Cloning and sequencing lab rep report examples

Health & Medicine, Cancer

\n[toc title="Table of Contents"]\n

\n \t

\n[/toc]\n \n

Introduction

The most widely used model when investigating the breast cancer is T47D cell lines. These cell lines are easily spread, easily manipulated genetically and when used in the experiment, it provides good results. They have been used to examine new therapies and the and the breast cancer pathobiology1.

Over many years HER2 has 25% - 30% of human breast cancers have been caused by gene amplification. The monoclonal HER2 and the EGFR are the two HER2-targeted therapies that are used for approval in the most clinics. During growth, HER2 is used to regulate the cell growth and assist in the cell differential at mammary growth and embryogenesis. Such that if HER2 is not performing the regulation task, it promotes breast tumorigenesis. Amplification of the HER2 is used as evidence in the administration of the HER2-targeted therapies2. Both Exon 20 and Exon 12 represent portions of Her2 protein targeted for anti-cancer therapy. These targeted therapies might be affected due to presence of mutations or SNP’s.

Exon 20 and Exon 12 sequence are used to assist in studying the breast cancer cell line; T47D. The cell line is preferred since it has normal amount of HER2 protein and has a better understanding of ERBB2 gene which will be used to interpret the result obtained in order to treat the breast cancer.

In the tyrosine kinase family, the ErbB has the following cell surface receptor; ErbB1, ErBB2, ErbB3 and ErbB4. These receptors can be mutated, amplified in various forms of cancer and over-expresses making them very significant therapeutic targets4. The amplification of the ErbB2 occurs in the breast and ovarian. The ErbB protein acts as both transcriptional and kinase regulators in the nucleus3.

Signaling of the ErbB is modulated by dense negative and positive feedback and feed forward loops by the newly synthesized miRNAs and proteins5.

Materials and Methods

Genomic DNA extraction: The purification kit (QIAGEN) was used to purify a total DNA from human breast cancer cells and followed the manufacturer's instructions. The T47D cell pellet was re-suspended in 200ul of PBS and then 4ul of RNAse A (120mg/ml) added. The mixture was then incubated for two minutes at room temperature. All cell contents will remove except the DNA will be available for extraction. 200ul of AL buffer were added into the mixture. 20ul of proteinase K was then added into the reaction mixture. The mixture was then vortexed and then incubated for ten minutes at 70˚C. 200ul of ethanol were then added to the sample and then vortexed. The white precipitate that formed was then applied to the DNeasy mini spin column. Addition of Ethanol precipitates DNA from the reaction mixture. The mixture and the precipitate were then added into the DNeasy mini spin

column and centrifuged for one minute at 8000 rpm. The flow through was then discarded. To the DNeasy mini-spin column was added 500ul of AW1buffer and then centrifuged for one minute at 8000 rpm and the flow through solution were then discarded. To the DNeasy mini-spin column was 500ul of AW2 buffer. The mixture was then centrifuged for three minutes at 14000rpm for drying of the DNeasy Membrane and the flow through solution was then discarded. The centrifugal step ensures that no residual ethanol is carried over to the next step. 100ul of AE buffer were then added into the DNeasy Column Membranes to eluted. The mixture was Then incubated for one minute at room temperature and then centrifugal for one minute at 8000 rpm. The step was repeated again and the elution from both steps combined with total elution of 200ul. 50ul of the DNA was then storied at -20˚C.

The DNA was quantified by spectrophotometer at optical density of 280 and 260 respectively by using quartz cuvettes. So, should be two quartz cuvettes. One for the blank would be with final volume 400ul of H_2O. The other one would be with final volume 400ul of the DNA diluted. HEX20 PCR & PCR purification: The T47D cell DNA was amplified by using Polymerase Chain Reaction. The reaction components were used for PCR with final concentrations of 1x pfx amplification buffer, 0. 3 mM of each dNTP, 1mM MgSO4, 0. 3uM of HEX20 primer for each primer forward and reverse, 200ng of DNA template, 1 unit of platinum pfx DNA polymerase, and distill water with 50ul final volumes of the reaction. For the negative control the same components concentrations and water instead to DNA. After the preparation of the reaction, the reactions were placed in a thermal cycler. The PCR

cycling parameters were 95˚C/2 minutes. There were 25 cycles of 94˚C/15 seconds (denaturation); 55˚C/ 15 seconds (annealing), and 72˚C/ 30 seconds (extension). The final extension is at 72˚C for 7 minutes, and hold at 4˚C. After that the products were analyzed by 1. 2% of agarose gel electrophoresis.

The QIAquick PCR purification kit (QIAGEN) was used to purify the PCR products of HEX20 exon from the ERBB2 gene of the T47D cell line and followed the manufacturer's instructions. Buffer PB was add to the PCR products at a ratio of 5: 1. The 0. 005mM sodium acetate was added to the buffer PB to level out the pH. The sample was applied to the QIAquick spin column and was centrifuged for 1 minute and the flow-through was discarded. Buffer PE was used to wash the sample and then eluted by added 50µl of Buffer EB.

Ligation: The ligation was prepared with two different ratios of the HEX20 and the pJet 1. 2. The first ratio had a 2: 1 and the second has an 8: 1 ratio. Purified of HEX20 PCR product was ligated to pJet 1. 2 blunted vectors. The ligation was achieved by adding of 1x T4 DNA ligase buffer, 1 unit concentration of T4 DNA ligase, and complete the reaction by adding distill water. The final volume of the ligation reaction was 10µl and then incubated at room temperature for one hour. After that the ligation products were put in ice. Transformation & plasmid DNA prep: Ligation reactions were transformed into the DH5α strain of E. coli. The isolated volumes of each ligation reactions were added to isolated volumes of the DH5α strain individually at a ratio of 1: 10 and mixing then incubated on ice for 30 minutes. The mixture samples were incubated in a 42˚C water bath for 20

seconds and placed back in the ice for 2 minutes before adding of pre-warmed LB media with the final volume of 1ml. After that the mixture samples were incubated at 37˚C for 1 hour. After the incubation the bacteria was centrifuged at 9000rpm for 3 minutes and the supernatant was discarded and resuspended in the remaining LB media then pelleted on an agar gel plate containing ampicillin. Plates were prepared by non-transformed DH5α cells on an agar plate with no ampicillin serving as positive control and pUC19 transformed DH5α on ampicillin containing agar plate serving as positive transformation control control. The negative control was prepared by contained ampicillin. Then, the plates were incubated for overnight at 37˚C.

The QIAprep Plasmid DNA purification kit (QIAGEN) was used to purify the Plasmid DNA of HEX20 exon from the ERBB2 gene of the T47D cell line and followed the manufacturer’s instructions by Resuspend the pellet in 250ul of P1 buffer then was added 250ul and mix the samples by inverting for 6 times. Buffer N3 was added about 350ul and mix them 6 times. After that centrifuged the samples for 10 minutes at 13, 000 rpm and applied the supernatants to the QlAprep spin columns. The samples were centrifuged for 60 seconds and the flow-through was discarded. The spin columns were wash twice with 500ul of PB buffer and 750ul of PE buffer. The DNA was eluted by adding 50ul of EB buffer. RE digestion analysis: The restriction digestions were performed in two different enzymes BglII and XBA. they were used on 5µl of plasmid DNA, 10x of the enzyme buffer, 0. 1x Bsa, and containing 1µl of the restriction enzyme in a final volume of 20µl by incubating at 37 ˚C for 1 hour. The restriction digests were analyzed by

electrophoresis on 1% agarose/TAE gels containing 0. 5 µg/ml ethidium bromide. Sequencing: The sequencing was done by a company for Sanger Chain Terminating or ddNTP sequencing and was used primers directed against the pJet 1. 2 blunt cloning vector.

Result a. Exon 20 (ENSEMBL 24) is 186 bp and has the sequence:

GGCCATGGCTGTGGTTTGTGATGGTTGGGAGGCTGTGTGGTGTTTGGGGGTGTGT
GGTCTCCCATACCCTCTCAGCGTACCCTTGTCCCCAGGAAGCATACGTGATGGCTG
GTGTGGGCTCCCCATATGTCTCCCGCCTTCTGGGCATCTGCCTGACATCCACGGTG
CAGCTGGTGACACAGCTTATGCCCTATGGCTGCCTCTTAGACCATGTCCGGGAAAA
CCGCGGACGCCTGGGCTCCCAGGACCTGCTGAACTGGTGTATGCAGATTGCCAAG
GTATGCACCTGGGCTCTTTGCAGGTCTCTCCGGAGCAAACCCCTATGTCCACAAGG
GGCTAGGATGGGGACTCTTGCTGGGCATGTGGCCAGGCCCAGGCCCTCCCAGAA
GGTCTACATGGGTGCTTCCCA

.

b.

pJet 1. 2 HEX20:

GGAAGCATACGTGATGGCTGGTGTGGGCTCCCCATATGTCTCCCGCCTTCTGGGC
ATCTGCCTGACATCCACGGTGCAGCTGGTGACACAGCTTATGCCCTATGGCTGCCT
CTTAGACCATGTCCGGGAAAACCGCGGACGCCTGGGCTCCCAGGACCTGCTGAAC
TGGTGTATGCAGATTGCCAAGGTATGCACCTGGGCTCTTTGCAGGTCTCTCCGGA

Figure4: a. the original sequence of HEX20. b. the result of pJet 1. 2 HEX20 sequencing.

- b.

c. d.

Figure2: transformation of DNA into bacterial colonies E. coli DH5α cells. Photographs of LB-Agar plates (a, b, c, and d) on which are grown transformed bacteria. DNA used for transformation was: (a) experimental ligation products of pJet-HEX20 PCR amplification ligated into pJet 1. 2 vector; (b) pJet control plasmid pJet (no insert); (c) DH5alpha pGAP; (d) positive transformation control containing pUC18 DH5alpha (no ampicillin).

Figure3: Restriction digests of plasmid DNA was run in 1% agarose gel electrophoresis. The samples were visualized by ethidum bromide staining. Two different enzymes were used. Sample 1; pJet HEX12 , sample 2; pJet HEX12 , sample 3; pJet HEX20, sample 5; pJet negative control , and sample7; PGAP = pJet 12 GAPDH.

Figure1: PCR product of T47D cells was run in 1. 2% agarose gel electrophoresis. The samples were visualized by ethidum bromide staining. Lane M; which is DNA marker, Lane 1; PCR product negative control, Lane 2; PCR product primer pair 1, Lane 3; PCR product primer pair 2, Lane DNA; genomic DNA , and Lane Etha; Ethanol precipitation.

Discussion

In this experiment, we were determining whether there was SNP or mutations when cloning two exons from the HER2 that was used to explain sensitivities to targeted therapies. First the Genomic DNA extraction was done from the human breast cancer purified and followed the manufacturer’s instruction. The T47D cell DNA was amplified by using Polymerase Chain Reaction.

The reaction components were used for PCR with final concentrations of 1x pfx amplification buffer, Purified of HEX20 PCR product was ligated to pJet 1.

2 blunted vectors. The ligation was achieved by adding of 1x T4 DNA ligase buffer, 1 unit concentration of T4 DNA ligase, and complete the reaction by adding distill water. Ligation reactions were transformed into the DH5α strain of E. coli.

The T47D cell line had a wild type on Exon 12 and Exon 20 to subdue the amplification of the normal sequenced and enriched amplification of mutant DNA. By using this T47D cell line it is easier to investigate the breast since it has normal amount of HER2 protein and has a better understanding of ERBB2 gene which will be used to interpret the result obtained in order to treat the breast cancer (1, 7).

References.

1." PLOS ONE: Molecular Profiling of Breast Cancer Cell Lines Defines Relevant Tumor

Models and Provides a Resource for Cancer Gene Discovery." PLOS ONE : accelerating the

publication of peer-reviewed science. Web. 2 Apr. 2013. http://www. plosone. org/article/info%3Adoi%2F10. 1371%2Fjournal. pone. 0006146

2. " Targeted Therapy in Breast Cancer: Current Status and Future Directions ." Oxford Journals | Medicine | Japanese Journal of Clinical Oncology. Web. 3 Apr. 2013. http://jjco. oxfordjournals. org/ content/40/8/711. full

3." Characterization of Estrogen Responsive Transforming Activity in Human Breast Cancer Cell Lines ." Cancer Research . Web. 2 Apr. 2013. http://cancerres. aacrjournals. org/content/446/4_Part_1/1707. short

4. The Human Homologue for the Caenorhabditis elegans cul-4 Gene Is Amplified and Overexpressed in Primary Breast Cancers ." Cancer Research .

Web. 3 Apr. 2013. http://cancerres. aacrjournals. org/content/58/16/3677. short

5. Du, Dr. Jinyan. " Signaling Pathways: ErbB/HER Signaling." Cell Signaling Technology. 1 Oct. 2012. Web. 2 Apr. 2013. http://www. cellsignal. com/reference/pathway/ErbB_HER. html

6. " Science. gov v5. 0 : Main View : Search Results for Full Record: t47d human breast." Science. gov: USA. gov for Science - Government Science Portal. Web. 2 Apr. 2013. http://www. science. gov/topicpages/t/t47d+human+breast. html

7. " ErbB2 Is Necessary for ErbB4 Ligands to Stimulate Oncogenic Activities in Models of Human Breast Cancer ." Genes & Cancer . Web. 2 Apr. 2013. http://gan. sagepub. com/content/2/8/792. full