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Research Article

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Parallel cloning, expression, purification, crystallization of human proteins for structural genomics

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ABSTRACT

100 human genes were study for testing targets for parallel cloning, expression, purification and crystallization. Proteins from these genes had a molecular weight of between 20 and 50 kDa, din not have a high percentage of hydrophobic residues and have unknown crystal structures and were not to be subunits of heterocomplexes. 100 expression clones have been constructed with the Gateway™ cloning system, the results showed that 79 clones were expressed as recombinant proteins in Escherichia coli strain BL21(DE3), 9 clones were expressed as recombinant proteins in Escherichia coli strain Rosetta(DE3), 21 were soluble and 12 have been purified to homogeneity. Crystallization conditions were screened for the purified proteins in 48-well plates by the sitting-drop. After further refinement with the same method or by the hanging-drop method, The crystal of COQ3 were grown.

Keywords: genomic proteins; expression; solubility identification; purification; crystallization

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INTRODUCTION

With the completion of human genome plan, biology is into the post-genome era. Understanding of protein function become a urgently demand for the biology research [1-3]. Because protein space structure can provide a lot of information for functional explanation and structure exploration, therefore structural genomics becomes a hot topic. Structural genomics are applied to discover, analyze, popularize the nature species of all proteins, RNA, DNA, sugar etc. Main research method is the use of X-ray crystallography, nuclear magnetic resonance (NMR) and electron microscope to the unknown structure of biological macromolecules or complex structure parsing, a higher resolution of space folding conformation. The present study mainly focuses on the protein molecules and protein, RNA, proteins, DNA, proteins, small molecules structure [4-7]. Due to the multidimensional nuclear magnetic resonance (NMR) having a large request sample of high purity, concentration, good stability, small molecular weight, the electron microscope requesting sample larger molecular weight, and lower resolution, the protein X-ray crystallography is still the main mean of parsing the protein structure [8-10].

EXPERIMENTAL SECTION

The target gene selection

We used bioinformatics software Vector NTI (invitrogen, U.S.A) for screening proteins. Selected the target gene was basically according to the following principles: the target gene expression protein prediction of molecular weight distribution is usually between 20 to 60 kda; Genes in the form of a cDNA clone can be purchased from human cDNA library, its coding proteins contained two reactions: BP system and LR reaction system, specific experimental operation procedure.

The PCR detection of expression vector

Because the pET21a - T7 existed on the DEST terminater sequence, we used the T7 with universal primers for PCR t of hydrophobic amino acid residues, it was a transmembrane protein without signal peptide; The proteins genes

encoded, the result showed that there was no high homology (30%) of protein crystal structure being reported.

GatewayTM construction of expression vector
GatewayTM technology was applied for building upstream and downstream primers. Detection theory results of T7 was to restructure locus sequence size, which was about 200 bp.

Level of agarose gel electrophoresis of nucleic acids
(1) according to the molecular size, choosing the appropriate concentration of gel

(2) take appropriate agarose, add appropriate amount of electrophoresis buffer to dissolve in the microwave oven.

(3) stay cool gel solution to about 60℃, pour into two end sealing with adhesive plaster having a comb at a horizontal position of the electrophoresis tank.

(4) after being solidified gel solution, removing the adhesive plaster, pulling out a comb, electrophoresis groove in the electrophoresis buffer, good circuit connected.

(5) sample and the buffer mix for electrophoresis, until the dye is close to the bottom of the gel, then take out the gel in the EB (including 0.5 g/mL) dye for 15 minutes (also can be in with glue when adding suitable amount of EB, you do not need to dyeing).

(6) remove the gel, gently wash gel (also don't rush), the uv gel imaging observe.

The expression of recombinant plasmid and testing
(1) The expression of recombinant proteins
Recombinant plasmid into e.c. with our fabrication: oli BL21 (DE3), and the coating containing ampicillin (Roth, Germany) 50 mu (g/mL), 100 mL LB culture medium containing 50 mg/mL ampicillin 37℃, 210 RPM. For overnight cultures, 50 ml liquid was added to the 1 l of fresh LB medium, develop to OD600 0.4 under 37℃, IPTG induction (Roth, Germany) was added untile concentration reached 0.5 mM, then continued to cultivate 16 hours under 18℃, liquid bacteria centrifuged at 5000 r/min.
(2)

(3) Expression of e.c. with our fabrication: oli BL21 (DE3)
Identification of recombinant protein without expression can once again into the our fabrication: oli Rosetta (DE3) under 18℃ trained 16 hours, and then express detection.

(4) Protein polyacrylamide gel electrophoresis (sds-page)

Express product soluble identification
100 mL bacteria liquid was induced, and after treating with ultrasound pyrolysis, 4000 g thalli precipitated in 2 mL HEPES buffer (20 mm NaCl 200 mm HEPES pH7.5) was collected, then the centrifuged 10 minutes and hanged off 100 mu l water, and added 30 mu l to precipitation, boiled 10 minutes under 100℃.

Protein purification method
Due to the selected forecast target protein isoelectric(PI) points were different from pH 7.5, HEPES buffer (20 mm NaCl 200 mm HEPES pH7.5) was used after ultrasound pyrolysis at 4℃ and 12000 r/min, then centrifuged for 30 minutes three times after cleaning untile the vast majority of bacteria precipitated, filtered through 0.22 mu m aperture and then stored for protein purification.

For protein could be carried in the end of the N 6 x His tag, nickel chelate chromatography column was used for purification with 500 mM imidazole gradient elution. then determined the target protein elution by sds-page detection. According to the target protein purification effect, further purification measures were measured, such as ion exchange chromatography (Hitrap S/Q), molecular sieve, etc. Hiload Superdex 75 (16/60) was applied for separation and purification. The rapid HPLC purification process system (FPLC) performed on the pre-loaded column. We used the purification strategies with Ni affinity chromatography and gel filtration.

Bradford method determination of protein concentration
The sample corresponds to the amount of BSA was the measured protein.
The sample concentration = (OD595 average/slope)/sample concentration.

Screening of protein crystal growth conditions by drop method
The gas phase diffusion primarily screened by drop method for crystal growth conditions. Protein was purified with enrichment tube concentrated by 10 mg/mL buffer, such as 20 mm) with a certain concentration of salt. Hampton Research Index, Crystal screen Ⅰ, Crystal screen Ⅱ were selected the kits such as Crystal growth conditions. Protein sample centrifuged (12,000 r/min) for 10 minutes. Under the microscope (preferably with polarizing microscope) observed, the condition of each droplet was recorded.

Optimization of protein crystals by hanging drop method
We used the gas phase diffusion drip crystal optimization suspension method. 1 u l protein solution with 1 u l liquid pool was used as hanging drop, a few days later under the microscope (preferably with polarizing microscope) observation, the condition of each droplet was recorded.

Diffraction data collection and processing
Crystal diffraction data was mainly collected by X-ray diffraction device (Bruker - Nonius FR591 rotating copper target generator and Bruker Smart 6000 CCD detectors) and synchrotron radiation devices (BSRF).

Bioinformatics retrieval and analysis
NCBI (National Center for Biotechnology Information) query nucleotide sequences, protein sequences and BLAST homologous comparison were used to find proteins related information. Rare codon GCUA analysis and ExPASy (Expert Protein Analysis System) could be used for protein sequence analysis, secondary structure prediction. PDB (Protein Data Bank) was used for viewing existing three-dimensional structure of proteins. Tertiary structure could be found in Dali on the server.

RESULTS AND DISCUSSION

Gene cloned by GatewayTM technology
Through the experiment we determined the PCR products, pDONOR201 plasmid, entry clone concentrations and optimal pET21a - DEST plasmid concentration ratio. PCR products, pDONR201 plasmid concentration and pET21a - DEST plasmid concentration did not exceed 150 mu g/mL, Entry clone plasmid moles was not higher three times than pET21a - DEST concentration. Finally, we successfully cloned 100 gene expression vector was constructed, the success rate was 100%.

The soluble protein expression detection and analysis
IPTG induced transformation of e.c. with our fabrication: oli BL21 (DE3) 100 expression vector, the results of 79 in the e.c. with our fabrication: oli BL21 (DE3), specifically indicated that there were nine purposes of gene expression, together a total of 88 purpose gene could express. 88 soluble genes were analyzed, the results were shown in table 1.

Table1 The results of the cloning, expression, soluble genes

	Target gene	Expression of target gene	Target gene expression of soluble protein
	100	88	21
percentage of success Compared to the previous step（%）		88	23
success percentage of all the gene（%）		88	21

Protein purification strategies
Ni affinity chromatography was suitable for rapid and efficient purifying protein with gel filtration. The crystal growth was a high-throughput genomics.

Crystal screen at optimal primary and x-rays diffraction
Optimization of crystal could be at two conditions of protein and crystallization. The optimization of protein samples: fused the tag, purified protein, restricted enzyme digestion for the stability of the protein fragments, methylated modification of lysine residues. The optimization of crystallization conditions:pH and precipitant concentration optimized, formed a two-dimensional array. The temperature of the crystal, hanging drop in the proportion of protein solution and molten pool could try to adjust. The influence of different additives on crystal growth could be explored by vaccination (seeding) method. Provided the crystal diffraction capability was not strong, also could crystallize by such as Annealing (Annealing), and Dehydration (Dehydration), crosslinking (Cross - linking) [11].

Screening high-throughput structural genomics

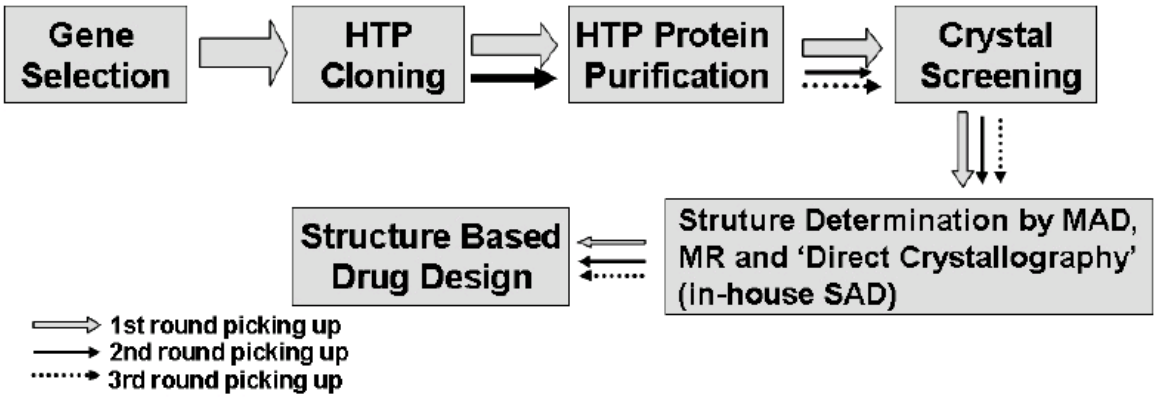

Figure1 the lab team structure genomics technology platform for process

As shown in Figure 1, the whole process was successful. The success rates of soluble protein expression and crystal growth were not high. The targeted gene eliminated at each step-down to save work, such as expression as inclusion body protein expression vector and express target gene using transformation strain method, the protein purification and purification condition optimization were screened.

CONCLUSION

GatewayTM technology could successfully constructed 100 unknown protein structure gene expression plasmid, though validating by PCR, constructing entirely correct, the chance of success reached 100%. By IPTG induction, the e.c. with our fabrication, oli BL21 (DE3), e.c and oli Rosetta (DE3) strain expression could successful build expression vector. The ultrasonic pyrolysis by sds-page detection could screen 21 a soluble protein. The Ni affinity chromatography and gel filtration purification filter were used for obtaining soluble protein, and successfully purified the high concentration of 12 target proteins. The crystal growth conditions were selected kits (Hampton Research, USA) by drop method. The COQ3 protein crystal growth conditions were determined by X-ray diffraction primary space group. The rapid high-throughput screening of unknown structure of protein crystals for structural genomics platform was established and optimized.

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