Construction of Modified pTRG Vector as pTRGQQ used in Bacterial Two Hybrid Assay for Protein-Protein Interaction Studies of Dengue Virus Type-2 Protease with Peptide Libraries

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Abstract: In molecular biology for cloning experiments, plasmid, a circular molecule is used as vector (carrier) to insert a desired gene or DNA fragment by ligating into it, after digestion of both with the compatible restriction enzymes. Then by transforming with a host (generally E. coli) strain of desired traits the screening for appropriate clone is done. A plasmid is having selectable marker in the form of antibiotic resistant gene which helps a molecular biologist for the proper selection of desired cloned while screening is done. Apart from having drug (antibiotic) resistance gene a plasmid also have an origin of replication and restriction sites where a gene or DNA can be inserted without interfering with plasmid replication or expression of the drug-resistance gene. For protein-protein interaction studies, yeast two-hybrid screening is traditionally used. It is however a tedious procedure, limited by the basic biology of the yeast. Yeast grows slowly, is difficult to transform efficiently and requires unique reagents and techniques. The bacterial two-hybrid system moves two-hybrid studies from yeast into E. coli – a much more convenient host with a fast growth rate, high transformation efficiency and easy manipulations. To carryout bacterial two hybrid assays two plasmids are required, one which carries a gene of interest bait (pBT), and another having a target gene (pTRG). Library which is a collection of multiple DNA fragments is screened for desired clones based on blue and white color appearance on X-gal indicator plate. In this article we report construction of a new modified plasmid vector pTRGQQ derived from pTRG vector which is highly compatible for blunt end cloning at SnaB1 restriction site of target gene into it to carry out Bacterial Two Hybrid Assay.

1. INTRODUCTION

Host and pathogens cross-talk through proteins, metabolites, small molecules, and nucleic acids, such as non-coding RNAs [1]. Protein–protein interactions (PPI) play a central role in biological systems [2–4] defining the interactome of an organism, or, as elegantly expressed by Robinson et al. the molecular sociology of the cell [5]. Many experimental approaches are used to investigate PPI, including yeast two-hybrid, [6,7] tandem affinity purification [8, 9] and mass spectroscopy [10]. Bacterial two hybrid assay to study protein-protein interaction has been emerged as a promising strategy in recent years, as the screening is faster and convenient in comparison with yeast two-hybrid assays. Here we report, construction of a modified bacterial two hybrid vector pTRGQQ, to enhance the cloning efficiency for protein-protein interaction studies.

2. MATERIALS AND METHODS

2.1 Construction of pTRGQQ plasmid

644 base pairs (bp) HBHA gene containing plasmid pUC57 was grown overnight in 100 ml Luria Bertani Broth (LB) supplemented with 100µg/ml ampicillin at 37°C. Preparation of DNA was followed by digesting HBHA gene fragment with Not1/Xho1 restriction enzymes. This 644 bp DNA fragment was cloned in Not1/Xho1 enzymes cut original pTRG vector which resulted in the formation of HBHA pTRG plasmid. The newly constructed HBHA pTRG plasmid was further digested with SnaB1 restriction enzyme and allowed to self ligate in an overnight ligation reaction which resulted in the formation of pTRGQQ plasmid. QQ was an addition to the name of SnaB1 digested self ligated HBHA pTRG plasmid to differentiate from other plasmids (pTRG and pTRGnn). PCR was
performed and sequencing of pTRGQQ plasmid was done to authenticate the cloning.

2.2 Cloning of genes in two hybrid vectors

To check the cloning efficiency of the pTRGQQ as two hybrid vector, Mycobacterial proteins CFP10 and ESAT6, that are known to form a tight 1:1 complex [11] and have been shown previously to interact using a bacterial two-hybrid system [12], were PCR amplified and digested with SnaB1 restriction enzyme. These genes were cloned into SnaB1 digested pBTnn and pTRGQQ, respectively, and used as positive controls for two hybrid assay. Dengue Virus (DV) type-2 protease NS3:2b gene was PCR amplified and the 751 bp PCR product was digested with SnaB1 restriction enzyme and cloned into SnaB1 digested pBTnn vector while Human cDNA lung library was already cloned in pTRG vector. NS3:2bpBTnn and Human cDNA lung library cloned in pTRG vector was used as test for bacterial two-hybrid assay. Empty pBTnn plasmid was used as negative control with ESAT6 pTRGQQ.

2.3 Bacterial two hybrid assay

*E. coli* reporter strain R1 was co-transformed with equal amounts of DNA of positive control (CFP10pBTnn and ESAT6pTRGQQ), test (NS3:2bpBTnn and Human cDNA lung library cloned in pTRG) and negative (pBTnn and ESAT6pTRGQQ) controls separately. The co-transformants were plated on X-Gal indicator plates containing kanamycin (50 mg/ml), chloramphenicol (30 mg/ml), tetracycline (12.5 mg/ml), X-Gal (80 mg/ml), Isopropyl b-D-thiogalactopyranoside (25 mM) and phenethyl-b-D-thiogalactoside (200mM). Appearance of blue color colonies on X-Gal indicator plate was the confirmation of interaction between proteins CFP10 and ESAT6 as positive control and interaction with Dengue NS3:2b protease with Human cDNA lung library as test was also confirmed. For further verification of the interaction, positive control(CFP10pBTnn and ESAT6pTRGQQ) and test plasmids plasmids (NS3:2bpBTnn and its unknown interacting partner from Human cDNA lung library cloned in pTRG vector) were separated by using chloramphenicol (30 mg/ml) and tetracycline (12.5 mg/ml) containing LB agar plates separately. The single isolated colony appeared at these plates were picked and re-grown overnight at 37°C in chloramphenicol (30 mg/ml) and tetracycline (12.5 mg/ml) antibiotic containing LB media. DNA was prepared and interaction was confirmed by repeated co-transformations.

2.4 Liquid β-galactosidase Assay

All interactions were further verified by performing liquid β-galactosidase assay. Individual colonies from each co-transformants from X-Gal indicator plate were selected and grown to mid-log phase in liquid culture in the presence of 10 μM IPTG and suitable antibiotics. Optical density (OD600) of each culture was measured at 600 nm. 10 part culture of negative control and one part culture of positive control was used while other (test sample) culture were taken as half of the positive control culture for performing the assay. The cell pellet was washed with 1 ml of Z buffer (60 mM Na2HPO4, 40 mM NaH2PO4, 10 mM KCl, and 1 mM MgSO4; pH 7.4) and resuspended in 150 μl of Z buffer containing 40 mM β-ME, 50 μl of chlorofrom, and 20 μl of 0.1% SDS. The mixture was vortexed vigorously. The assay was initiated by adding 700 μl of pre-warmed ONPG (Sigma) substrate from 1 mg/ml (~3M) stock solution (prepared in Z buffer with β-ME). The tubes were incubated at 30°C for appropriate time and the reaction was quenched with the addition of 0.5 ml of 1 M Na2CO3. The reaction mixture was centrifuged at high speed in a table top centrifuge for 10 minutes at room temperature and absorbance of the clear supernatant was measured at 420 nm. Enzyme activity (in Millers unit) was calculated using the equation; Millers Unit = [1000 x A420]/ [Time (in minutes) x Volume (in ml) x A600].

3. Results

1) Schematic representation of construction of pTRGQQ vector

[Diagram of the construction process]
3) **Bacterial two hybrid assay & Liquid β-galactosidase assay**

Figure 3. BacterioMatch two-hybrid reporter strain R1 was co-transformed with individual colonies from co-transformant plates which were patched on X-gal indicator plate in duplicates: EA1pTTRGQQ+CFP10pBTnn as positive control; cDNApTTRG (Human cDNA library+NS3:2bpBT as test; along with ESAT6pTTRGQQ+pBTrim as negative control.

4. DISCUSSION

In our efforts to carryout bacterial two hybrid assay for protein-protein interaction studies of Dengue virus type-2 protease NS3:2b with Human cDNA lung library, we needed highly efficient two hybrid vectors for blunt end cloning. Vector pBT and pTRG to clone gene of interest and target gene are used for bacterial two hybrid assays with required modification. In our case for blunt end cloning, we have been earlier using vector pBTnn (with SnaB1 restriction site) which is derived from pBT vector but we were facing problem with earlier used pTRGnn vector (with SnaB1 restriction site) derived from pTRG vector. So, we decided to further modify pTRG vector for our experimental suitability to study protein-protein interaction. We were successfully able to clone and re-clone target as well as gene of interest in newly modified pTRGQQ vector at SnaB1 site.

5. CONCLUSION

In an attempt to improve protein-protein interaction studies based on bacterial two hybrid assay, we successfully modified pTRG vector resulting in new version of it as pTRGQQ for blunt end cloning at SnaB1 restriction site. Due to its tenacity, the cloning efficiency of this vector was found to be very much improved as we cloned ESAT6 gene into it, which is known to interact with CFP10 protein. We were also able to found a new unknown interacting partner of Dengue virus type-2 NS3:2b protease from Human cDNA lung library, which has to be further studied. Liquid β-
galactosidase assay results further proved the successful creation of modified pTRGQQ vector for molecular cloning used in protein-protein interaction studies.

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COMPETING INTERESTS

No competing interest.

REFERENCES


