Mutational analysis of *TEP1*, a budding yeast homologue of the human prostate cancer factor *PTEN*

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Abstract

The phosphate and tensin homolog (*PTEN*) gene is a tumor suppressor gene in humans that encodes for a phosphatase protein involved in the regulation of the cell cycle. Mutations of the *PTEN* gene have been linked to aggressive forms of prostate cancer (PC), which calls for further characterization of the gene. The goals of this experiment are to generate a *Saccharomyces cerevisae* FY86 strain lacking the yeast *PTEN* homologue, *TEP1*, via Leucine2 homologous recombination, to clone the *TEP1* gene into a bacterial plasmid using OneShot TOP10 transformed *E. coli* cells, and to perform side-by-side growth experimentation between WT and *TEP1* knockout FY86 cell cultures, all with the purpose of characterizing the function of the *TEP1* homologue to provide insight on *PTEN*'s involvement in tumor growth and development. The results of the experiment indicated that two successful *TEP1* knockout FY86 strains were successfully generated and preserved. Preliminary quantitative growth tests revealed that that the WT and *TEP1* knockout strains are able to survive and grow in a pH rang of 7.0-8.0 at 37°C in order to simulate human physiological environments. After repeated trials, the cloning of the *TEP1* gene into a bacterial plasmid was determined unsuccessful after analysis of the agarose gel bands.

INTRODUCTION

For decades prostate cancer has been a hot topic for cancer research. There have been scientific breakthroughs involving prostate cancer ranging from novel early screening tests to less invasive medical procedures. Despite this progress, prostate cancer still results in more than 32,000 deaths and 200,000 new cases annually, in the United States, making it the second leading cause of cancer related deaths in men (American Cancer Society, 2013). Of all prostate cancer cases among men 65 years and older, about 60% are diagnosed and this further increases to about 97% in men of 50 years and older (American Cancer Society, 2013). Early screening and surgical procedures have greatly increased the likelihood of early diagnosis and survivability of prostate cancer, but more research must be conducted to characterize genes related to prostate cancer in hopes of developing novel treatments.

Advancements in genomics and bioinformatics have made it possible to identify and verify many of the genes involved in the initiation and progression of prostate cancer. Among the various genes identified as being related to prostate cancer, *PTEN* (MMAC1/*TEP1*) stands out as a candidate gene for research due to its involvement in advanced cases of prostate cancer when lost or mutated (Huang *et al*., 2001). Mutations of the *PTEN* gene have shown to be prevalent in prostate cancer cases, with studies discovering *PTEN* mutations in over 55% of tumor DNA samples analyzed (Pesche *et al*., 1998). Functional mutations in the gene have also been observed and studied in various other forms of cancer such as bladder, breast, and cervical cancer (Cancer Index, 2014).*PTEN* is a tumor suppressor gene that encodes for phosphatase and tensin homologue, a protein that participates in the regulation of the cell cycle by limiting the rate of cell growth and division. This enzyme, found in almost all tissues, removes phosphate groups from other proteins and lipids in part of a chemical pathway that ultimately signals the cell to halt division and initiate apoptosis. In addition, research suggests that the *PTEN* protein product is also involved in cell migration, adhesion to surrounding tissues, and in angiogenesis (Genetics Home Reference). Mutations of this gene, based on these known characteristics, make it likely to promote tumor growth. The purpose of this experiment is to characterize the *TEP1* yeast gene homologue of the human *PTEN* gene in order to gain further insight on its involvement in cell growth and division suppression, particularly relating to PC.

Materials and Methods

FY86 Yeast Strain

The yeast strain chosen for this study was *Saccharomyces cerevisae* FY86. This yeast strain serves as a reliable model for studying human gene homologues, which is why it was chosen to study the telomerase protein component (*TEP1*) yeast homologue to the human *PTEN* gene. FY86 cultures were grown in yeast growth media (YPD) overnight at 30°C and stored in -4°C until needed. Strains with Leucine2 gene transformation were grown on SC Leucine dropout plates for growth specific selection.

YIplac128, YIplac195, and YIplac211 Bacteria Strains

Initially, YIplac128 was the bacteria strand used in the experiment, which contains the Leucine2 and ampicillin gene used for plate selection on either LB + amp or SC – Leu plates. Later, YIplac195 and YIplac211 were used in the experiment, which have similar Leucine2 and ampicillin gene characteristics as YIplac128. All bacterial cultures were incubated at 37°C overnight to allow for growth.

Plasmid DNA Purification

Overnight bacteria cultures grown in 5mL LB+amp broth were pelleted and the plasmid DNA was purified using the QIAprep Spin Miniprep Kit protocol and microcentrifuge. The overall procedure yields a total of 50µl of EB buffer containing bacterial plasmid DNA. The DNA was then stored at -20°C until needed.

Genomic DNA Isolation

Overnight FY86 cultures grown in 5mL SC – leucine were pelleted and the YeaStar^{TM} Genomic DNA Kit was used to reliably and efficiently isolate the genomic DNA from the cells. The standard procedure yielded a total of 20µl of EB buffer containing genomic yeast DNA. The DNA was then stored at -20°C until needed.

Polymerase Chain Reaction (PCR)

Two sets of primers were used during the experiment. The first set of primers, Leu2 Lower and Upper, were designed to be long to contain the *TEP1* gene in order to amplify the Leucine2 gene in the YIplac128, YIplac195, and YIplac211 bacterial genomes with *TEP1* flanking either side of the product (Figure 1). The second set of primers, XbaI L and U, were designed contain XbaI restriction sites in order to amplify the *TEP1* gene from the FY86 yeast genome with XbaI restriction sites flanking either side of the product (Figure 2). Similarly the last primer, BamHI U, was designed to amplify *TEP1* from the genome with BamHI restriction sites on the upper end and to be used with the XbaI L primer for double digestion (Figure 1).

Primer Sequences	
Primer Name	**Sequence**
TEP LEU2 L	**5'-**AAA GTA CCA TAC AAG AAT ATT GAA GAA AAG GAA AAG AAT ACA TAA TAT AGC GTC TCC GGG AGC TGC TGC ATG TGT CAG**-3'**
TEP LEU2 U	**5'-**TCT AAA GCT TTG TAA GGA TTA GGA AAG CGC CAC AAA ATC GAT GAG AAG TAT TTC CGC GCA CAT TTC CCC GAA AAG T**-3'**
TEP L XbaI	**5'-**CTC CTT GGT TCT CAT ATA TTT CTT CCT GTT TC**-3'**
TEP U XbaI	**5'-**TTT TCT AGA TTT GAA TAT AAA AGA GTT CAT AAA TGA TGA TGA**-3'**
TEP U BamHI	**5'-**TTT GGA TCC TTT GAA TAT AAA AGA GTT CAT AAA TGA TGA TGA**-3'**

Figure 1: Sequence of Primers

Gel Electrophoresis

Gel electrophoresis was performed on PCR products in order to identify specific band lengths of interest to be extracted and isolate the DNA. This was done to ensure that the DNA being used in subsequent experiments was correct. Gel electrophoresis was also performed as a diagnostic technique to determine the presence or absence of the *TEP1* or Leucine2 gene of interest in various transformed plasmids.

Agarose Gel Extraction

Following gel electrophoresis, bands containing DNA of interest were cut from the gel using a razor blade and then melted in QG buffer (Qiagen). The DNA contained in the melted agarose gel was then purified using P1 and EB buffer through a spin column. The three buffer solutions were purchased from the manufacturer, Qiagen, and standard protocol was used. This yielded a total of 30 µl of EB buffer containing DNA, which was stored at -20°C until needed.

Spectrophotometer DNA Quantification

Using an EPOCH spectrophotometer, any DNA samples contained in EB buffer were quantified in order to determine the concentration of DNA in units of ng/µl. This step was important for not only determining presence of DNA, but also necessary for determining volume of DNA needed for reactions that require a specific range of DNA mass.

Enzymatic Digestion of Vector and Insert

In order to setup ligation reactions, isolated bacterial plasmids and genomic yeast DNA were digested by enzymes to generate vectors and inserts, respectively. Initially the DNA samples were double digested with BamHI and XbaI, but following further experimentation it was decided a single digestion was appropriate using only the XbaI enzyme. Setup enzyme reactions were incubated in a 37°C bath overnight to allow for digestion to proceed to completion.

Calf Intestinal Alkaline Phosphatase (CIP) Treatment of Plasmid

After enzymatic digestion plasmids were CIP treated, which removes the 5' and 3' phosphorylated ends of the DNA to prevent them from religating with one another. This step was necessary prior to ligation reactions to ensure that the digested plasmid's 5' and 3' ends would still be available for the insert to bind to. The CIP treatment was performed on 30µl of DNA at 37°C for one hour

Ligation of Vector and Insert

Ligation reactions were setup using XbaI digested insert DNA and XbaI and CIP treated vector plasmid DNA. A 5:1 ratio of insert to vector was used when setting up the reaction and control reactions were setup lacking insert DNA. The reactions were left overnight at 16°C and stored at -20°C until needed. The ligation products were then used in subsequent transformation reactions.

Lithium Acetate Yeast Transformation

To incorporate the leucine2 gene into Yeast cells, Super-high efficiency LiAc transformation was utilized. This process includes treating overnight FY86 cultures with 1X TE/1X LiAc and using snap-coiled salmon sperm with insert DNA to transform the yeast cells. This procedure allowed for the generation of FY86 cultures lacking the *TEP1* gene.

Transformation of OneShot TOP10 Competent Cells

In order to incorporate the *TEP1* gene into a bacterial plasmid, OneShot TOP10 Competent Cells were utilized. The *E. coli* cells were purchased and came already chemically treated to be able to accept plasmid DNA. Once transformed these cells were LB + amp plated and stored at -4°C until needed.

Growth Rate Experimentation

To test the effects of losing the *TEP1* gene, Wild-type FY86 cells were compared to transformed colonies 1 and 6 lacking the *TEP1* gene. YNB plates were made to have pH values of 7.0, 7.2, 7.45, 7.7, and 8.0 using a pH meter and adding NaOH drop-wise into the pre-autoclaved agar. Cell cultures were grown overnight at 30°C and 37°C on the plates by streaking, pipetting and spreading 500µl of broth cells, and performing 1:100 dilutions of cells to plate.

Results and Discussion

Generation of *TEP1* Knockout Strain: Creating Leu2 cassette for transformation of FY86 yeast cells

The first goal during this experiment was to generate a FY86 cell line lacking the *TEP1* gene, so that the mutant knockout cell cultures can be compared side-by-side with wild-type FY86 cell cultures.

Respectively, plasmid DNA and genomic DNA, were isolated from YIplac128 and FY86 cell cultures grown overnight, following standard protocol as described in the materials and methods. The isolated YIplac128 plasmid DNA was amplified using PCR with the Leu2 upper and lower primers, which were designed to contain the entire *TEP1* gene so that the final product would be the leu2 gene flanked on either side by the *TEP1* gene. To ensure sufficient concentrations of PCR product for later transformation, five PCR reactions were setup.

Following PCR amplification, the DNA products were run through Agarose gel electrophoresis to determine if the products are the desired length, so that they could be gel extracted and isolated. All the PCR products were extracted and isolated from the gel and then their concentrations were determined using an EPOCH spectrophotometer (Figure 2). This step was necessary to ensure that the correct amount of DNA would be added to the reaction mixture for the lithium acetate yeast transformation procedure.

DNA Quantification of PCR Products		
Sample #	Concentration 1 (ng/µl)	Concentration 2 (ng/µl)
Leucine2 TEP1 Flanked #1	134.345	121.595
Leucine2 TEP1 Flanked #2	119.444	131.641
Leucine2 TEP1 Flanked #3	56.182	49.009
Leucine2 TEP1 Flanked #4	95.936	97.207
Leucine2 TEP1 Flanked #5	120.993	122.874

Figure 2: DNA Quantification of PCR Products

The spectrophotometer measured the absorbency of a blank sample. The blank sample for this experiment was EB buffer, which is what each PCR product is contained in following DNA isolation after gel extraction. Using the EB buffer as the blank background, the absorbencies of two samples for each PCR product were measured (Figure 2). The closeness of these two values to one another indicates the accuracy of the results for the concentration. The concentration values were averaged for each product to determine how many microliters of each sample were needed for the individual transformation reactions (Figure 4).

Lithium Acetate Transformation of FY86 Yeast with Leucine2 Cassette

Naturally, FY86 yeast cells would not be capable of up taking a free floating DNA in the environment and become transformed. In order to incorporate the amplified Leu2, *TEP1* flanked gene segment into the FY86 genome, lithium acetate

transformation of FY86 yeast cells was performed using standard protocol. This process chemically treats the FY86 cells, in their midlog phase, to make them acceptable to the snap coiled gene segments loaded in salmon sperm. Once the Leu2 *TEP1* flanked gene segment was inserted into the FY86 cells homologous recombination was left to occur (Figure 3). This process involves the crossing over of the FY86 genomic *TEP1* gene with the *TEP1* capped ends of the YIplac128 Leu2 gene segment. The probability of this homologous recombination event is low, but the resulting product are FY86 cells that have recombined to remove their genomic *TEP1* gene and contain a newly acquired genomic Leu2 gene sequence (Figure 3).
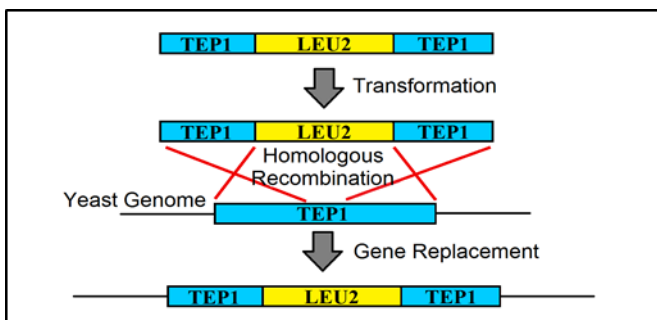

Figure 3: Homologous Recombination of FY86 genomic *TEP1* with Yiplac128 Leu2 flanked by *TEP1* gene sequence, resulting in the knockout of FY86 genomic *TEP1* by Leu2.

In total, eight FY86 5mL broth cultures were grown overnight to be used in transformation reactions. Four of the cultures were grown from an existing YPD plated FY86 line (labelled P1-P4, "P" to denote the culture was grown on a plate) and the remaining four cultures were grown from an existing YPD broth FY86 line (labelled L1-L4, "L" to denote culture was grown in liquid broth) (Figure 4). This step was taken to ensure that the growth technique used for the existing cultures would not effect the transformation process. From the five PCR products made containing Leucine2 flanked by *TEP1*, four were used. PCR product #3 was not used due to its low DNA concentration (Figure 4). The transformation reactions were setup as described in the table below (Figure 4).

Cell Line	Cassette
Plate #1 and Liquid Broth #1	Leucine2 TEP1 Flanked #1
Plate #2 and Liquid Broth #2	Leucine2 TEP1 Flanked #2
Plate #3 and Liquid Broth #3	Leucine2 TEP1 Flanked #4
Plate #4 and Liquid Broth #4	Leucine2 TEP1 Flanked #5

Figure 4: Table of the combinations of Cell lines and Cassettes used in the LiAc Transformation procedure.

Following Lithium Acetate transformation of FY86 yeast cells, eight plates were grown overnight on synthetic complete minus leucine plates. At random, ten colonies were selected from across the eight plates to determine the presence or absence of the *TEP1* gene (Figure 5). Each of the selected colonies was labelled #1-10 on their original plate and a swabbed sample for each was individually grown in broth overnight. The ten colonies selected were QIAprepped to isolate their DNA and then each sample was amplified using the Leucine2 lower and XbaI upper primers. If the Leucine2 gene is not present in the yeast genome then the Leu2 primer would not be able to bind and amplify the DNA and similarly if the *TEP1* gene is not present in the genome, the XbaI primer has no place to bind. Using this as a diagnostic technique, the Leu2 L and XbaI U PCR products were run through agarose gel electrophoresis (Figure 5).
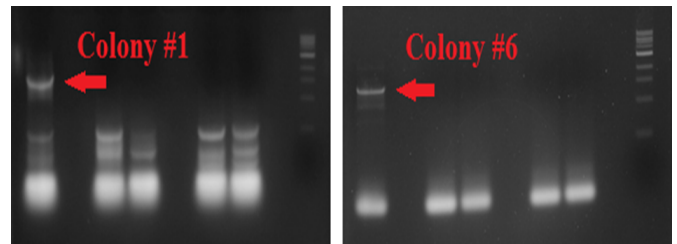

Figure 5: The wells above from left to right are colonies 1-10, which were selected at random from the eight plates grown from the Lithium Acetate FY86 transformation. The bands on the far right of each image is the 1 kb ladder, which correspond with colonies #1 and #6 at the expected length of between 1.0 and 1.5 kilobases.

The two lone bands in the far left well of each image indicate the presence of the Leucine2 gene between 1 to 1.5 kilobases, as expected. There is no indication that the *TEP1* gene is present, due to no band present with a length greater than that of Leucine2. The results from this diagnostic test are strongly indicative that the *TEP1* gene was successfully removed from the YIplac128 yeast genome and replaced with a functioning Leucine2 gene via homologous gene recombination.

Colonies #1 and #6 from their original plates were swabbed and grown on individual plates to be used later. As well, the confirmed *TEP1* knockout yeast colonies were frozen at -70°C in cryogel to remain as a permanent sample to be propagated in any future experimentation.

Cloning of the *TEP1* Gene into Bacterial Plasmid: PCR Amplification of FY86 *TEP1* and YIplac195/YIplac211 Leucine2

The second overall goal of the experiment was to insert the *TEP1* gene into a bacterial plasmid to produce unlimited copies, with the intentions of performing Alanine single point mutations on the suspected functional regions of the *TEP1* gene sequence to characterize the effects on protein functionality.

In order to clone the *TEP1* gene into a bacterial plasmid, first a bacterial plasmid vector and *TEP1* DNA were generated to contain XbaI restriction sites. This was accomplished by using the XbaI upper and lower primers, which were designed to contain XbaI restriction sites in their sequence and to amplify the *TEP1* gene from the yeast genome. The final PCR product from the FY86 yeast genome was used as the insert in the ligation reaction and contained the *TEP1* gene segments flanked on either side by XbaI restriction sites. Once the samples were amplified overnight they were run through gel electrophoresis to extract and isolate the XbaI flanked *TEP1* gene product.

Digestion, CIP Treatment, and Ligation

In preparation for the ligation reaction, both the amplified FY86 *TEP1* insert and the YIplac195 and YIplac211 leucine2 vectors were XbaI individually digested. Initially the plan was to double digest the bacterial plasmid with the enzymes XbaI and BamHI to remove leucine2 and expose a ligation site for the insert, but there were complications with BamHI resulting in double gel bands that were not the correct length. Tests were performed to diagnose the problem, but nothing was able to be pinpointed exactly (Figure 6). It was later decided to simplify the digestion to using only XbaI, since the restriction sites were already in desired positions to perform digestion. The XbaI digested plasmid vector was CIP treated to prevent the two exposed ends from religating with one another prior to the ligation reaction including the insert DNA. To increase the likelihood of the ligation reaction being successful, the maximum amount of insert DNA (10µl) was used in the reaction mixture.
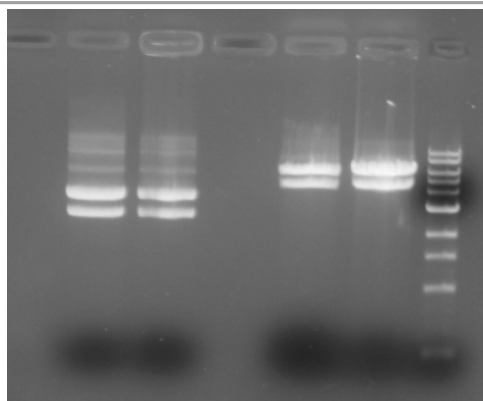

Figure 6: Single digest was performed with BamHI to determine if it may be involved in the complication involving successfully transforming OneShot TOP10 cells.

OneShot TOP10 Transformation

The bacterial plasmids, YIplac195 and YIplac211, were used to replace YIplac128 due to repeated complications with YIplac128 as the vector. Analysis of the OneShot TOP10 transformed cells revealed that the *TEP1* gene was not successfully incorporated into the plasmid when YIplac 128 was used as the vector (Gel image not shown). It was suspected, that due to the age of the YIplac128 cryogel sample used to generate the cell lines may no longer be useful in the experiment. YIplac195 and YIplac211 were chosen because they contained all the same significant gene sequence regions as YIplac128 and their cryogel samples were made more recently.

Following the transformation of OneShot TOP10 cells using YIplac195 and YIplac211 as the vectors, 10 Colonies were selected, at random, to be tested for the presence of *TEP1* in the bacterial plasmid. This was done by digesting the miniprepped cell plasmid DNA with XbaI and running the digest through gel electrophoresis (Figure 7). Unfortunately, after multiple trials and repeats of transformation procedure there was no success in cloning the *TEP1* gene into the bacterial plasmids. The two gel images are of the digested plasmid DNA from the ten colonies randomly selected from the last cloning trial conducted during the experiment (Figure 7). There are no bands indicating the presence of the *TEP1* gene in the digested bacterial plasmid (Figure 7). The expected band length was roughly 3600 bases long, which was not present when comparing with the reference 1kb ladder. These results strongly indicate that the cloning of the *TEP1* gene into the YIplac195 and YIplac211 bacterial plasmids was unsuccessful.
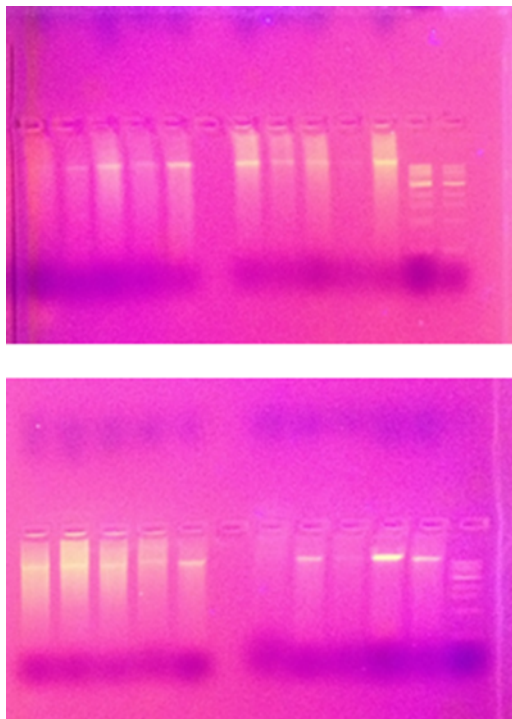
Figure 7: The agarose gel bands above are of the XbaI digested isolated DNA from the colonies selected at random following the final OneShot TOP10 transformation experiment conducted

Growth Comparison of Wild-type FY86 with Transformed FY86 *TEP1* Knockout under Ranging pH Conditions

        The final objective during the experiment was to perform side-by-side growth comparison experiments between the wild-type FY86, containing the *TEP1* gene, and the transformed FY86, containing a *TEP1* knockout by Leucine2. The purpose of this is to study the effects of losing complete function of the *TEP1* gene and how that may influence tumor growth under ranging physiological conditions. The transformed colonies #1 and #6 were used as the transformed cultures and the original propagated FY86 plate was used as the WT. It was only possible to perform qualitative tests by observing if the WT and mutant cultures could even grow in a range of pH conditions (pH 7.0, 7.2, 7.45, 7.7, and 8.0) at 37°C (Figure 10). After one day of incubation all plates had sufficient growth to conclude that it is possible to grow both WT and *TEP1* knockout FY86 cell cultures under pH conditions ranging from 7.0 and 8.0 in a 37°C incubator (Figure 10).

Conclusions

FY86 *TEP1* Knockout Strain

The results from gel electrophoresis performed on the

        Though only a qualitative test could be performed to test the possibility of growing WT and *TEP1* knockout FY86 in a pH range of 7.0, 7.2, 7.45, 7.7, and 8.0 at 37°C, these results are important. The growth of colonies on all of the plates indicate that it is possible to perform growth experimentations using FY86 as a model system for testing the effects of lacking the *PTEN* homologue, *TEP1*, under a wide range of physiological conditions.

        The next step, based on the results, could involve performing growth rate experimentation by making 1:100 dilutions of the WT and knockout cells and plating a small amount on each plate under the range of pH's. Incubate all of the plates at 37°C to mimic human body temperature and take images of the plates periodically over a period of time. Software can then be used to measure the area of cell coverage and this can be used to calculate the growth rate of the colonies under each condition. Testing the rate of growth of knockout cells under the range of pH values would provide insight to how a loss of function of the gene may be involved in prostate cancer and how aggressive the tumor's growth may be.

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