Gene identification and Expression of BH3 Interacting Domain Death Agonist

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Abstract

Molecular biotechnology is becoming increasingly integral to modern industrial processes involving living organisms, such as food production, medical applications, and even fuel production. As an educational exercise to investigate this budding field, a recombinant plasmid containing an unknown GFP-tagged gene was inserted and ultimately expressed in U20S cells. Two identical trials were carried through the entire experiment. Prior to transfection, the plasmid DNA was amplified by selection of ampicillin resistant transformed bacteria, purified by anion exchange chromatography on silica resins, brought to known concentration after determining an initial concentration of .955 μg/μl (Exp 1) and 1.285 μg/μl (Exp 2) via 260/280nm spectrophotometery, and evaluated by agarose gel electrophoresis of PCR products and BsmB1 and Kas1 restriction digests. For BsmB1 digest, a single fragment of 7260bp was observed, while fragments of 5488bp and 1772bp were observed with Kas1 digest. Double restriction digest lengths were 5488, 945, and 827 bp, and the PCR amplified a 800bp gene. These electrophoresis results eliminated three out of the four possible identities for the unknown gene, thereby identifying it as the encoding for BID, a mitochondrial-associated protein in the apoptosis pathway. The BID gene was then transfected into U20S cells. Optical microscopy revealed localization of the GFP-tagged protein to the mitochondria for cells stressed by TNFα, further verifying gene identification. During the transfection process, a NFkB reporter luciferase plasmid was also transfected, and the TNFα was observed to triple luminometry measurements of the lysates, implying TNFα influence on the NFkB pathway. The study was concluded by measuring GFP expression by an immunoblot of cytoplasmic proteins subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis. A band of 50kDa was noted, consistent with BID-GFP complex. Decreased GFP detection was also noted for the TNFα lysates, possibly due to BID localization within the mitochondria rather than the cytoplasm under TNFα stress.

Keywords: Plasmid purification, gene cloning, restriction digest, polymerase chain reaction

Introduction

The ability to move genes from one organism to another has profound applications. For this reason, recombinant DNA has become a widely adopted technology in a variety of disciplines. The pharmaceutical industry has benefited greatly from recombinant organisms programmed to express genes for medically relevant proteins, such as human insulin [1]. Gene transfer is not limited to single celled organisms, however. Modern agricultural developments include crops genetically programmed to excrete insecticides, be more easily harvestable, or more nutritious [2]. The ability to tag recombinant genes with GFP has lead to major developments in this industry over the past 15 years [3].

This technology boom has made recombinant DNA procedures, and the associated techniques for purifying and identifying genetic material, a highly relevant subject for engineering education. Accordingly, a general procedure for the transfer of a commercially bought recombinant plasmid into a mammalian cell line is discussed. The end goal of this study is to verify the identity of an unknown

gene using a variety of biotechnology techniques.

The process consists of several amplification, testing, and purification procedures, followed by analysis of gene expression. The plasmid gene was identified from gel electrophoresis of restriction digest and PCR products, successfully transfected into a mammalian cell line, manipulated with external stimuli, and analyzed for gene expression.

Materials and Methods

Bacterial Transformation and Culture

To increase the quantity of the DNA of interest, competent bacteria were transformed with the plasmid and then cultured. An aliquot of 30 µL of 'competent' DH5α cells combined with 2 µL of plasmid DNA was incubated on ice for 30 minutes. The aliquot was then placed into a 42 °C water bath for 45 seconds then back on ice. Next, 450 µL of SOC broth were added to the mixture then incubated in a 225 rpm shaker at 37 °C for 30 min. LB plates were prepared using ampicillin to kill any bacteria that did not uptake the DNA plasmid. Two plates of transformed bacterial cells were then prepared using 20 and 50 µL of solution respectively. A third plate, positive control, with no ampicillin was prepared using 20 µL of transformed bacteria solution; this assured the transformed bacteria grew normally under nonselective conditions. A forth plate, negative control, was carried out in which the plasmid DNA was replaced with TE buffer and then plated on LB/ampicillin. The negative control assured the non-transformed bacterial cells were susceptible to ampicillin. Plates were then incubated overnight at 37 °C. The transformed bacterial colonies were then harvested after several days at 4°C into a 2 mL LB/ampicilin broth and cultured in a shaking bacterial incubator for 5 hours. After the incubation 40 µL of the starter cultures were added to 20 mL of LB/ampicilin broth and incubated for 16 hours at 37°C in the shaking bacterial incubator.

DNA Purification

The transformed bacterial cultures described above were lysed releasing our plasmid, allowing for extraction and purification of DNA. To begin DNA purification, 14 mL of cell suspension was pipetted out from each harvested colony and centrifuged for 15 minutes at 6000*g at 4 °C. Supernatant liquid was then removed and the cells were resuspended in 4 mL of Buffer P1 (resuspension buffer with LyseBlue reagent). 4 mL of Buffer P2 (alkaline lysis buffer) was added to the mixture, followed by 4 mL of Buffer P3 (neutralization buffer) and left to set for 10 min. This lysed the cells releasing the plasmid DNA along with all other cell components. A QIAGEN-tip 100 was primed with 4 mL of Buffer QBT (equilibration buffer, 750 mM NaCl) then the cell mixture was poured into the QIAGEN-tip. After the QIAGEN-tip was drained it was washed twice with 10 mL of Buffer QC (wash buffer, 1.0 M NaCl). The positive charge of the QIAGEN-tip bonds with the negatively charged DNA while the less charged RNA, proteins and other impurities are rinsed through the filter. The DNA sample was then eluted with 5 mL of Buffer QF (elution buffer, 1.25 M NaCl) and eluted DNA was collected. The elution buffer carries a highly negative charge which bonds strongly to the QIAGEN-tip thus releasing the less negatively charged DNA. Next 3.5 mL of isopropanol was added to the eluted DNA and mixture was centrifuged at 15000 X g for 25 minutes at 4 °C. The supernatant was then decanted, 1000 µL of 70% ethanol was added to the pellet and centrifuged for 5 minutes. The ethanol was removed and purified DNA pellet was allowed to dry for 15 minutes. Finally the DNA was dissolved in 100 µL of TE buffer and stored at -20 °C. This purification procedure harvested the DNA that the bacteria had amplified, vastly increasing the amount of DNA available for testing.

Optical Density

Purified plasmid DNA concentration and purity were then measured using light absorbance readings from a UV/Vis spectrophotometer. The machine was set for 260 nm and 280 nm wavelength readings. After the machine was calibrated, a diluted DNA sample of 6 µL of DNA

and 600 µL of dH_2O was analyzed and results were recorded. This test showed the degree of protein contamination as well as the concentration of the DNA. Based upon the optical density measurements, the DNA was diluted with TE buffer to create a 0.5 µg/µL DNA mixture to standardize the two samples for further use. This was achieved by adding 74.6 µL to sample 1 and 138.16 µL to sample 2.

Restriction Digest

Uncut Digest

An aliquot of 43 µL of dH_20, 5 µL of 10X NEB buffer 4, and 1 µg purified plasmid DNA was created. The mixture was incubated in a 37 °C water bath for 1 hour. After incubation, the reaction was terminated with 10 µL of 6X DNA loading dye, pulse spun then stored at -20 °C. This digest formed the control sample for the total plasmid length.

Kas1 Digest

The restriction digest Kas1 was used to selectively cut the DNA on specific sites to identify genes from the fragment lengths. Kas1 was chosen by analysis of the provided plasmid base sequence. An aliquot of 41.5 µL of dH20, 5 µL of 10X NEB buffer 4, 0.5 µL of 10 mg/mL BSA, 1 µg purified plasmid DNA, and 1 µL of 4 U/µL Kas1 digest was created. The mixture was incubated in a 37 °C water bath for 1 hour. After incubation, the reaction was terminated with 10 µL of 6X DNA loading dye, pulse spun then stored at -20 °C.

BsmB1 Digest

The restriction digest BsmB1 was also used to selectively cut the DNA on different sites, providing a variety of fragment lengths to aid gene identification. An aliquot of 42.0 µL of dH20, 5 µL of 10X NEB buffer 4, 1 µg purified plasmid DNA, and 1 µL of 10 U/µL BsmB1 digest was created. The mixture was incubated in a 55 °C water bath for 1 hour. After incubation, the reaction was terminated with 10 µL of 6X DNA loading dye, pulse spun then stored at -20 °C.

Kas1 and BsmB1 Digest

We combined BsmB1 and Kas1 for a final digest to obtain more fragment lengths for gene identification. An aliquot of 41.5 µL of dH20, 5 µL of 10X NEB buffer 4, 0.5 µL of 10 mg/mL BSA, 1 µg purified plasmid DNA, and 1 µL of 4 U/µL Kas1 was created. The mixture was incubated in a 37 °C water bath for 1 hour. Next 1 µL of BsmB1 was added and sample was moved to a 55 °C water bath for 1 hour. After incubation, the reaction was terminated with 10 µL of 6X DNA loading dye, pulse spun then stored at -20 °C.

Polymerase Chain Reaction

This process amplifies the gene of interest between the two primers. A mixture was made of 1 µL of "BD-forward" primer at 50 µM concentration, 1 µL of "BD-reverse" primer at 50 µM concentration, and 20 ng of purified plasmid DNA, 1 µL 10 mM dNTPs, 1 µL Taq DNA Polymerase, 5 µL Taq Buffer with Mg^{+2}, and dH_2O to a 50 µL total volume. A negative control was prepared in parallel that replaced the 20 ng of plasmid DNA with an dH_2O. All mixtures were then placed into the PCR machine. The machine was set for 32 cycles of 15 sec at 94 °C (denaturation), 30 sec at 56 °C (annealing), 1 minute at 72 °C (polymerization). PCR amplified the DNA segments by denaturing (separating) the two DNA strands, annealing the primers onto the DNA strands, then creating the compliment DNA strands starting from the primer location. After the 32 cycles were completed the PCR mixture was stored at 4 °C.

Agarose Gel Electrophoresis

Electrophoresis separates DNA segments by size by electrostatically pulling them through a porous gel. The restriction digests and PCR were run in a Agarose Gel Electrophoresis to measure the segment lengths. The gel was prepared by pouring 40 mL liquid 1% agarose in 1X TBE/Ethidium Bromide (EtBr) into the gel apparatus. After solidification, 300 mL of 1X TBE buffer was poured into the gel box. Each lane was then loaded

as follows: 12 μL of λ/HindIII DNA marker, 8 μL of X174 marker, 20 μL each of uncut, Kas1, BsmB1, and Kas1 and BsmB1 cell digests, and 12 μL each of PCR negative control, PCR DNA #1, and PCR DNA #2. The gel was then run for 35 minutes at 100 Volts. Using the (Biochemi) Gel doc system, the gel was imaged under UV light. UV light fluoresces the enthidium bromide bonded to the DNA fragments showing bands for restriction digest and PCR fragment lengths.

DNA Transfection

To identify of our gene of interest, the purified DNA was transfected into mammalian cells to see where it localized and allow for further testing. To begin the transfection, U20S cells were plated into a 6-well dish and cultured overnight. Aliquots of 3 μg DNA sample (either DNA#1, DNA#2, pDEST, or pDEST-GFP), 7.5 μL PEI to assist DNA uptake, and 1 μg of reporter luciferase with NFkB promoter region. The pDEST and pDEST-GFP act as negative and positive controls, respectively, for GFP expression in U20S cells. After a 15 minute wait for PEI to create complexes with the DNA, the aliquots were added to the cell cultures that had been recently washed with 1ml PBS and given 2ml of fresh DMEM. After two days, TNFα was added to one sample each of DNA#1 and DNA#2, as well as both controls.

Luciferase Detection

To evaluate if our gene or TNFα affected the NFkB pathway, we measured the luciferase activity of the cells that expressed the transfected luciferase reporter. To first lyse the cells, we added 100 μL of commercial lysis buffer strong enough to lyse the cells but not enough to denature proteins. After 15 minutes incubation on ice and 5 minutes of cold centrifuge, 100 μL of luciferin substrate was added to 20 μL of the lysate sample for the luciferase to activate. The gene expression via luciferase activity was then measured by luminometer. One negative sample was also tested without lysate products to measure background luminescence.

SDS-PAGE of Cell Lysates and Western Blot

We completed the study by performing a Western blot on SDS-PAGE lysate samples to evaluate our gene expression in the U20S cells with and without TNFα. We added 20 μL of 'blue' 2X reducing sample buffer to 20 μL of each lysate. A acrylamide gel was then run at 100V for one hour with 25 μL each sample and a protein molecular weight marker. After the electrophoresis, the gel was washed with 150mL of 1X Transfer Buffer. The proteins were then transferred onto similarly washed nitrocellulose paper in a semi-dry transfer apparatus at 15V for 30 minutes. A blocking buffer of 5% non-fat dry milk in 1X TBST was then added to the nitrocellulose paper to block nonspecific antibody binding. A 10ml 1:10,000 solution of rabbit polyclonal antibody in .5% milk was then added to bind to the GFP and uncubated for 1.5hrs. After 3 washes in 1X TBST, a 10ml 1:50,0000 solution of HRP-linked rabbit anti-mouse immunoglobulin in .5% milk to bind to the earlier rabbit antibodies for detection. After one final rewashing, 4mL of Luminol and peroxide buffer were added to make the proteins luminesce.

Results and Discussion

Optical Density

A common method for determining the concentration and quantity of purified DNA is to measure the absorbance of 260 nm light in a dilute sample using a spectrophotometer. The Lambert-Beer Law relates the absorbance of light to its concentration by the equation $A = \varepsilon l C$, where A is the absorbance at a specific wavelength, ε is the extinction coefficient, l is the pathlength of light through the sample, and C is the concentration. Given ε = 0.020 $(\mu g/ml)^{-1}$ cm^{-1} for double stranded DNA, the dilution factor, and the measured absorbance of the samples, the concentrations of the two samples were found to be 0.955 μg/μl and 1.285 μg/μl (Table 1).

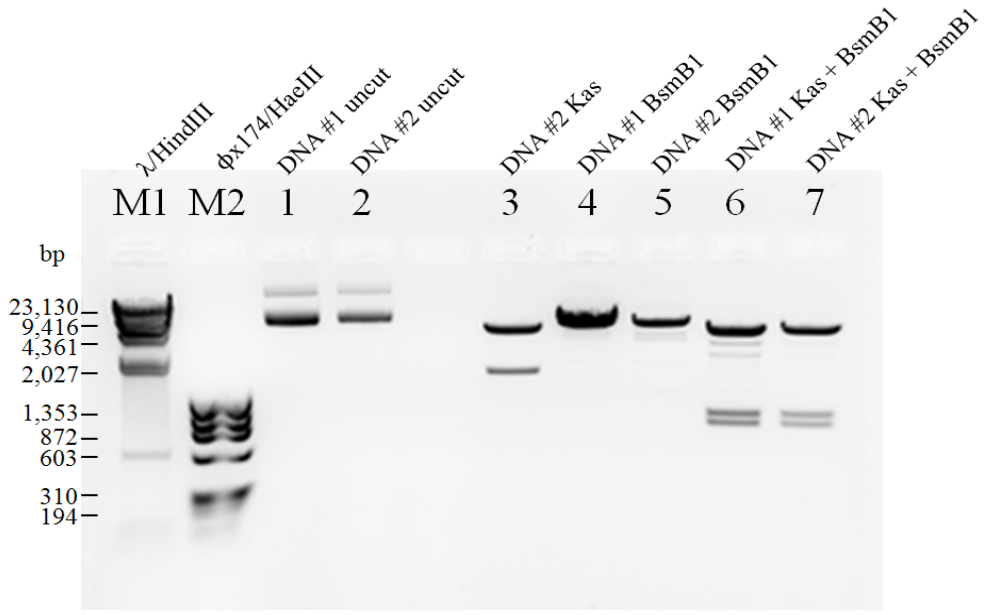

Figure 1: Agarose gel electrophoresis of restriction digest products. The first two lanes in the inverted image are molecular weight markers. Lanes 1 and 2 contain uncut plasmid samples. Lane 3 has been digested by Kas1, lanes 4 and 5 by BsmB1, and lanes 6 and 7 by both restriction enzymes. The observed bands indicate a BID-containing plasmid. Note that the DNA #1 Kas sample is missing due to a preparation error.

In order to verify the quality of the sample, data was taken on the absorbance of 280 nm light, which is more heavily absorbed by proteins. Both samples had A_{260}/A_{280} ratios of around 1.8, corresponding to appropriately clean samples. The absorbance values, A_{260}/A_{280} ratios, and calculated concentrations are shown in Table 1. Note that the samples were subsequently diluted to 0.5 µg/µl.

Table 1
Optical density measurements of purified DNA samples.

	Absorbance			
	A_{260}	A_{280}	A_{260}/A_{280}	C (µg/µl)
DNA #1	0.191	0.106	1.802	0.955
DNA #2	0.257	0.137	1.876	1.285

Agarose Gel Electrophoresis

Agarose gel electrophoresis was used to identify DNA fragment lengths of restriction digest and PCR products. The data from these gels were compared to expected fragment lengths for each of the possible genes of interest as found using the nominal DNA sequences and NEBcutter from New England BioLabs (Table 2).

Figure 1 shows the gel with the restriction enzyme products. M1 and M2 are molecular weight markers with fragments of known lengths. These bands were used to estimate the lengths of the other samples. Lanes 1 and 2 contain uncut plasmid samples. There are clear bands corresponding to DNA with a length of approximately 7500 bp. Lane 3 has been digested by the restriction enzyme Kas1. The two bands indicate that the circular plasmid has been cut twice, resulting in two, linear fragments, one that is around 5500 bp long and the other ~2000 bp. The DNA in lanes 4 and 5 have been cut by the restriction enzyme BsmB1 at a single site, resulting in linear fragments ~7500 bp long. Lanes 6 and 7 have been digested by both Kas1 and BsmB1. This results in three bands with lengths of ~5500 bp, ~1000 bp, and ~900 bp that correspond in size to the predicted bands of 5388 bp, 945 bp, and 827 bp. This suggests a plasmid with restriction enzyme sites as shown in Figure 2, matching the expected results for a plasmid vector with the BID gene.

Table 2
Expected fragment lengths for double digests of possible genes.

Gene		Fragment Lengths		
BID		827	945	5488
TNFR1	407	827	945	5742
Cyclin d1	177	827	945	5451
Rassfla	593	827	945	5214

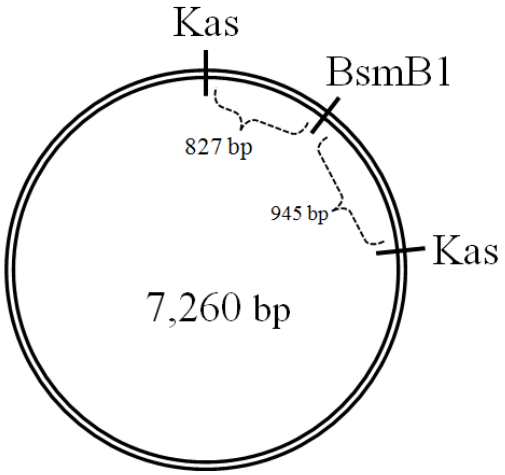

Figure 2: Restriction enzyme sites. The basic structure of the plasmid of interest is illustrated.

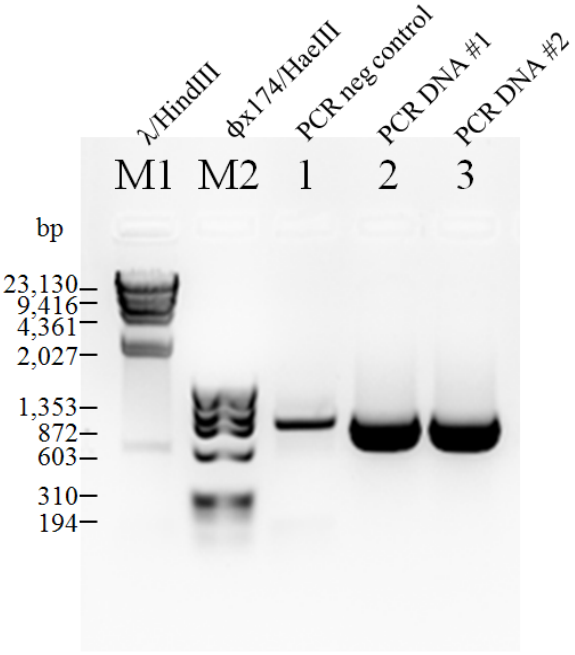

Figure 3: Agarose gel electrophoresis of PCR products. The first two lanes of the inverted image are molecular weight markers. The prominent band in the negative control (Lane 1) may have resulted from accidental contamination with template DNA. Lanes 2 and 3 show DNA samples after PCR. The ~800 bp bands are in line with amplification of the BID gene.

Figure 3 illustrates the PCR results. M1 and M2 are again known molecular weight ladders used for comparison. Lane 1 is a negative PCR control sample. There should be no visible bands; however, the there are clearly fragments present ~800 bp in length. This suggests that the control was contaminated with a small amount of template DNA. Optical density measurements of our samples revealed a successful purification of the plasmid DNA from the cell remnants, indicating that the error was probably caused by contamination during the PCR sample preparation. Lanes 2 and 3 contain the PCR products. There are very large bands indicating the successful amplification of a fragment approximately 800 bp long. This is close to the expected length of the BID (844 bp), although it is hard to rule out Cyclin D1 (984 bp). These base pair lengths were obtained by counting the base pairs between the forward and reverse primers. (Table 3)

Table 3
Expected DNA lengths for PRC for possible genes.

Gene	Fragment Length
BID	844
TNFR1	1505
Cyclin d1	984
Rassfla	1163

DNA Transfection

With the DNA transfected into the U20S cells it was now possible to see the expression of the gene under UV light. The microscope images (Figure 4) show glowing of the cells under UV light, suggesting that GFP linked proteins were successfully transfected into the cells. Cells without TNFα appeared to have a diffuse light emission throughout the cell with a few localized spots. The cells that had TNFα, a known apoptotic inducer (source), appeared to have GFP more localized in the cell. This supports the identification of BID as the gene of interest because the BID protein moves from cytoplasm to the mitochondrial membrane when a cell receives apoptotic signals.

Luciferase Assay

In unstimulated cells, NFkB remains sequestered in an inactive state in the cytoplasm (as controlled by IkBs). When under harmful stimuli, the inhibitors free the NFkB proteins, allowing them to enter the nucleus and act as a transcription factor leading to a given cellular response. In this case,

NFkB acts as a promoter for transcription of luciferase from the transfected plasmid.

Table 4
Luciferase assay results which are correlated with activation of NFkB.

Sample	Light Intensity
Background	201
- Control	24,089,314
+ Control	14,881,240
Exp 1 no drug	1,200,253
Exp 1 +TNFα	5,274,314
Exp 2 no drug	3,254,567
Exp 2 +TNFα	16,630,910

The luminosity results of the luciferase assay are found in Table 4. The difference in rest response between the two experiments can be attributed to a difference in DNA purity and transfection effectiveness. However, the both experiments clearly indicate a three to five-fold increase in luciferase luminescence when TNFα is added to the transgenic cells. This implies that the addition of TNFα affects the NFkB pathway, an expected result given NFkB's known involvement in cellular response to apoptotic stimuli [5].

Unfortunately, this experiment is inconclusive as to the effect of BID in the NFkB pathway. Because the "controls" do not have the same transfected plasmids, they are not an appropriate control for the effect of BID. This lack of a suitable comparison makes it difficult to analyze the luciferase assay results with certainty. Ideally, this luciferase experiment would include an identical control plasmid without the gene of interest, cultured in U20S cells with and without TNFα to isolate the two possible effects.

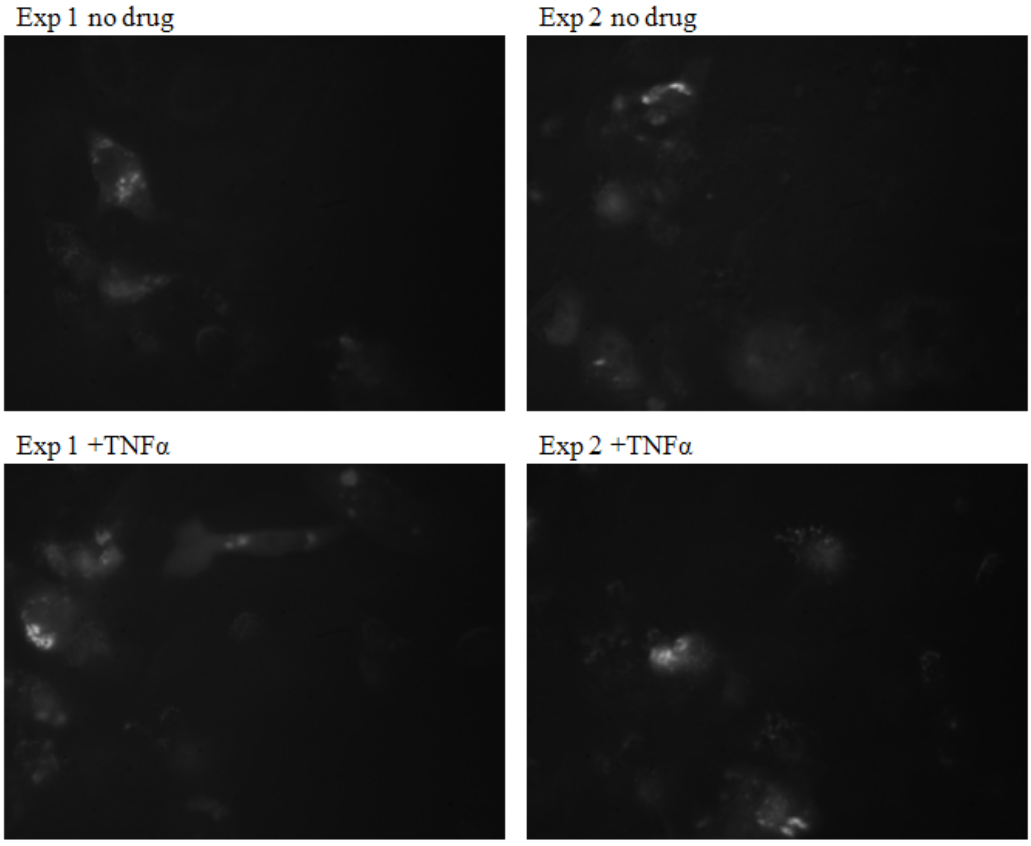

Figure 4: Optical microscopy of GFP-tagged proteins in tranfected cells. Normal expression of GFP-tagged BID is shown in the top images. The bottom images show BID expression for cell stressed by TNFα. The cells treated with TNFα appear to have slightly more localized protein expression

Western Blot

Our final method of gene identification was a western blot of the expressed GFP linked protein. All the lanes of our western blot showed a band at ~65 kDa except for our standards lane. This includes the positive and negative control, neither of which were expected to have any

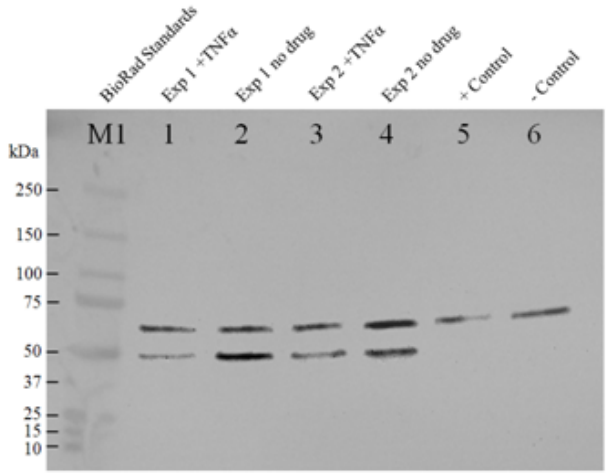

Figure 5: Western blot of expressed gene of interest. The band at 50 kDa in the first four lanes (Experiments 1 and 2 with and without TNFα) corresponds well with the expected 49 kDa molecular weight of the BID protein with GFP marker. Note that the TNFα lanes have a lighter band, consistent with the fact that BID, under drug stimulation, will move to the mitochondria and not be released in the lysate. The single band across all lanes at 65 kDa is most like some unintended reaction between the antibodies and normal cell proteins.

bands expressed; suggesting an unintended reaction between the antibodies and the proteins in the U20S cells, or some contaminating protein in our western blot.

In addition to the ~65 kDa band, the four lanes containing transfected cell culture all showed lines at ~50 kDa. This strongly suggests BID as the gene of interest since it is clearly closest to the protein weight, 49 kDa. The other proteins estimated lengths were not close to the 50 kDa found in the western blot the closest being Cyclin d1 at 60 kDa. Estimated protein lengths were determined by adding the gene of interests' protein length to the GFP protein length, 29 kDa [6].

Table 5
Expected protein lengths for Western blot for possible genes [6].

Gene	Protein Length	Protein Length with GFP added
BID	22	49
TNFR1	55	82
Cyclin d1	33	60
rassfla	39	69

The western blot also showed darker lines in the experimental samples without the TNFα than in the treated cells. This could be caused by the BID moving into the mitochondria as described earlier; if BID had entered the mitochondria the BID would not be in the final lysate because the mitochondrial membrane is not lysed in the SDS-PAGE preparation. This theory further supports that BID was the gene of interest.

Conclusion

Our restriction digest and PCR results are consistent with a circular plasmid vector containing the gene for BID. This identification is further verified by the observation of localized GFP florescence, increased NFkB promotion, and decreased cytoplasmic concentration under TNFα. These results are consistent with the known characteristics of the BID protein. Under apoptotic signaling, BID interacts with the BAX, which then attaches to the mitrochondrial membrane [7]. NFkB is a known apoptotic promoter, causing the BID to localize to the mitochondria.

This procedure has so far proved an effective overview of applied biotechnology and lab practice. The results certainly underscore the importance of proper contamination prevention, yet are robust enough to provide a convincing case for the gene's identity and characteristics. The plasmid was successfully amplified, purified, and identified using a variety of lab techniques.

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