EnPress Publisher

ORIGINAL RESEARCH ARTICLE

Cloning of Mouse β-actin Gene

Chengjun Ji,Mingli Wang,Xuedong Wen

Life Science College, Shantou University of Science and Technology, Guangdong, China

ABSTRACT

β-actin gene is a kind of actin, in a variety of cells and tissues, β-actin gene expression is relatively stable [1], and β-actin gene in nuclear DNA has multiple copies, increased The detection rate of nuclear DNA [2], which is often used as an internal reference in PCR. In this experiment, the β-actin gene with good purity was obtained by PT-PCR, amplification, cloning, screening and PCR identification and restriction enzyme digestion of the RNA extracted from the mouse liver, which laid the foundation for the later experiment.

KEYWORDS: RT-PCR, cloning, digestion

1. Introduction

B-actin in different longevity conditions and different stages of stable expression, which is often used as an internal reference gene is widely used for β-actin gene research also has a lot of experiments [3], but the paper came to the end of shallow We do not only understand the role of β-actin gene, while the experimental operation ability greatly enhanced, more importantly, the β-actin gene, We gained the ability to think independently and understand the importance of teamwork. This is the original intention of our big experiment.

2. Experimental equipment, reagents and materials

2.1. Experimental apparatus:

(Anke TGL-16B, Shanghai Anting Scientific Instrument Factory), tissue grinding rods, DEPC water treatment EP tubes and tips, pipettes (0.5-10 µl, 10-200 µl, 100- (2720 Thermal Cycler, AB Applied Biosystems); constant temperature water bath (HWS 26-type electric constant temperature water bath, Shanghai Heng Technology Co., Ltd.), a one-time glove, ophthalmic tweezers, (DYY-8C-type electrophoresis instrument, Beijing sixty-one instrument factory), the UV analyzer (Beijing), the Shanghai Science and Technology Co., Ltd., (CAS, SGSP-02, Huangshi City Hengfeng Medical Devices Co., Ltd.), ice box, incubator (model, SGSP-02, Huangshi City Hengfeng Medical Equipment Co., Ltd.) , Shaker (air bath thermostat oscillator, SHZ-82A, Jintan Honghua Instrument Factory), glass tube, centrifuge, glass rods; coater, sterilization test tube.

2.2. Reagents and Materials:

(CH3) 2CHOH, Jiangsu Qiangsheng Chemical Co., Ltd., Shanghai Runjie Chemical Reagent Co., Ltd. distributor), isopropyl acetate [(CH3) 2CHOH, Jiangsu Qiangsheng Chemical Co., Ltd., 75% ethanol (DEPC treatment of water preparation), anhydrous ethanol [(CH3) 2CHOH, Jiangsu strong Chemical Co., Ltd. produced, Shanghai Runjie Chemical Reagent Co., Ltd. distributor], 5 × RT Buffer, dNTP Mixture, RNase Inhibitor, AMV Reverse Transcriptase, Oligo dT-Adapter Primer; MgCl2, 10x RT Buffer, RNase Free H2O, dNTP Mixture, RNase Inhibitor, AMV Reverse Transcriptase, Oligo dT-Adapter Primer, 5x PCR Buffer, dH2O, dNTP Mixture, TaKaRa Ex Taq HS (10 pmol / L), gel recovery kit, TAE Buffer, electrophoretic loading buffer, EB, DNA marker, pMD19-T vector kit, 0.1M CaCl2, strain and culture medium [Escherichia coli DH5α LB (Amp +) plate, LB (Amp +) plate containing LB and Ampiclate; LB (Amp) liquid medium, PCR Buffer, dNTP mix, Primer 1 #, Primer 2 #, T Aq DNA polymerase, H2O, agarose, TAE electrophoresis buffer, loading buffer, DNA dye; overnight culture of plasmid DNA containing DH5α strain LB bacteria 1.5mL, solution [50 mmol / L glucose, 25 mmol / L (0.2 mol / L NaOH, 1% SDS), solution III [(potassium acetate solution): 5 mol / L KAc 60 mL, 10 mL / L EDTA (pH 8.0) Acetic acid 11.5mL, H2O 28.5mL, constant volume to 100mL, and autoclave, the final concentration of solution: K + 3mol / L, Ac 5mol / L, isopropyl alcohol, ethanol, 70%

Copyright © 2017 -. This is an Open Access article distributed under the terms of the Creative Commons Attribution-NonCommercial 4.0 International License (http://creativecommons.org/licenses/by-nc/4.0/), permitting all non-commercial use, distribution, and reproduction in any medium, provided the original work is properly cited.

ethanol, enzyme identification Reagents (restriction enzyme digestion required reagents: plasmid DNA, EcoR I, HindIII and digestion reaction buffer, agarose gel electrophoresis required reagents: 1% agarose gel, TAE electrophoresis buffer, 10 × loading buffer, DNA dye.

3. Methods and results analysis

3.1. Total RNA extraction in mouse liver

3.1.1 Experimental steps

Dissemination of mice, taking their liver, divided into six copies, take one into the already containing 500ul Trizol DEPC-treated 1.5ml EP tube;

Rapid grinding, plus 500mlTrizol grinding, room temperature 5min to fully dissolve;

Centrifuge at 12000 rpm for 5 min, transfer the supernatant to a new DEPC treated 1.5 mL EP, add 200 μL of chloroform, shake and mix for 3 min at room temperature;

Centrifugation at 12000 rpm for 15 min;

After centrifugation the mixture is divided into three layers: the lower layer of red phenol - chloroform layer, the middle layer, the upper layer of colorless water layer. RNA is present in the water sample without exception. Carefully remove the upper layer of colorless water, transferred to another new EP tube;

Add 500 μL of isopropyl alcohol at room temperature for 15 min;

12000 rpm centrifugal 15 min, then the bottom of the EP tube to see the white precipitate; gently discard the supernatant;

RNA pellet was washed by adding 1 mL of 75% ethanol (prepared with DEPC treated water)

7500 rpm centrifugation 5 min, discard the supernatant; the EP tube inversion, dry, volatile the remaining ethanol;

Dissolve RNA from 20 μl of DEPC-treated water.

3.2. RT-PCR, amplification product purification and agarose gel electrophoresis identification

3.2.1 Experimental steps

3.2.1.1 RT-PCR

Mix the reverse transcription reaction solution (5 × RT Buffer 2 μL + dNTP Mixture 4 μL + RNase Inhibitor 0.5 μL + AMV Reverse Transcriptase 1 μL + Oligo dT-Adapter Primer 0.5 μL + test sample RNA 2 μL) , And then reverse transcription reaction (42 30min, 99 5min, 5 5min);

3.2.1.2 PCR

PCR solution (10 × PCR Buffer 5 μL + ddH2O 28ul + dNTP Mixture 4 μL + TaKaRa Taq HS 1 μL + upstream PCR primer 1ul + downstream PCR primer 1ul), then PCR reaction [94 2min, (94 ° C 30 sec, 61.5 ° C 30 sec, 72 ° C 1.5 min) × 30 Cycle, 72 ° C 7 min, 4 ° C]

3.2.1.3 Agarose gel electrophoresis

1g agarose + 100mlTAE buffer, microwave oven heated for 2 minutes after melting, to be cooled to the hands of the temperature can be tolerated by adding 5ulEB mix, and then poured into the installed glue tank (A large plastic two small plastic). (50ul sample + 10ul Loading Buffer, mix, feel that 50ul comb hole cannot fit, then add only 40ul, the remaining added to the second hole). Electrophoresis (109mA), the indicator went to half the time to stop the glue;

3.2.1.4 Purification of the amplified product

After taking the photo, take the UV light and observe the required DNA stripe in the 1.5mlEP tube + 200ul Binding Buffer (xp2) 59.9 water bath. The melt was transferred to a well in a 2 mL collection tube in a HiBind DNA Mini column at 10,000 rpm for 1 min. Discard the filtrate, + 300ul Binding Buffer (xp2) 10000 rpm Centrifuge 1min. Discard the filtrate, + 700 μl SPW Wash Buffer 10000 rpm for 1 min, repeat once. Discard the filtrate, 13000 rpm centrifugation

2min. Place the column in a new EP tube, + 20 μl Elution Buffer 13000 rpm Centrifuge the DNA for 1 min, repeat the time.

3.2.1.5 Run electrophoresis

5ul sample + 1ul loading Buffer, 128mA electrophoresis, because running too slowly, halfway to 143mA, 103v. Electrophoresis to take pictures to take pictures.

3.2.2 Analysis of results

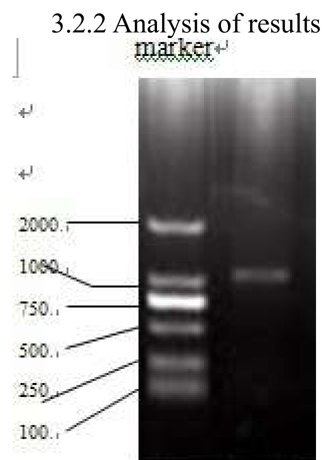

Figure 1. shows the gel electrophoresis of the amplified product

This is the purified display strip, compared with Figure 1, visible DNA content of the band is basically the same, to prove that the purification effect is better, and further identified the strip does show that the DNA content, to achieve the desired effect.

3.3. 3.3 RT-PCR product clone

3.3.1 Experimental steps

3.3.1 Linking the PCR product to the T vector: The experimental group (dd H2O 2 μL + Solution I 5 μL + pMD19-T vector 1 μL + PCR product 2 μL) and the control group (dd H2O 3 μL + Solution 5 μL + pMD19-T carrier 1 μL + Control Insert 1 μL), light mixing, 16 connection reaction for more than 30min.

3.3.1.2 Preparation of Escherichia coli competent cells: Inoculate 10 μL of glycerophilus to a tube containing 2 ml of medium (Amp-) and incubate overnight at 37 ° C with shaking. 30 μL of glycerophilus was inoculated into 3 ml of LB medium (Amp-) at 1% of the inoculation amount and incubated at 37 ° C for 3 hours (the A600 value of the bacterial solution reached 0.3 to 0.4). The cells were centrifuged at 5000 rpm for 2 min at room temperature. In the clean bench with the tip of the tip to learn to discard, and then 100μL pre-cooled 0.1M CaCl2 solution to resuspend the bacteria (heavy operation when the light), ice bath placed.

3.3.1.3 Transformation of Escherichia coli (aseptic): The ligation product (test group and control group) was added to the prepared competent cells, repeatedly beaten with a sample gun, the plasmid was mixed with the competent cells, and the ice Bath for 10 min; 42 water bath heat shock 2 min, time to quickly after ice cooling 10 min; add 1mL LB medium (Amp -), 37 standing for half an hour; 5000rpm centrifugation 2min, in the clean bench The cells were suspended with the remaining liquid and the suspension cells were all pipetted and transferred to LB (Amp +) plates containing X-gal and IPTG, coated with sterile glass rods uniform, 37 Inverted culture overnight.

3.4. 3.4 Screening of transformants and identification of PCR

3.4.1 Experimental steps

3.4.1.1 Preparation of blue-white plate

1L beaker by adding: Tryptone 10g, Yeast Extract 5g and NaCl 10g, add about 800mL of deionized water, stir well and mix well. Add 5N NaOH (about 0.2 mL) and adjust the pH to 7.0. Add the deionized water to set the medium to 1 L, add 15 g Agar. High temperature autoclave, cooled to about 60 degrees. Add 1 mL of Ampicillin (100 mg / mL), 1 mL of IPTG (24 mg / mL), 2 mL of X-Gal (20 mg / mL) and mix well. Plates were plated (30-35 mL medium / 90 mm

dish). 4 degrees away from light preservation, the general storage after 24 hours to start using. The next day from the 37-degree incubator to remove the plate observation, blue colonies that is initially identified as untransformed bacteria, white colonies were initially identified as transformants.

3.4.1.2 Colonies PCR

A conversion plate was randomly selected at room temperature in the presence of PCR reaction system (10X PCR Buffer 2 μL + dNTP mix 1.6 μL + Primer1 # 1ul + Primer2 # 1 μL + Taq DNA polymerase 0.5 μL + dd H2O 13.9 μL) On the transformant, with a small amount of sterilized small tip to pick a small number of bacteria; first small tip into a tube containing about 3mL LB (Amp +) medium washing tube 2-3 times, and then the same gun (2), but without the transformation of the sub-template, as a negative control. The control tube was used as a negative control for the operation of the PCR tube. After the above two PCR tubes were centrifuged, they were placed in a PCR instrument and reacted at 94 ° C for 5 min (94 ° C for 30 sec, 61.5 ° C for 30 sec, 72 ° C for 1.5 min) x 30Cycle, 72 ° C for 7 min, 4 ° C. Take a small amount of 10 μL PCR reaction product, 1% agarose gel electrophoresis analysis. The size of the amplified fragment was observed and recorded under UV detector. Note: Electrophoresis requires a standard DNA molecular weight Marker and a negative control sample without template. In the above-mentioned operation (2), the test tube was washed and incubated at 37 ° C and overnight (12 to 14 hours) in an air shaker in a test tube.

3.4.2 Results analysis

Figure 2. blue white spot plate colony map

Observation of the blue and white plate can be seen on the plate there are many colonies were cloned out, colony covered the entire plate, indicating that our flat coating is very uniform; while most of the colony is white (transformants), only a small amount or even no Is blue bacteria (untransformed bacteria), indicating that we have done in the transformation of this step was successful; but the afternoon and then observe the blue mushroom seems to have some longer, probably because the colonies grow slowly, but also may be Because of the effects of contamination on the table exposed to air pollution, these differences suggest that our experiments in the morning may be somewhat less error-prone, such as picking blue unchanging but not yet showing blue colonies Take these colonies to train, then, will result in the failure of experimental results.

Figure 3. shows the gel electrophoresis of the PCR reaction product

No. 1,2 hole plus the PCR reaction product, we can see the first hole effect is more ideal, the second hole strip slightly lighter color, may be picked up the colony of the gun in the LB medium tube too much washing, so that The tip of the tube and then put into the PCR tube when the remaining colonies at this time has been very little; there may be picked on the plate when the colony picked the colonies too little, but more agar picked up , Dilute the concentration of DNA in the solution, so the DNA content is low, the strip shows lighter. Of course, the first hole is too bright, then there may be too many because of the colony, causing non-specific gene amplification. The reason why the recombinant sub-PCR showed only 1.2kb instead of 3.9kb band, because the primers are only specific amplification of the target gene [4].

3.5. 3.5 Recombinant plasmid extraction and restriction enzyme digestion

3.5.1 Experimental steps

3.5.1.1 Recombinant plasmid extraction

Take 1.5mL of culture solution twice into 1.5mL EP tube, 12000r / min centrifugation for 1 minute, discard the supernatant, the tube inverted on the absorbent paper for several minutes, so that the liquid flow. Add 100μL solution, with the tip of the full resuscitation of bacteria precipitation. Add the newly prepared solution 200μL, cover the mouth, immediately gently up and down 4-5 times. (3min or so, do not exceed 5min) by adding 150μL solution, cover the mouth, up and down the EP tube 5-10 times. Centrifuge at 12000 r / min for 5 minutes. Carefully transfer the supernatant to a new EP tube (400ml, 400ml and 430ml respectively) and add an equal volume of phenol chloroform (lower) to mix. Centrifuge at 12000 r / min for 2 minutes. The supernatant was transferred to a new EP tube (350ml, 250ml and 400ml, respectively). Twice the volume of absolute ethanol was added and centrifuged at 12000r / min for 5 minutes. Add 1mL 70% ethanol, gently with the tip of the brush to wash the precipitate, be careful not to break the sediment or suck away. 12000r / min centrifugal 5 minutes, be careful to abandon the supernatant, be careful not to precipitate down. The EP tube inverted on the absorbent paper, in addition to ethanol, room temperature naturally dry. The extract was dissolved in 20 μl of RNase A-containing sterile distilled water and allowed to stand at room temperature to dissolve the DNA sufficiently.

Enzyme digestion

The enzyme was digested in the EP tube (plasmid DNA 10 μL + 10 × digestion reaction buffer 2 μL + EcoR I 1 μL + HindIII 1 μL + sterile distilled water 6 μL). Centrifugal mix, 37 water bath reaction 3-4 hours. A 1% agarose gel plate was prepared. After digestion, 10 μl of all DNA samples were added to the sample buffer and mixed with 2 μl. The samples were subjected to agarose gel electrophoresis (131 mA, 103 U). Electrophoretic plastic camera, UV monitor observation.

3.5.2 Results analysis

Figure 4. plasmid digestion identification map

The samples 1, 2, and 3 of the electrophoregram are samples 5-1, 5-2, 5-3, and 4, 5, and 6, respectively, which are not digested, respectively. -2 ', 5-3', the left is the marker and the size of each band. It can be seen from the figure, there is no digestion of the first hole and the corresponding cut 4 hole electrophoresis after no band display, analysis of the reasons that the largest possible because the first tube that is 5-1 is the first to do, It may be because the time is too long and lead to degradation of DNA, but there may be less solution to the amount of DNA does not release or solution is not a good neutralization of the previous alkaline solution and make the plasmid DNA refolding becomes insoluble The The molecular weight of the recombinant was 3.9 kb, of which the plasmid was 2.7 kb and the target gene was 1.2 kb. However, the 2, 2, and 2, 2, 2, 2, 2, 2, 2, 2, 2, 2, 3, 2, 2, 2, 2, 2, 2, 2, 2, 2, 2, 2,

The location of the bands of the second and third channels is biased, which is related to the conformation of the plasmid DNA. There are three kinds of plasmid DNA: covalent closed-loop DNA, often in supercoiled form; open-loop DNA, One of the two DNA chains has one or more breaks; (3) linear DNA is caused by the cleavage of the two strands of the plasmid at the same site. Three forms of plasmid DNA Swim velocities: supercoil> linear> open loop. And thus the positional deviation of the same material strip on the lane occurs.

Look at the fifth channel, after digestion, the good separation of the 2.7kb and 1.2kb band, and no 3.9kb band appears, indicating that this is a good complete removal; and the sixth channel The phenomenon is similar to that of the second and third channels, which is clearly affected by the degradation of the target gene of the second pore, but the 3.9kb band of the 6th pore is also significantly stronger than that of the second pore, taking into account that the fifth hole does not appear to be 3.9 Kb, which fully demonstrates that the samples of the second and third holes are degraded in the time of the digestion reaction. Of course, there are many factors that affect the effect of digestion, in addition to enzyme reaction conditions, the most important is the purity of DNA. For example, the sample contains a lot of RNA impurities, some of the protein is not in addition to net and combined with DNA, will affect the digestion effect. As for the extraction process is not removed by the various factors, such as trace of chloroform, SDS, EDTA, phenol, EB, ethanol or agarose gel sulfate ions.5

4. 4 Conclusion

The results of this experiment showed that most of the recombinants of the mouse beta-actin gene and plasmid were not excised and completely excised, and no satisfactory results were obtained, indicating that our experimental ability and team spirit remained to be Strengthen, warning us in the course of the experiment should pay attention to some details and time control. Fortunately, we still have a hole in the strip clearly shows the complete removal of

the plasmid and the gene, to prove that our ability is still some, the lack of practical ability, as long as more hands, I believe we will do better later. In general, the first time to do such a big experiment, we have such a result, or worthy of encouragement.

References

1. The specific heat of the PCR reaction system of mouse housekeeping gene β-actin was determined by DSC. The Institute of Food and Cryogenic Biotechnology, Shanghai University of Science and Technology, Food Science - Bioengineering 348, Section 10 Period, 2007, vol.28.
2. Yuan Zhengjie, Zhang Gengsheng, Sun Rui, et al., the internal reference method in the mitochondrial DNA purity identification of the application, Journal of wheat crop, 2009,29 (6): 1088-1093.
3. Yang Aifu, Zhou Zunchun, Dong Ying, et al. Comparative analysis of the expression stability of cytb and β-actin genes in the genus Eriocheir sinensis, China Agricultural Science and Technology Herald, 2010.12 (1); 79-84.
4. http: //www.baidu.com Baidu Encyclopedia
5. http://jpkc.gdpu.cn/2jpfzswx/Course/Content.asp?c=345\u0026a=587\u0026todo=show plasmid DNA extraction and purification of alkaline lysis of Guangdong Institute of Molecular Biology Course website.