to express a desired protein using genetically modified bacteria cells. The process begins with the acquisition of a gene of interest and a backbone plasmid. Then, a recombinant plasmid is constructed that contains the gene of interest and the backbone plasmid. The plasmid is then cloned into bacteria cells in a process called bacterial transformation. The transformed bacteria will then express the desired protein, and the protein will typically be purified using a variety of purification methods, including column chromatography, Ni2+ affinity chromatography, and other methods. After this step, an SDS-PAGE gel will usually be run to verify the purification of the protein.

The first uses of protein manufacturing with recombinant plasmids began in the 1970s, with drug manufacturers producing therapeutic proteins, but the

discovery of the existence of DNA outside of the nucleus was made decades before then, in the 1940s. It was not until around the 1950s that the term “plasmid” was coined by scientist Joshua Lederberg to describe extrachromosomal DNA.

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Visualization of plasmids, which are extrachromosomal pieces of DNA

Since their initial implementation in the pharmaceutical industry in the 1970s, plasmids have entered new fields, including human genetics, vaccine production, and even in ecology. They continue to be used routinely in the pharmaceutical industries, and have become much more advanced in their capabilities and uses in this industry, as illustrated in a study in which an important human immune system protein, interleukin-15 (IL-15), was manufactured using bacteria that had been transformed with a plasmid encoding for the IL-15 gene (Ahmed, et al., 2021).

This research led to the first-ever report of functional recombinant IL-15 being expressed in *E. coli.* The IL-15 gene was cloned into a plasmid, and transformed into multiple strains of *E. coli* to manufacture the protein. Two of the strains, Rosetta 2 and Rossetagami 2, were modified *E. coli* strains that had enhanced the expression of target proteins in past studies. Rosetta 2 supplies tRNAs of several infrequently used codons in *E. coli*. Rosetta Gami 2 also has this property and was also able to produce properly folded heterologous proteins. The results of the experiment indicated that the Rossetagami 2 produced the highest levels of IL-15.

Another instance of protein manufacturing with recombinant plasmids was done with human growth hormone (HGH). Similarly, HGH was expressed using plasmids that directed synthesized polypeptides to the *E. coli* periplasm and contained the gene for HGH, a protein found in the human body. After the bacteria were transformed, HGH was manufactured and then purified using Ni2+ affinity chromatography, which was enabled through the use of poly-histidine tags. These scientists manufactured a bioactive, crucial human protein that can and likely will be used in a variety of medical treatments for growth hormone-based therapy (Sockolosky et al., 2013).

This study aims to manufacture and purify recombinant human erythropoietin (rHuEpo), a hormone that stimulates red blood cell production in the bone marrow. This study begins with the construction of a recombinant plasmid made from a gene of interest and a backbone plasmid using Gibson

Assembly. After this step, the correctly assembled DNA construct is transformed into bacteria to allow for the uptake of the recombinant plasmid. After this, the transformed bacteria are spread on LB agar plates to multiply and produce the protein. After this, erythropoietin is expressed and the protein is purified. This process begins with the lysing of the transformed bacteria cells, and the use of nickel beads to isolate the desired erythropoietin. After the purification is verified by SDS page gel, it will be confirmed that the protein was isolated.

Erythropoietin, or EPO, a glycoprotein cytokine secreted mainly by the kidneys in response to hypoxia, stimulates the production of red blood cells. It is also produced at a steady rate to compensate for regular red blood cell decay. Appropriate conditions that may cause an increase in EPO levels include Chronic obstructive pulmonary disease (COPD) and high altitude, which cause hypoxia, and inappropriate conditions that may cause an increase in EPO levels include cancer of the liver or kidney. Because EPO raises oxygen levels in the body by stimulating the production of red blood cells, its synthetic version has been used in doping in the olympics.

In this study we plan to propose the utilization of synthetic EPO's blood cell production properties to treat Sickle Cell Disease patients, who struggle with low oxygen content in their blood due to anemia. The process by which EPO leads to the production of red blood cells begins with the production of EPO by interstitial fibroblasts in the kidneys. EPO, which is soluble in water, will then circulate in the body and only interact with cells that have EPO receptors. When EPO gets to the bone marrow, it will interact with red blood cell precursors which have EPO receptors to prevent them from undergoing apoptosis, and instead cause them to differentiate into matured blood cells.

The cell signaling interaction that red blood cell precursors go through in order to differentiate into mature red blood cells is schematized in the image below.

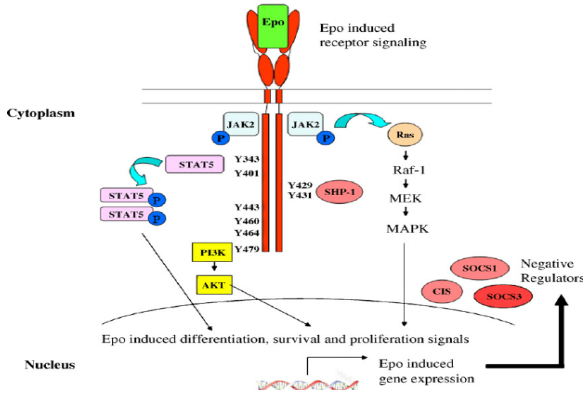


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Diagram depicting EPO cell signaling pathway

As shown in the image, precursor red blood cells have EPO receptors. Upon Epo binding to its receptor, a signal is transmitted through Janus Tyrosine Kinase (JAK) proteins on the intracellular domain of the receptor Signal transducer and activator of transcription (STAT) proteins, which are transcription factors, work with JAK

proteins to transmit the signal. If EPO does not come into contact with red blood cell precursors, the cells will undergo apoptosis. If EPO comes into contact with its receptors on these cells, the EPO receptors will dimerize, which will lead to the JAK proteins to come together and become phosphorylated. In one of the cell signaling pathways, the STATs will also come together with the JAK proteins and also become phosphorylated. Then, the activated STATs will go to the nucleus to alter the gene expression of the red blood cell precursor so that many proteins, most importantly B-cell lymphoma-extra large (BCL-X), are produced by the cell. BCL-X is anti-apoptotic, so it allows for the red blood cell precursor to become a red blood cell instead of undergoing apoptosis.

EPO's synthetic version, recombinant human erythropoietin, or rHuEPO, has been produced in a number of scientific studies. In one study, rHuEPO was manufactured using recombinant plasmids that were transformed into yeast cells. To construct the plasmid, the researchers first amplified a plasmid containing the EPO gene that was in *E.coli* cells. Then, they designed primers to amplify the gene in the plasmid using PCR and add on ECOR1 and factor XA restriction sites. They manufactured the plasmid backbone that the gene was to be cloned into using *E. coli* cells, and then they double digested the purified gene and the backbone plasmid with ECOR1 and Xba1 to create the recombinant plasmid containing the EPO gene. After this new plasmid was purified and verified, it was transformed into pichia pastoris cells. Then, the rHuEpo was manufactured and purified (Celik, et al,. 2007).

RHuEPO has also been used to treat a wide variety of diseases, including cancer. One experiment used recombinant human erythropoietin to treat MCF-7 breast cancer cells. The results of the experiment indicated that the rHuEpo administered had a "cytostatic" effect on the MCF-7 breast cancer cells, and they suggest that the recombinant protein could be used in the future to treat breast cancer. In the experiment, MCF-7 cells were grown in a 3D model, and a separate group of them was grown in a 2D model. After the cells were incubated and prepped, they were treated with rHuEpo. Then, the cells were observed and tested using multiple assays. The results indicated that rHuEpo prevents the proliferation of MCF-7 cancer cells in a 3D model (Hareth et al., 2021).

**Results**

In order to construct the recombinant plasmid containing the gene for rHuEPO, the Gibson Assembly method was used. The schematic of the assembled plasmid and the EPO gene with 6-his tag, shown in figures 1 and 2, respectively. Figure 1 shows the fully

constructed plasmid visualized in the software *SnapGene,* and figure 2 shows a zoomed in part of the plasmid containing the gene of interest, Hig-tag, and homology arms for cloning, also visualized in *SnapGene*.

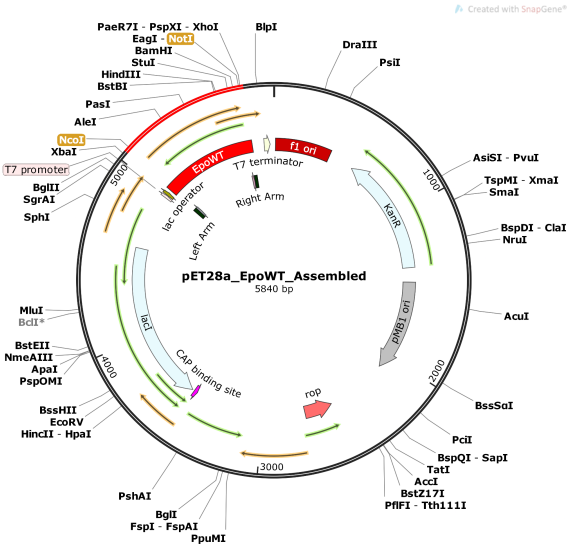


**Figure 1: Constructed Recombinant plasmid containing EPO gene**

The fully assembled plasmid was visualized in SnapGene. It portrays the entire plasmid, complete with the gene of interest and the restriction enzyme sites, and it shows the total number of base pairs of the plasmid (5840).

**Figure 2: EPO gene within recombinant plasmid**

A zoomed-in section of the plasmid shows the EPO gene insert within the plasmid. The visualization also shows the modified parts of the insert, including the his tag on it that would later be used for the purification of the expressed rhEPO

In order to verify that the recombinant plasmids were constructed correctly, the plasmids underwent DNA gel electrophoresis and DNA sequencing. Figure 3 shows the results of the DNA sequencing, and figures 4 and 5 show the result of the DNA gel electrophoresis. Both figures show verification of the plasmids, but figure 3, which depicts the DNA sequence of the plasmids, shows more specific information that better verifies the plasmids. In figure 3, the sequence of the individual nucleotides from most of the plasmid samples matches up identically with the actual sequence of the plasmid of interest.

In figure 4, all of the wells in the gel but one were loaded with the same plasmid, so the banding pattern of the samples are nearly identical. 10 plasmids that were extracted from bacteria cells were placed in the gel, so each well corresponds with a plasmid sample. The one well that was not loaded with the plasmid sample was loaded with a molecular weight marker to provide a reference point for the results. The gel shows the correct banding pattern of the plasmid, indicating that the plasmids are the size that they are supposed to be. In figure 5, before being run on the gel, the plasmids were cut using restriction enzymes to allow for the gene of interest's banding pattern to be visualized on the gel. Like the gel in figure 4, all of the lanes but one were loaded with the same samples, which

contained the digested plasmid, so each lane shows nearly identical results. The results show the banding pattern of the gene of interest in the red rectangle. The band is at the correct size on the gel, indicating that the gene of interest in the plasmid is the correct size.

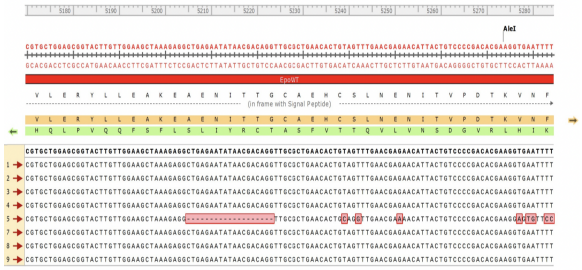

**Figure 3: DNA Sequencing of recombinant plasmids**

DNA sequencing was very useful in verifying the recombinant plasmid. The results showed that the nucleotide sequence of the samples run on the gel were nearly identical to what they should look like. The red highlighted letters indicate a base pair that was not correct.

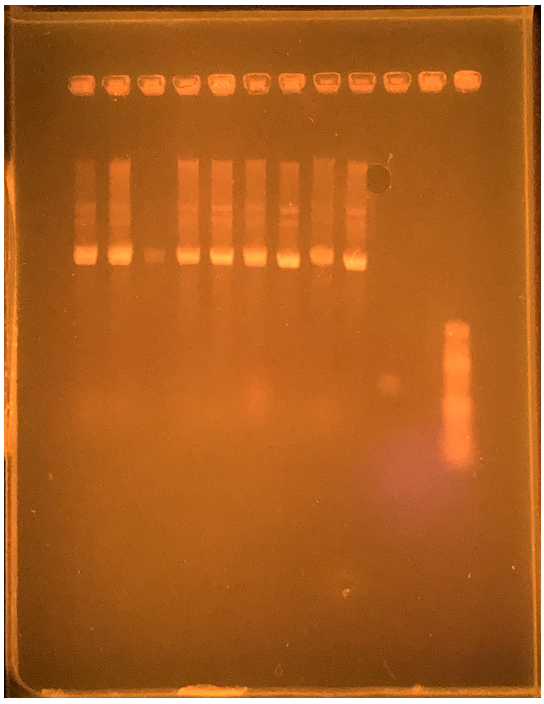

**Figure 4: Gel electrophoresis result of verified ligated plasmids**

Each well except the molecular weight marker well was loaded with the same ligated plasmid sample. The gel shows the banding pattern of the plasmid at the correct length, indicating that the plasmid to be used in transformation was the correct length. Before entering the gel electrophoresis chamber, the plasmid samples were cut to allow them to be a string of DNA instead of a circle.

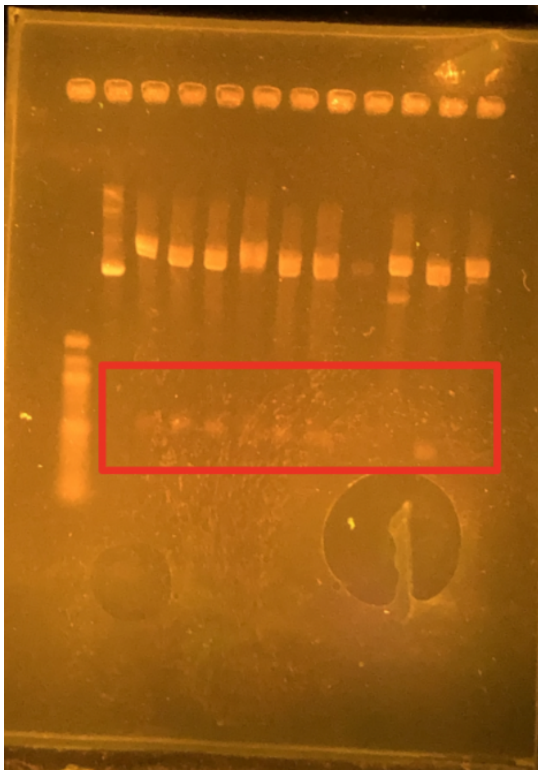

**Figure 5: Gel electrophoresis result verifying digested plasmids and gene insert**

In this gel, the plasmid samples, all of which were nearly identical, were first digested using restriction enzymes to separate the gene of interest from the rest of the plasmid. This allowed for verification of the gene of interest because the gel separated the gene of interest from the rest of the plasmid based on their sizes. The red rectangle shows the banding pattern of the gene of interest, and the result is very similar to the ideal banding pattern.

The results of the transformed bacteria containing the plasmid of interest are depicted in figure 6. The figure shows 10 agar plates containing the

transformants, all of which were treated with the same plasmid. Although it is difficult to make out from the image, the transformants did manufacture the rhEPO thanks to their recombinant plasmids, indicating that the plasmid was successfully transformed into the bacteria. The plates contained LB agar and 5% kanamycin solution, and the bacteria would only be able to grow on the plates if they had a gene that made them resistant to the kanamycin. They were able to grow on the plates because the recombinant plasmid that was transformed into the bacteria not only contained the gene to express rHuEPO, but also a gene that coded for kanamycin resistance. The bacteria growth on the plates suggests that the plasmid was successfully transformed into the *E.coli* cells.

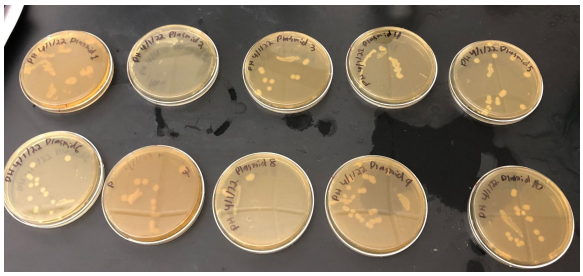

**Figure 6: Bacterial transformants**
The transformed bacteria were grown on ten different LB+kan plates to maximize the chances of growing transformants. The plates had kanamycin on them to verify successful transformation. After the bacteria were treated with the plasmid, they incubated overnight and were then transferred to the plates above.

In order to verify the purified rHuEPO that was purified using Ni2+ affinity chromatography, an SDS PAGE gel was performed. An SDS PAGE gel is a technique used to separate proteins by their size, so it is useful in identifying specific proteins. The results of the SDS PAGE gel are depicted in figure 7. The banding patterns on the gel show that the protein of interest was purified from the cell lysate in the affinity chromatography. The first well in the gel was loaded with the molecular weight marker to provide a reference point for the rest of the samples. Without it, it would not be possible to relate the banding results of the samples back to something to draw conclusions. The next three wells, going from left to right, are from the same sample of purified protein from different stages of protein elution. The banding pattern shows the correct size of the purified rhEPO, verifying that the protein was successfully purified, as the samples containing the purified protein showed the correct size of the protein on the gel. The fourth lane contains a molecular weight marker to provide a reference point to the next three lanes, which contain the purified protein. The next three lanes also verify that the protein was purified and that pure rhEPO was acquired.

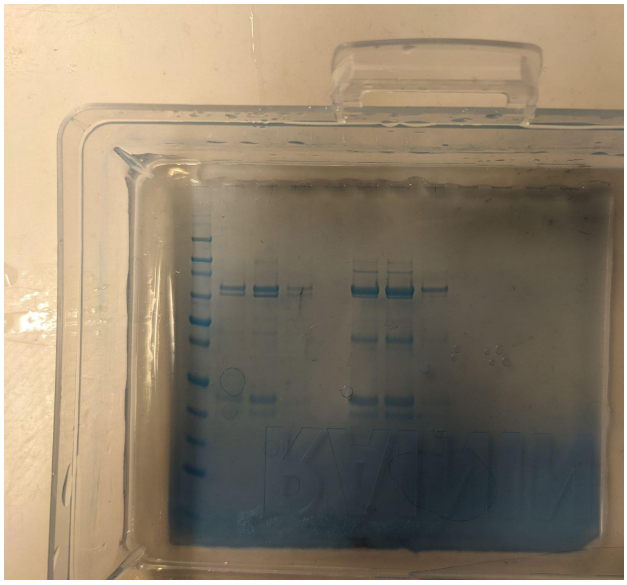

**Figure 7: SDS PAGE gel verifying purified protein**

The gel shows the banding pattern of the molecular weight marker and of the purified protein. Most wells were loaded with the same contents to maximize the chances of successfully verifying the protein.

## Discussion

The goals of this experiment were to create a recombinant plasmid containing the HuEPO gene, manufacture rHuEPO using *E.coli* cells that had been treated with said recombinant plasmids, purify and verify the manufactured protein, and use an assay to test the treatment potential of the rHuEPO. The results indicate that the recombinant plasmid containing the rHuEPO gene was successfully constructed, the desired protein was manufactured by transformed *E.coli* cells, and the manufactured protein was purified and verified. Most of the goals of the experiment were met and it was successful on the whole.

The strongest strengths in this experiment were the extensive verification processes that were used to verify results. In both the verification of the constructed recombinant plasmid and the purified protein, detailed and precise verification methods were used, which allowed for more accurate, trustworthy results. In the case of the recombinant plasmid, the verification process included both multiple DNA gel electrophoresis gels and DNA sequencing. The DNA gel electrophoresis was imperative in verifying the plasmids, as it displayed both the size of the constructed recombinant plasmid, and the size of the EPO gene within the plasmid. The DNA sequencing accomplished both of these tasks as well, but in a much more

precise manner. It was able to identify the specific nucleotide sequence of the constructed recombinant plasmid, and compare it to the ideal specific nucleotide sequence of the desired plasmid. This was extremely beneficial to the experiment as it boosted the trustworthiness of the sample that was being tested that it would behave in the correct way in future parts of the experiment.

One of the key weaknesses of the design of the experiment was that the bacterial transformation procedure had to be repeated multiple times. The procedure utilized the heat shock method, which required the samples containing the recombinant plasmid and the bacteria cells to be put on ice for fifteen minutes, then “shocked” in a hot heat plate for forty five seconds, and then put back on ice for one to two minutes. This process was difficult because, due to the number of samples that had to be “shocked”, it was hard to have each of them start at the same time. The extended “shock” of the first few samples likely damaged the bacteria cells and led to multiple failed transformations, which caused the experiment to have to have been redone multiple times. The transformation procedure also called for the transformants to be immediately spread onto plates to grow immediately after they were mixed in LB broth. This prevented the transformants from being able to incubate overnight in the LB broth to multiply, and prevented them from growing on the plates when they were immediately transferred to the plates.

If this experiment were to be replicated, many of the weaknesses of its design and execution would be avoided. The transformation procedure would have been altered in order to allow for all of the samples to be “heat shocked” at the same time. This would have allowed for the plasmids to enter the bacteria cells more easily, and would have prevented the bacteria cells from dying due to overheating. The procedure would also be altered in order for the transformants to incubate in the LB broth to grow before being spread on to the LB+kan plates. By implementing these revisions to the design of these experiments, the accuracy and efficiency of the study would be greatly improved.

Although most of the goals of the study were accomplished, the treatment potential of the purified rHuEPO was not tested. Future experiments to progress this study would include completing a red blood cell assay to test the erythropoiesis stimulating potential of the purified rHuEPO. This would begin with an RBC cell line to study the rHuEPO effects on the RBCs. If the results of this experiment show that the rhEPO was able to stimulate erythropoiesis, the next phase of the study would be to test the protein on animal subjects at an offsite location.

**Experimental Procedures**

To construct a recombinant plasmid containing the EPO gene and a plasmid backbone, a slightly altered version of the gene was purchased and the backbone was acquired. The gene was specifically designed to have a 6xHis-tag and homologous ends to the backbone plasmid, so that it could be easily cloned into the backbone plasmid using Gibson Assembly. A polyhistidine tag on the gene would be necessary for future poly-histidine tag enabled purification by Ni2+ affinity chromatography. In order to clone the gene into the backbone plasmid, the backbone was first cut using restriction enzymes in order to use it as a double-stranded linearized piece of DNA. Once the backbone was prepared, the gene was cloned into the backbone using Gibson assembly and the recombinant plasmid was constructed.

The plasmid that contained the assembled EPO gene and then transformed into bacteria cells in order to store the plasmid was extracted from the bacteria cells using a plasmid miniprep. The extraction process began with the administration of a buffer to the cells. Then, the cells were applied to a QiaQuick column and centrifuged in order to bind DNA. The flow through from the centrifuge was then discarded and the sample was washed in the column using a buffer and then centrifuged. Then, the DNA was eluted using a buffer and centrifuged.

Once the plasmids were extracted from the cells, half of them were digested using restriction enzymes, and all of them were run on a gel electrophoresis to verify the plasmid. The purpose of cutting half of them with restriction enzymes was to observe whether or not the gene of interest in the plasmid was the correct length. The length of the insert was determined by looking at the results of the gel and seeing if there was a band where the protein of interest would be.

After the gel electrophoresis verified the plasmids, the plasmids were transformed into bacteria cells using the heat shock method. The samples containing *E.coli* cells and plasmids were first put on ice for fifteen minutes and then shocked in a 42ºC bath for 45 seconds. They were then transferred back to the ice and administered LB broth to grow the transformed bacteria. After being incubated overnight, the transformants were transferred to LB+kan plates to grow and manufacture the recombinant erythropoietin.

In order to purify the protein that was produced by the bacteria that had undergone transformation, the transformants underwent poly-histidine tag enabled purification by Ni2+ affinity chromatography. To prepare for this, the transformants that had been growing on LB+kan plates were administered PBS and lysosome (to lyse the cells) and transferred to new tubes labeled

“lysate”. Once the cell lysate was acquired, the nickel beads that were to be used to purify the protein using affinity chromatography were prepped and washed with a PBS buffer.

After the cell lysate was made and the nickel beads were washed, the purification process began. First, the cell lysates were centrifuged and the supernatant of one of the samples was removed and transferred to the nickel beads. Then, this tube was rocked to wash the supernatant in the nickel beads. The supernatant of this sample was transferred to a new tube and labeled flow through. The sample was then washed twice with PBS and administered an elution buffer and rocked to wash the sample in the elution buffer. The supernatant of the sample, which was theoretically the purified protein, was transferred to a new tube and the nickel beads were kept.

In order to verify that the protein of interest was purified, an SDS page gel was conducted. The samples from the affinity purification (SUP (supernatant), FT (Flow Through), W1 (Wash 1), W2, E (Eluate) and E2), were prepped by first adding sample buffers and then heating at 95ºC for 5 minutes. The samples were then loaded into an SDS PAGE gel box along with a molecular weight marker ladder for determining the band size of the samples. After the gel finished running, it was stained using coomassie blue stain and destained to better visualize the banding pattern of the gel.

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