Biotechnology lab report examples

Science, Genetics

Analysis of Culture Cell KU812F with Three Protocols

Abstract

Protein analysis for cancer cell research has been found to be extremely reliable. The culture cell KU812F (chronicle myeloid leukemia cell line) was used for this laboratory. Globin gene switching was observed by evaluating the gene expression in the cells. Gene expression and protein folding were provoked. Gene expression is the process of transcribing DNA to cDNA and then used qPCR to study gene expression. cDNA was translated into mRNA which in turn is translated into a protein. mRNA concentration was analyzed. The three protocols were Reverse Transcription Polymerase Chain Reaction (RT-PCR), Enzyme-Linked immune sorbent assay (ELISA), and Fluorescence Activated Cell Sorting (FACS). The experiment analyzed Gamma Globin gene switching which codes for fetal hemoglobin (HbF). The PCR analysis results showed that the Gamma Globin/GAPDH (ratio) of 0. 6 concentrations was the highest when the cells were treated with 0. 5 mMol NaB. The fold change for Gamma-globin expression reached its highest level of 6000 when treated with 0. 5 mMol NaB. The ELISA technique results measured protein level of Hbf when the cells were untreated (approximately 4090) treated with 0. 2 mMol NaB (approximately 4095), treated with 0. 5 mMol NaB (approximately 4010) and treated with 5. 0 mMol NaB (approximately 2090). The ELISA results demonstrated overlapping Standard Deviations (S. D.) for the untreated and the treated at 0. 2 mMol and o. 5 mMol. The ELISA results showed overlapping S. D. for the two highest NaB treatments of 0. 5 mMol nad 5. 0 mMol NaB. The largest numbers of cells were in the Go/Gi portion of their life cycle as demonstrated by the results of the FACS analysis. The

three methodologies were used to observe and measure different cellular levels of gene expression using cell culture KU812F.

Introduction

The culture cell KU812F (chronicle myeloid leukemia cell line) was used for this laboratory. A sample was evaluated for cDNA using three different methodologies PCR. ELISA was used to evaluate antigens and Fluorescence Activated Cell S was used to evaluate the viability of the cells. The reverse transcription PCR (RT-PCR) is used to detect gene expression. The PCR methodology separates the protein from cells. Expression switching was quantified by initiating expression swithching. The purpose of the qPCR portion of the lab was to quantify gamma-globin gene expression as the gamma globin/GADPH ratio. The harvesting of the cKU812 cells was carried out in order to do an RNA extraction by standard kits and gel electrophoresis, and then reverse transcription resulting in the generation of cDNA. Enzyme-Linked Immunosorbant Assay (ELISA) is a solid phase enzyme immunoassay that recognized antigens. The ELISA assay is done on the basis of Antigen-Antibody binding for quantitative determination of certain antibodies in the sample. ELISA is a simple test that can be used to identify types of venom in snakebites for example, because in general antibodies are used that exhibit specificity for a particular antigen. The plats have several wells composed with a solid phase embedded on them that contains ligating agent, so essentially ELISA is a ligand binding assay.

The ELISA Immunoassay was prepared to measure Human Fetal Hemoglobin (HbF) at different concentration of sodium butyrate (NaB). These are commonly used to evaluate the inhibition ability of cancer cells by activating

mutations or in other words activating expression switching. [1] [5]

Cancerous cells were treated for 72 hours and using a range of concentrations from 0 to 5. 0 µMol sodium byutrate, NaB) to reverse transcribe RNA, ELISA to quantify total histone levels and “ cell cycle phase distribution was examined by flow cytometry” [2]

Fluorescence Activated Cell Sorting (FACS) is an experimental methodology that has been developed by adapting basic flow cytometry (FCM) to supply fluorescent readouts. FCM is a commonly used for analysis the properties of individual cells within heterogeneous population of cells. FCM is used for many applications such as cell counting, immunophenotyping, and GFP (Green Fluorescent protein) expression. Chung, McHale and Louis (2012) have used a fluorescence technique to determine the speed of the transition pathway in proteins and determined that in terms of the protein folding activity, fast and slow protein folding take approximately the same amount of time. The technique is widely used in industry because the estimates of viable bacterial population are detected rapidly and counted rapidly. [3] In this experiment the FACS analytical methodology was used in order “ to sort cells from within mixed cell populations and to determine the level of expression of protein on the cell surface . . . or in the cell.” [4] Propidium Iodide is used to analyze the cell cycle of cells on the basis of the number of DNA copy number and intensity. Propidium Iodide (PI) was prepared to use as the staining agent because it helps identify non-viable cells by red fluorescence. The PI stain enters the membranes of cells that are dying or dead and then intercalates (the action of ligands that are good nucleic acid stains to position themselves between base pairs of DNA).

Materials and Methods

Note: Each measurement repeated in triplicate.

Note: A control group (the untreated cells) was used for each analysis.

KU812 Cell Induction and RNA Isolation for qPCR analysis

Materials

-Culture cell KU812F (chronicle myeloid leukemia cell line)

-Sodium butyrate (NaB)

-Chloroform, isopropanol, 70% ethanol

-Distilled water

-RNase free water

-Nuclease free water

-Stat-60

Methods

The cells KU812F (chronicle myeloid leukemia cell line) were cultured using IMDM medium, 10 % fetal bovine serum (FBS), Penicillin and Streptomycin (antibiotics). Trypan blue dye (o. 4%) was applied to aid in the counting in order to determine viability of the cells. The cells were treated with 0. 2 mMol, NaB, 0. 5 mM NaB and 5 mMol NaB. A fourth portion of the cells was left untreated. The cells were then incubated for 48 hours at 37° C, 5% CO2. And then the cells were harvest for RNA extraction in other words the RNA was isolated using the RNA Extraction Protocol. 4ml of cells were centrifuge for 5 min at 2, 000 rpm, and then 1ml of stat-60 was added to the cells and mixed well after the supernatant was removed. After 5 min, 200µl of chloroform was added and vortexed briefly. 500µl of isopropanol was added to the aqueous phase after it was transferred to a new labeled tube. The

tube was maintained at -70oC for 24hr. The pellet was rinsed with70% Ethanol after the isopropanol was removed and then centrifuge for 3 min at 14, 000 rpm. The resulting pellet was (re)suspended in RNase free water for measurement of its Optical Density at 260 nm.

Reverse Transcription PCR

Materials

- Extracted RNA
- Promega kit
- Oligo(dT)-primer
- Vortex , ice, and PCR tubes
- Prepared master mix

Method

1µl of oligo(dT)-primer and 4µl of RNA was mixed to make the total volume of 5µl in the labeled PCR tubes. Then the tubes were vortexed and centrifuged. The RT-Program was setup at 70oC for 5 minutes and then cool down to 4oC for 5 minutes. RT-Master Mix was prepared by adding ImProm-II (5X) buffer, MgCl2 (25mM), dNTP mix (10mM), RNasin Inhibitor (40U/µl), ImProm-II RT, RNAse free water. The total volume of each tube of master mix (15µl) was add to each tube of 5µl of oligo(dT)-primer and RNA. RT2-Program was run, in which incubation at 25oC for 5 mins (annealing), 42oC for 60 min (extension) and 70oC for 15 mins (termination). Then the cDNA sample was stored at -20oC for quantitative PCR analysis.

Detail

The RNA Extraction Protocol required using a clean pipette to put 4 ml of cells into a clean 15 ml centrifuged tube. The centrifuge was set at 2000 rpm and the tube with the cells was centrifuged at that speed for 5 minutes. After the 5 minutes was over the tube was removed and a supernatant has formed. The supernatant was not necessary for the experiment so it was removed and placed in a waste container. 1 ml of Stat-60 was added to the cells and mixed by using pipetting as the means of mixing. Next vortex for 5 seconds. Afterwards the mixture was again placed into the centrifuge but a much faster rate, 14, 000 rpm was used to centrifuge the mixture for 15 minutes at 4° C. This time the centrifuge resulted in making an upper aqueous phase that must be transferred into tubes (that were labeled while waiting). 500 µl of the alcohol Isopropanol was pipette into the aqueous phase not sitting in the tubes – vortex well at this point. Then the tubes with the aqueous phase mixed with isopropanol were placed in a freezer with the temperature of -70 ° C; the mixture in the tubes was left in the freezer overnight.

In the laboratory the next day the tubes were removed from the freezer and again centrifuged at 14, 000 rpm for 4 minutes at 4° C which forms a pellet of what was originally the aqueous upper phase. The isopropanol is no longer needed so it was carefully pipette out of the tube. The isopropanol needed to be removed without disturbing the pellet. (Possibility of error at this point if not done super carefully.) With the pellet remaining in the tube 200 µl of a different alcohol, this time 70% ethanol was added to the tube as a rinse to rinse the pellet. The tube was ‘ vortexed’ for 5 seconds in order to rinse the

pellet completely. Then the tube with the pellet and ethanol were centrifuged for 14, 000 rpm at 4°C for 3 minutes.

When the 3 minute centrifuge was over the ethanol was removed again with a pipette again very carefully so that the pellet would not be disturbed. The pellet (known as the sample) dried by standing at room temperature in the tube for 5 to 10 minutes. After the drying period the pellet was resuspended, this time using 20 µl of RNase free water. And then the optical density was measured at the 260 nm wavelength.

RT reaction

The reverse transcription reaction needed to be accomplished now so that the cDNAs would be produced from the RNA; the cDNA can be measured by the qPCR. Tubes were labeled both on the top and on the side. Added to each tube was 1 µl Oligo(dT)-primer and 5 µl RNA (1 µg total) resulting in a total volume in the tube of 5 µl. In order to form a good mixture the tube was vortexed and given a spin down then place on ice.

The last step of the RT1-program was to allow the mixture to incubate for 5 minutes at 70° C and then at the temperature at 4° C for 5 minutes, and then held until ready to add the appropriate reagents to the tube.

The RT-Master Mix was prepared by adding ImProm-II (5X) buffer = 4 µl; MgCl2 (25mM) = 2. 4 µl; dNTP mix (10mMol each dNTP) = 0. 5; RNasin Inhibitor (400 µl/µl); ImProm-II RT = 1. 0 µl andRNase free water = 6. 1 µl. The tube will be holding 15 µl after the additions have been made the resulting final concentrations of the reagents were thn ImProm-II (5X) buffer 1X; MgCl2 (25mM) = 3 mMol; dNTP mix (10mMol each dNTP) = 0. 5 mMol; and RNasin Inhibitor (400 µl/µl) = 1 U µl.

The RT- Master Mix was added in 15 µl amounts to each of the following the tube containing the RNA oligo(dT) –containing PCR so that the following resulting mixture was in the tube: 5 µl RNA oligo(dT) [1X] and 15 µl Master Mix after which the tube holds 20 µl.

The PCR machine needs to be set for the program RT2 before running the samples.

The process annealing required incubation at 20°C for 5 minutes

The process extension required incubation at 42°C for 60 minutes

The process termination required incubation at 70°C for 15 minutes

Now is the time to (at end of the RT reaction to store the product, cDNA, samples in a the cold at -20° C

The samples are now ready for the qPCR analysis

Quantitative PCR (qPCR)

Materials

Primer set

2 tube for each

BP 274 – BP 276 for alpha globin

BP 291- BP 292 for GAPD

BP300 – BP 301 for beta globin

Group 1:

Tubes -12 gene samples

0. 2 mMol NaB

0. 5 mMol NAB

5 mMol NaB

Apparatus:

BioRad iCycler IQ

Methods

The qPCR protocol for Reverse Transcription was followed (a process with several steps at varying temperatures (heat shock and durations of incubation). Standard curve dilutions and Gene primers were prepared. Measurements were done with a BioRad iCycler IQ. KU812f cell culture (chronicle myeloid leukemia cell line) were used to determine the gamma-globin gene expression when induced with penicillin and streptomycillin. Three 1. 5 mL microcentrifuge tubes were prepared with GAPDH, γ-globin and β- globin and the appropriate primers. Seventeen tubes were prepared for gene detection. A negative control was incorporated into the methodology. (a) The RNA is isolated (used a standard kit) and (b) gel electrophoresis was performed and the staining agent ethidium bromide was used to discern the bands. The bands 185 and 28s were studied because those two bands have the biggest population of RNA. Briefly the RNA was extracted in order to isolate the cells, gene expression was induce with reverse transcription reaction resulting in the product, cDNA and a fluorescent probe (qPCR) was used for gene expression because a PCR reaction results.

Nuclease free water was used for all the dilutions. The standard curve dilutions were prepared (see a, b, and c below) four dilutions were necessary to prepare each standard. Then each standard had to prepared with an appropriate forward concentration [10 pm] and an appropriate reverse concentration [10 pm] for all three standards resulting in a total of 6 ‘

primers’

(a) the gamma-globin standards

(b) the beta-globin standards, and

(c)the GAPDH standards

The set-up for each gene by wells in the tray

Negative controls 2 wells

Standards (w/ duplicates) 8 wells

Unknown samples for Group 1 required 12 wells

Note: the negative controls consisted of the reaction mix with water the purpose being to negate the background fluorescence

(Labeled R1 & R2)

The Standard Curve required 8 wells because for the 4 standard curves a duplicate was run for each.

The experimental samples for Group 1 required 12 wells. The cDNA which had been prepared earlier was used because now the mRNA level of the target genes needed to be measured (purpose of the qPCR)

Sybergreen Master Mix was prepared. Group 1 prepared 25 samples using (note 22 plus 3)

Sybergreen Master Mix = 312. 5

Primer 1 (10 pM) = 25

Pimer 2 (10 pM) = 25

RNase free water = 137. 5

resulting in a Total = 500 µl

Clean Eppendorf tubes were used to prepare 17 tubes labels as neg.

(negative control), D4, D3, D2, D1, and 6-17 for the unknown samples U1 to U17

Next Eppendorf tubes were prepared for the negative control (40 µl master mix plus 10 µl Nuclease free water) for a total of 50 µl

The solution for the Standard Curve was prepared using 40 µl master mix plus 10 µl plus the dilutions prepared earlier D1 to D4 (duplicates of the 4 dilutions) for a total of 50 µl

Unknown preparation (U1 to U12) with 20 µl master mix plus 5 µl of cDNA for a total of 25 µl

The Bio RAD iCycler was used to gain the quantativePCR data in real time.

ELISA immunoassay

Materials

Hman Fetal Hemoglobin (HbF)

Coating Antibody

Post-coat solution

Standards samples

HRP Conjugate Antibody

IMB Substrate Solution

Sulfuric acid

Method

The ELISA Immunoassay was prepared for Human Fetal Hemoglobin (HbF). Group 1 worked on GPDH. 50 MI capture to 5 MI used as the coating buffer. 100 MI of the 50 MI capture Ab was added to each well with a clean Eppendorf pipette and then the plate (or tray) was incubated for 25 minutes. After the incubation 4 automatic washes were preformed. And then 200 MI

blocking buffer was added to the wells, after the wells had each been treated with the blocking buffer the completed ELISA assay tray was again incubated for 30 minutes after which the tray (wells on the tray) was again washed four times. finally 270 ml was added to each tube (G1 to G12)

The protocol for preparing the HbF was followed. The first step was the and 270 ml of the resulting solution was added to each tube for the Bradford assay. The Bradford assay was done to determine the protein concentration. The HbF/Total Protein was calculated for the unknown solution under 4 different conditions: untreated 0. 2 mMol NaB, 0. 5 mMol NaB and 5 mMolNaB.

FACS

Materials

Propidium iodide (PI) staining solution

PBS

ice

DNAse-free RNAse A

Apparatus

centrifuge

Incubator

Flow cytometer

Method

The cells were centrifuged at 2000 rpm at room temperature for five minutes. After the medium was removed the cells were resuspended with 10

ml of cold PBS. The cells were pelleted by returning them to the centrifuge and for five minutes at 2000 rpm. The cells were then resuspended in 1. 2 ml PBS on ice. Chilled 100! ethanol was added by drops until a total of 2. 8 ml had been added. The cells were fixed by leaving on the ice for thirty minutes Propidium Iodide (PI) was prepared to use as the staining agent. 500 ml of the PI staining solution was prepared by mixing 1 ml PBS, 2 µg PI and 4 µl 10% Triton X-1000. The cells were carefully transferred to a tube which was covered with foil. Cells were incubated for 30 minutes at 37° C. A flow cytometer was used to make the measurements.

Results and Discussion

The purpose for doing a PCR analysis was to predict the gene expression of Gamma globin and GaPDH. The aim was to gain the ratio, Gamma-globin/GADPH in order to determine any change in the switching of gamma and beta expressions. [5] The data used to calculate the Gamma-globin/GADPH ratio were taken from the gamma SQ and the GAPDH SQ that was generated in the laboratory. (See Table 1) The results were calculated from the standard curves after the data were plotted onto graphs. (See table 1) The data was generated by the qPCR analysis. Fold change for Gamma-globin expression with four varying concentrations of NaB has been organized into a bar graph which also displays the standard deviations. (See fig. 1) Another bar graph which compares the fold change between the different environments used in the laboratory: untreated, 0. 2 mMol NaB, 0. 5 mMol NaB and 5 mMol NaB. (See fig. 2)

The Gamma-globin/GAPD ratio which was calculated for the four different environments: untreated, 0. 2 mMol NaB, . 5 mMOl NaB, and 5 mMol NaB.

The standard deviations are denoted over the top of the bars. (See fig. 1)

The PCR analysis results demonstrate that the Gamma Globin/GaPDH (ratio) of 0. 6 was the highest when the cells were treated with 0. 5 mMol NaB. (See fig. 1) After the peak was reached at 0. 5 mMol NaB the standard deviation increases significantly indicating that the 0. 5 mMol NaB concentration is the best to use. The fold change for Gamma-globin expression reached its highest level of 6000 when treated with 0. 5 mMol NaB. (See fig. 2) which is another indication that 0. 5 mMol NaB is the best concentration to use for gene expression of the KU812F when using the PCR analysis technique. The information given was for change detection of the proteins in the sample but not specifically for the detection of antigens. Antigens are necessary for the production of antibodies in a cell.

Figure 1. Ratio of Gamma-globin to GAPD ratio with four varying concentrations of NaB. T. The bar graph above is a representation of the Gamma-globin/GAPD ratio which was calculated for the four different environments: untreated, 0. 2 mMol NaB, . 5 mMOl NaB, and 5 mMol NaB. the standard deviations are denoted over the top of the bars. The data was generated by the qPCR analysis.

Figure 2. Fold change for Gamma-globin expression with four varying concentrations of NaB. This is the bar graph which compares the fold change between the different environments used in the laboratory: untreated, 0. 2 mMol NaB, 0. 5 mMol NaB and 5 mMol NaB.

The ELISA analytical technique results measured protein under four different conditions when the cells were untreated, treated with 0. 2 mMol NaB, treated with 0. 5 mMol NaB and treated with 5 mMol NaB and then the

optical density of the prepared samples was measured at 595 nm. (See Table 2) Each of the measurements was done in triplicate and the resulting data of the Optical Density measurement next to the concentrations gives no conclusive information. Therefore the Bradford Assay (or similar assay) is an essential part of the ELISA laboratory. The results of the Bradford Assay are reported in Optical Density (at 595 nm) in reference to the concentrations of the trials. The Bradford Assay results showed that the concentrations for the four environments were similar in value for the untreated, 0. 2 mMol NaB and the 0. 5 mMol NaB environments but the fourth sample measured a concentration of approximately 35 percent less than the highest OD measurement of 4. 230. (See Table 3)

The ELISA results are reported as protein concentration. The highest concentration was with the 0. 5 mMol NaB treatment (graphed at approximately 4090 µg/ml) treated with 0. 2 mMol NaB (approximately 4095), treated with 0. 5 mMol NaB (approximately 4010 µg/ml) and treated with 5. 0 mMol NaB (approximately 2090 µg/ml). The Elisa results demonstrated overlapping Standard Deviations (S. D.) for the untreated cells, the cells treated at 0. 2 mMol NaB and the cells treated at 0. 5 mMo NaB. The Elisa results showed overlapping S. D. for the two highest NaB treatments of 0. 5 mMol and 5. 0 mMol NaB. See fig. 3) The Sodium Butyrate concentrations (from 0 to 5 mMol NaB) are compared and their standard deviation is noted in a bar graph format. (See fig. 3). Protein concentration in µg/ml is graphed on the y-axis; the treatment regimes (untreated, 0. 2mMol NaB, 0. 5 mMol NaB, and5 mMol NaB) are graphed on the x-axis. The

standard deviations overlap in each of the four treatment regimes indicating that the ELISA method does not have a high sensitivity.

The Sodium Butyrate concentrations are compared and

their standard deviation is listed.

Figure 3. ELISA Results depicting the protein concentration under four different concentration regimes. Protein concentration in µg/ml is graphed on the y-axis; the treatment regimes (untreated, 0. 2mMol NaB, 0. 5 mMol NaB, and5 mMol NaB) are graphed on the x-axis.

The FACS analysis was the third and final analysis of the laboratory. A table of the mean and standard deviation for each of the four treatment regimes were reported in relation to the four life cycle phases that were present Dead, Go/Gi, S, G2/M using the Histogram Statistics. (See Table 4) The largest numbers of cells were in the Go/Gi portion of their life cycle as demonstrated by the results of the FACS analysis. Graphs were created from the Histogram Statistics in order to calculate the mean and standard deviations. The results from the FACS analysis of protein detected were graphed as percentages on the y-axis and as the life cycle stage (dead, Go/Gi, S, and G2/M) on the x-axis. (See fig. 4) Trends can be identified by studying the bar graph. The highest percentages of Go/Gi were identified with 0. 2 mMol NaB concentration; the highest population was in the untreated sample, after the next highest population in the 0. 5 mMol NaB treatment environment, and thirdly the 0. 2 mMol NaB environment was lower than the first two mentioned treatment environments but all three contained a similar amount of Go/Gi. For the ‘ dead’ population the trend starts from low and moves to high with the 5 mMol NaB treatment of ‘ dead’

population close to the same found in the 5 mMol NaB treatment of Go/Gi. The population in S is very low and trends from the fewest in the untreated environment to the highest in the 5 mMol NaB (the highest concentration of Sodium butyrate treatment). The G2/M population is also very low and occur in each of the four treatments at approximately the same amounts.

for the 4 treatment regimes (dead, Go/Gi, S, and G2/M) catalogued by life cycle stage.

Figure. 4. FACs results graph created from the Histogram Statistics listed in Table 4 and graphed as percentage on the y-axis and the life cycle stage (dead, Go/Gi, S, and G2/M) on the x-axis.

Conclusion

Each of the three methods allowed for the observation and measurement goal of determining information about gene expression and gene switching induced in a cell culture of KU812F (chronicle myeloid leukemia cell line). Fetal hemoglobin (HbF) was used to explore the KU812F gene expression for the fetal γ-globin and adult β-globin genes. The RT-qPCR analysis resulted in the the highest Gamma-globin/GAPD ratio was measured in the cells indicating the usefulness of the streptomycillin and/or the penicillin as inducers of gene expression and protein fold change. The highest protein concentration was observed at approximately 4090 µg/ml using the ELISA immunoassay coupled with the with the Bradford Assay. The protein detection capability of the ELISA immunoassay was found to be the least sensitive. The PCR cannot give direct measurements for antigen detection so the FACS was used. Together the three methods can give practical, usable

data about the protein but perhaps it would be best to combine them into a more efficient method. Gene expression and gene switching can be induced in a KU812Fcell culture using HbF as an inducer. This has positive implications for treating sickle cell carriers with antibiotics from the penicillin family.

References

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Appendix

Figure Appendix A. Group 1 protein concentrations