The Production of Human Leptin Using Cell Free Protein Synthesis

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Human leptin is a hormone that suppresses hunger, also known as satiety. It is produced by the adipose (fat cells) in order to help regulate energy balance. Obesity is often attributed to a decreased sensitivity to leptin because of the inability to tell when one is satiated despite high energy stores. Our goal is to engineer a plasmid that would cause non harmful bacteria to produce leptin for use in weight loss dietary supplements or for hormone therapy. Our plan to do this begins with us cloning the leptin gene into the plasmid pP454-SR and mass producing the plasmid using PCR. We will then insert the plasmid into cell free lysate where it will begin to produce measurable amounts of leptin that can be quantified by ELISA. Then, we plate *Escherichia coli* BL21(DE3) cells with the cloned plasmid and amplify using PCR to make multiple copies of the leptin gene construct with a T7 promoter and T7 terminator. Then, we will insert the plasmid with the human leptin gene construct into the cell free lysate and test if it has produced any leptin with the ELISA test, which can detect small traces of the hormone. We believe that using this construct, we can produce at least 2 pg/mL of leptin because that is the minimum amount the ELISA test can detect.

Keywords: ELISA, Leptin, Cell Free Protein Synthesis, Lysate, TXTL

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Obesity is a very common and pressing health concern in many developed countries across the world. In an attempt to offer more options for doctors and pharmaceuticals treating patients with obesity, our team has decided to try to express the human leptin gene in a Cell-Free Protein Synthesis environment. We hope that the process can be performed at an industrial level some time soon. Leptin is a hormone that is produced by the body to inhibit the desire to consume food and we believe that leptin can be created using bacterial cell lysates and mass produced similar to the production of pharmaceutical insulin. It could then be packaged in dose-appropriate amounts in gel caps for patients with obesity and other hunger related issues. The cell free product could be dosed appropriately using mouse models and eventually used in human clinical trials. The experiment we performed will allow researchers to consider using cell free protein synthesis techniques to create leptin in large quantities that would be used in health care protocols with human patients. Human leptin has been produced in bacteria before, using a leptin-cloned plasmid (Li et al., 2010). The resulting hormone was used in mouse models and yielded positive results. Our project will use cell-free lysate, effectively bypassing the cellular constraints like resource distribution, genetic and protein cross-talk and genetic mutation that sometimes can interfere and slow down the research and development process (Figure 1).

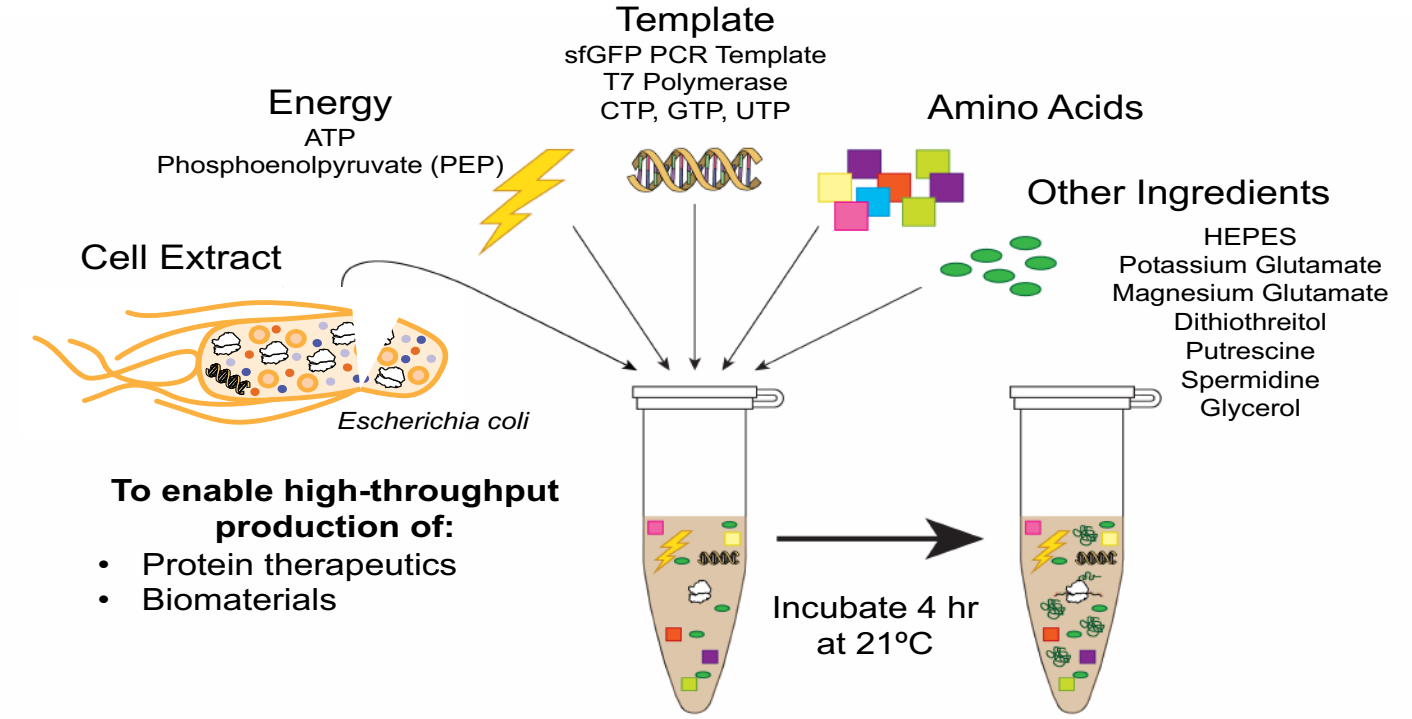

Figure 1. Cell-free protein synthesis is a combined translation/transcription reaction driven by ATP and catalyzed by lysate (Carlson, et al., 2011; Goshima, et al., 2008). Image adapted from Jennifer Schoborg. Copied with permission from Jessica Stark and Jennifer Schoborg.

Materials and Methods

Preparation of the Plasmid

We came up with the idea of putting a leptin gene, a T7 promoter, and a T7 terminator in a plasmid. The plasmid was designed and ordered from ATUM (Newark, California). The genetic construct consisted of a T7 promoter, human leptin gene coding sequence (GenBan Accession BC060830), and a T7 terminator sequence (BBa_B0013), cloned into plasmid pD454-SR (Figure 2). The plasmid was shipped freeze dried and rehydrated with 20 µL nuclease free water and incubated at room temperature for 10 minutes.

Competent Cell Transformation

The first step in our project was to transform CaCl2 competent cells of the *E.coli* strain BL21(DE3) star, provided by the Jewett Lab at Northwestern University, with the student-designed recombinant plasmid pD454-SR Leptin, provided by ATUM, and grown on Teknova LB agar plates supplemented with 100 µg/mL of ampicillin and incubated overnight at 37°C. In each of the stock agar plates there were BL21(DE3) star cells to enhance the probability of a successful transformation. We used a standard heat shock protocol employing a 90 s incubation in a water bath set at 42°C. We added 2 µl of 2-5 µg of designed plasmid per transformation. The results of our transformations indicated relatively low transformation frequencies with two or three transformed colonies per plate. Individual colonies from our stock plates were selected and grown overnight in 4 tubes of 5 mL LB broth supplemented with 100 µg/mL of ampicillin, mixed in an Argos tube roller at 22 RPM. After rolling for 24 h, the cells were centrifuged at 15,000 RPM for one minute to form a pellet and was then mini-prepped using a kit protocol designed by Omega Biotek. The plasmids were quantified at 33.3 ng/µL as compared to a nuclease free water blank using Nano-Drop technology. The extracted plasmids were stored in a refrigerator at 4°C until needed for the cell-free portion of the experiment.

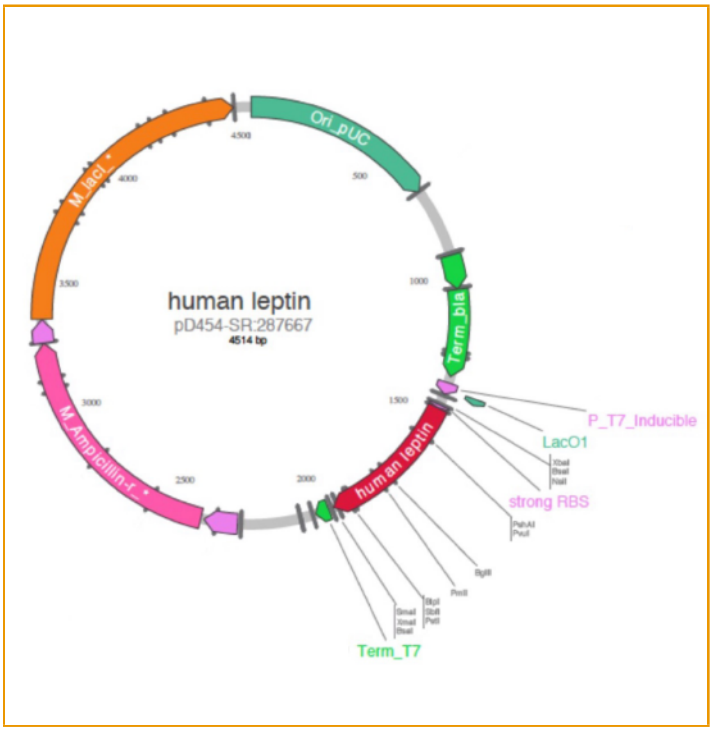

Figure 2. Plasmid pD454-SR with T7 promoter leptin ORF and T7 terminator cloned genetic construct.

In vivo Product Comparison

Our project was initially designed to test the protein produced by two different DNA constructs, one plasmid-based and the other linear-based, in a cell-free protein format. We later decided to add a third element for comparison by measuring out the leptin production by the originally transformed BL21(DE3) star cells using SDS-PAGE (BioRad).

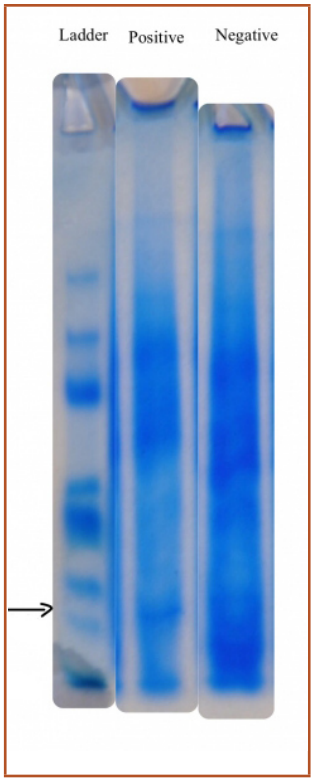

Figure 3. In vivo SDS-PAGE gel showing a 16 kDa leptin band for the positive control sample.

We cultured our transformed cells on LB agar supplemented with 100 µg/mL ampicillin, 40 µL 0.1M IPTG and a liquid culture of 4 mL LB/amp/0.1M IPTG broth. The IPTG was added to activate the production of leptin by the DNA construct. We scraped off the fresh cells from the agar plate and added them to 150 µL Laemmli buffer with DTT and then added 100µL of the cells from the broth into 150 µL of the Laemmli buffer with DTT and heated at 95°C for five minutes prior to adding the contents to the polyacrylamide gel for separation. We did all this in order to determine how the two different approaches to leptin production would compare in terms of measurable protein output. The leptin band was measured to a mass of 16 kDa on a 4-20% mini-protein gel (BioRad) run at 125 V for 90 minutes. Our gel used 20 µL sample in each of 6 wells to enhance band visibility. Our gels used a Kaleidoscope protein ladder (BioRad) for the estimation of protein band mass. We decided to compare a negative control of BL21(DE3) star cell protein banding quantity versus a positive control of BL21(DE3) star cells transformed with our plasmid and activated by IPTG (Figure 3).

Inoculation of Cell Lysates

We chose to perform the the cell-free protein portion with a lysate comparison approach. For the negative control,we hydrated freeze-dried bacterial lysates from BL21(DE3) star cells with 15 µL nuclease free water (Promega) mixed by brief centrifugation and we then incubated the mix. The positive control tube was inoculated with 6 µL pD454-SR leptin plasmids purified by mini-prep and 9 µL nuclease free water to balance the volume at 15 µL total for both trials. Both lysates were incubated in a water bath at 30°C for 20 h and frozen at -80°C until SDS-PAGE could be performed.

In Vitro Product Comparison

We used freeze dried BL21(DE3) star lysates for comparison of leptin production during the in vitro plasmid phase of the experiment. After the 20 h incubation period, the lysates were combined with 15 µL Laemmli buffer with DTT, mixed by flicking gently, then added into the SDS-PAGE gel. Six 5 µL samples of negative control and six 5 µL samples of positive control were added. The samples were run under 125V for 90 minutes. The negative control lysate had no plasmid added while the positive control lysate contained plasmid (Figure 3).

Observations and Measurements

Proteins produced by cell-free TX-TL and in vivo protein synthesis were identified first by SDS-PAGE analysis. Two samples of lysate were run along with a negative control using only BL21(DE3) star lysate and 15 µL nuclease-free water and a positive control using BL21(DE3) star lysate and 6 µL 33.3 ng/µL plasmid DNA and 9 µL nuclease-free water for a total volume of 15 µL. Both 15 µL lysate samples were mixed with 15 µL Laemmli buffer with DTT added to bring the total volume to 30 µL. The samples were incubated in a water bath at 95°C for 5 minutes prior to gel analysis. 5 µL aliquots were added to the SDS_PAGE gel and run at 125 V for 90 minutes, stained with Coomassie stain for 24 h and destained with several rinses using diH20 over a 24 h period. Protein bands were photographed and then later quantified with an ELISA test (Invitrogen). The in vivo results were previously described. Expression results were intended to compare protein yield using a plasmid-based approach versus an in vivo approach. Data was compared against a standard curve and appropriate controls were used.

PCR of the genetic construct was performed and the T7 promoter, leptin ORF and T7 terminator were bracketed by forward and reverse primer built by IDT. The primers consisted of 19 oligonucleotides and had a melting temperature of 55°C. PCR tubes were loaded with 2.5 µL 10 µM nuclease free water. The thermocycler was set to 98°C during the denaturing phase, 51°C for the annealing phase, and 72°C during the extension phase. After 35 cycles in the thermocycler, a 1% agarose gel was run. After confirmation of the 655 bp fragments, DNA was cleaned up with a kit from Omega Biotek and a new confirmation gel was run. The results clearly show triplicate bands at 655 bp indicating appropriate fragment lengths (Figure 4).

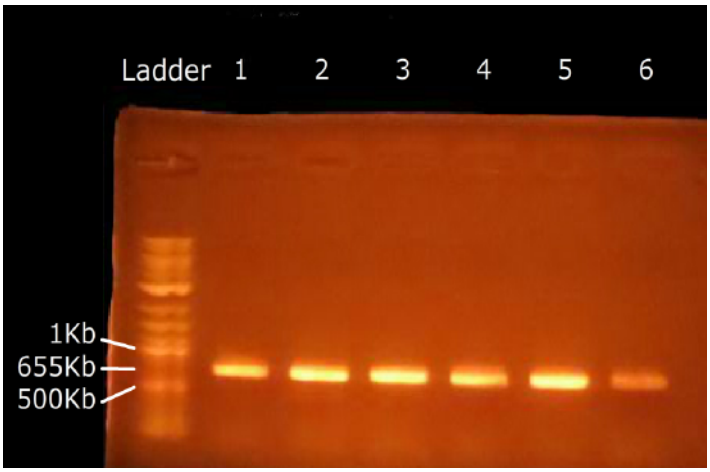

Figure 4. Linear PCR amplicons of the leptin DNA construct.

Amplicon DNA was quantified using nano-drop technology and the yield was 24 ng/μL as compared to nuclease free water used as blank.

Cell-free lysates of BL21(DE3) star cells were inoculated with 8.4 μL linear DNA, 2 μL GamS and 4.6 μL nuclease free water for a total volume of 15 μL. A negative control lysate was inoculated with 2 μL of GamS and 13 μL of nuclease free water. The lysates were incubated for 20 hours at 30°C and placed in a -80°C freezer until SDS-PAGE gels and an ELISA test were run to quantify the product.

A leptin ELISA test (Invitrogen) was performed for all three experimental conditions, in vivo, in vitro plasmid-based and in vitro linear DNA based expression. In vivo negative control cells BL21(DE3) star were grown in LB media while positive control cells BL21(DE3) star+plasmid were grown in LB amp IPTG broth to 0.220 OD600. 100 μL cells were lysed with 100 μL 1% SDS and vortexed for 10 s prior to loading into the ELISA plate. BL21(DE3) star negative control lysate and BL21(DE3) star lysate with plasmid were incubated and loaded into the ELISA plate. Finally, BL21(DE3) star negative lysate and BL21(DE3) star lysate with linear DNA were incubated and loaded into the ELISA plate (Figure 5).

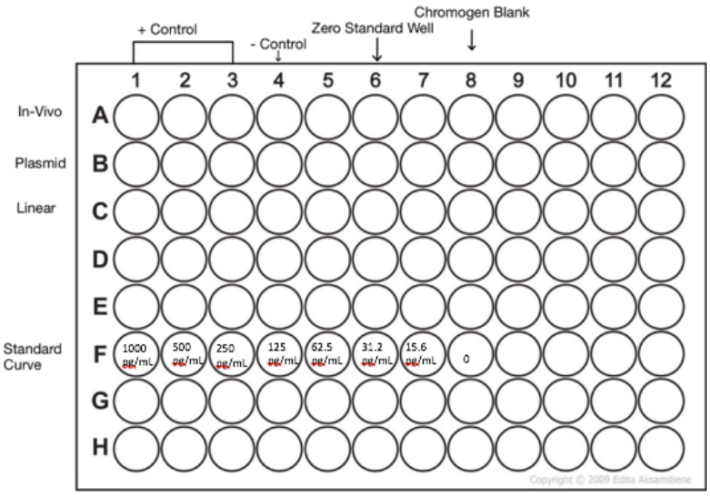

Figure 5. ELISA plate setup

The ELISA plate was loaded with all three cell/lysate conditions in triplicate and with negative control samples. Samples were all loaded with 10 μL cell/lysate volume diluted with 990 μL standard diluent buffer. The ELISA test also used three wells of chromogen and stop buffer used as a blank for the plate reader. A standard curve was also established through serial dilutions to serve as a comparison for our data versus a known data set. The ELISA 96 well plate results were measured by a plate reader at Northwestern University and analysis of results follows.

Environmental and Safety

BL21(DE3) non-pathogenic *E.coli* were used during this experiment and standard aseptic techniques were followed during the course of the experiment. Containers and inoculating loops were treated with 10% bleach for 20 minutes following use. The cells, lysates, and DNA pose no health risks to the experimenters or the environment, however, proper eye and skin protection were used throughout this experiment. All lab surfaces and equipment were cleaned with 10% bleach following each portion of the experiment.

Results and Discussion

Our experiment featured two protein production processes, one in vivo process and two in vitro. The in vivo BL21(DE3) star, IPTG induced leptin genetic construct worked well as was indicated by a protein band at the 16 kDa location on an SDS-PAGE gel (Figure 6). This evaluation was our positive control, which was repeated six times, and in each gel lane there was a leptin band at the 16 kDa location proving that the leptin genetic construct worked as anticipated. The negative control featured non-transformed BL21(DE3) star cells and when applied to an SDS-PAGE gel yielded no leptin protein band at the 16 kDa location thus supporting our successful leptin production genetic construct in the transformed cells. The negative control was also repeated six times in the SDS-PAGE gel. The visible bands were relatively easy to see and our comparison showed an obvious difference in protein production. Using SDS-PAGE proved to be a bit of a challenge as we had several rounds of gels that had inconclusive smearing of protein bands. We mitigated this issue by changing the sample volumes, modifying the Laemmli and DTT quantities, and slowing running time in order to produce the appropriate gel quality needed for our evaluation. A human ELISA test was performed to quantify leptin concentration for each of the test samples. The ELISA test has a sensitivity of 2 pg/mL and proved to be a more reliable way to quantify leptin as compared to using SDS-PAGE.

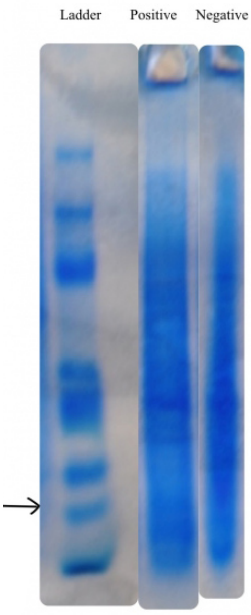

Figure 6. In vitro SDS-PAGE gel showing a plasmid-based 16 kDa leptin band.

The in vitro component of our experiment was the more interesting protein evaluation format because the cell-free process is new and untested. We used the exact same freeze-dried lysates derived from BL21(DE3) star cells for our positive and negative controls. The positive control lysate, which was supplied with our genetic construct cloned into plasmids worked surprisingly well and yielded a strong leptin band at the 16 kDa location on the SDS-PAGE gel. This leptin band illustrated that the plasmid was a quality vector and the cloned genetic construct worked as anticipated. The negative control without the plasmids did not produce a leptin band in the gel at the 16 kDa position thus proving that the leptin was a direct result of the added plasmid. We were surprised to see the quantity of protein produced by the TX-TL system and it could perhaps be a candidate for increased scale for industrial manufacture. As was seen with the in vivo gel process, getting the proper amount of protein Laemmli buffer and DTT proved difficult.

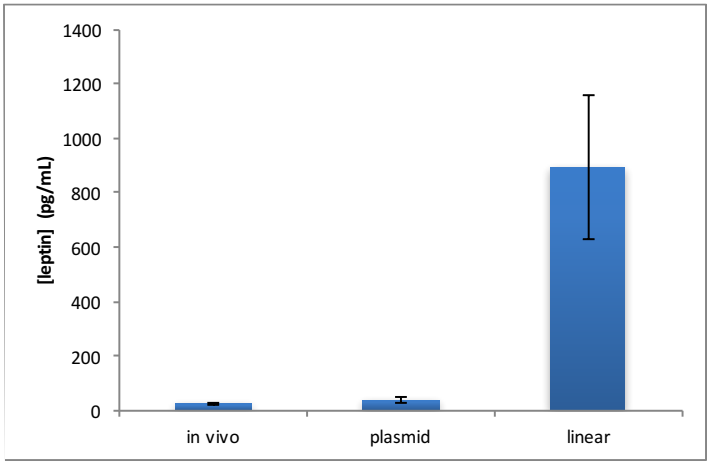

Figure 7. Results from the ELISA test showing a comparison between in vivo, in vitro plasmid, and in vitro linear protein production.

The performance between the in vivo and in vitro protein production systems were different than expected (Figure 7). The protein product measured by the ELISA test indicated that the in vivo and plasmid-based techniques yielded far less leptin as compared to the linear DNA technique which was opposite of our expectations. There are potential reasons for this result. Perhaps the whole cell in vivo technique needs longer lysis and vortex time for adequate removal of leptin from within the cells. Perhaps the plasmid-based technique required too many cell resources or had possible plasmid folding issues. Perhaps the linear DNA construct produced leptin with a more appropriately shaped conformation that could bind more frequently with the ELISA wells. There are several potential reasons for the results that we measured and these conditions could be investigated more thoroughly in future experiments. Our main goal was to evaluate the in vitro TX-TL system to see if we could get meaningful results that could be scaled up in an industrial application. We were pleasantly surprised with the amount of leptin created by TX-TL, especially using linear DNA. The next logical step would involve creating a concentration assay and optimizing the linear DNA and lysate concentrations for maximum production output.

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