

ORIGINAL ARTICLE

Construction of Yeast Two-Hybrid Vectors for Screening Novel OTULIN-, OTUB1- and OTUB2-Interacting Protein from Human cDNA Library

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ABSTRACT

Introduction: OTULIN, OTUB1 and OTUB2 are deubiquitinases, the enzymes responsible for reversing ubiquitination process that occupies key roles in numerous cellular processes. The ubiquitination protein-protein interaction (PPI) network has been extensively explored in order to unravel the complexity of ubiquitin pathway. However, many significant challenges remain to develop a network-based understanding of the ubiquitination complexity including incompleteness of human interactome. Therefore, we aim to construct a pair of yeast two-hybrid (Y2H) vectors using pDEST32/pDEST22 vector system as a preparation for screening OTULIN-, OTUB1- and OTUB2-interacting proteins from human cDNA library, with ultimate aim of expanding the PPI network in human ubiquitome. **Methods:** *OTULIN*, *OTUB1* and *OTUB2* were cloned into entry vector using pCRTM8/GW/TOPO[®] TA Cloning[®] system and shuttled into pDESTTM32 bait vector by LR recombination reaction. To generate Y2H prey library clones, cDNA library was synthesized from HEK293 cells and cloned into donor vector pDONRTM222 before transferred into destination vector pDESTTM22. **Results:** DNA sequencing analysis confirmed the correct sequence of *OTULIN*, *OTUB1* and *OTUB2* inserts in pDEST32. Meanwhile, generation of cDNA library in pDEST22 produced 5.2×10^6 clones. Randomly picked pDEST22-cDNA clones showed that the recombination rate was 83% and gel electrophoresis indicated that the inserts length ranged from 0.45 to 3.4 kb. **Conclusion:** *OTULIN*, *OTUB1*, *OTUB2* and cDNA library were successfully cloned into Y2H bait and prey vectors. The clones have been transfected into competent yeast *Saccharomyces cerevisiae* strain MaV203 and Y2H experiment to screen novel OTULIN-, OTUB1- and OTUB2-interacting protein from human cDNA library is underway.

Keywords: OTULIN, OTUB1, OTUB2, cDNA library, Yeast two-hybrid vectors

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signal (5). Thus, DUBs could spare the substrates from the functions they supposed to commit, a characteristic that makes DUBs an appealing target for therapeutic interventions.

INTRODUCTION

Ubiquitin pathway or ubiquitination is a reversible post-translational modification process that is critical in numerous biological and pathological processes (1-3). In this pathway, ubiquitin molecule is attached to the lysine residue of a target substrate through a highly regulated, hierarchical combination of ubiquitin activating enzyme (E1), ubiquitin conjugating enzyme (E2) and ubiquitin ligase (E3) (4). Substrates tagged with ubiquitin, either single or several ubiquitin moieties, will be endowed with particular function depending on the ubiquitin chain topology. The ubiquitin pathway is negatively regulated by the antagonistic action of deubiquitin enzyme (DUB), which cleave off ubiquitin(s) from the substrate thereby reversing the ubiquitination

OTULIN, OTUB1 and OTUB2 are DUBs of ever-expanding ovarian tumor (OTU)-domain family. Being annotated in 2016, OTULIN is the latest addition to the family and is known to be involved in angiogenesis and NF- κ B regulation through Met1-polyubiquitin signaling (6,7). Not much is known regarding other roles of OTULIN especially in diseases, only, its loss-of-function mutation promotes a severe autoimmune condition termed OTULIN-Related Autoinflammation Syndrome (ORAS), which is normally suppressed by OTULIN-LUBAC interaction in no mutation condition (8). Meanwhile, OTUB1 and OTUB2 were the first two OTU proteins found to display *in vitro* DUB activity (9,10). OTUB1, which has remarkably similar fold with OTULIN (6) has emerged as important regulators in disease-related pathways since its discovery as a

tumor suppressor p53 regulator (11). Following that, many studies have reported that OTUB1 is directly associated with numerous cancer-related proteins and involved in the development of various malignancies including breast cancer, colorectal cancer, gastric adenocarcinoma, lung cancer, ovarian cancer, glioma and prostate cancer (12). Interestingly, OTUB2, the closest structural homologue of OTUB1 does not share the same ability in promoting cancer development and progression. Nevertheless, both proteins negatively regulate virus-triggered type I interferon (IFN) induction pathways and cellular antiviral response via interaction with TNF receptor associated factor (TRAF) protein family, TRAF3 and TRAF6 (13).

Protein-protein interactions (PPIs) are fundamental for all biological processes as proteins perform their functions by interacting and communicating with each other (14,15). According to 'guilt by association' paradigm, two proteins that interact with one another are also likely to be involved in related cellular functions (16). Thus, discovering novel PPI can provide important insights into the protein's potential interaction partners and decipher its roles in cellular biology. To study PPI, a powerful high-throughput technique such as yeast two-hybrid (Y2H) is very efficient in identifying novel partners of the proteins of interest (17). Y2H works by manipulating two separable domains of yeast Gal4 transcription factor, the DNA binding domain (BD) and the activation domain (AD). In Y2H screen, two hybrid proteins, BD-protein X fusion known as the 'bait' and AD-protein Y fusion known as the 'prey' are constructed in separate yeast expression plasmids with independent selectable markers. Upon transfection and expression of both proteins in the yeast nucleus, interaction between protein X and protein Y will bring the BD and AD in close proximity and reconstitute transcriptional activation that will result in expression of the reporter gene (18).

In the case of DUBs, discovering novel PPI is especially relevant since DUBs are often found in complexes with various proteins that eventually affect the DUBs activity (19). Previously, Y2H has been adopted to explore the complexity of PPI within human E2, E3 and DUBs (20-22). These studies revealed various combinatorial preferences within ubiquitination networks that confer substrate specificity, and thereupon, affect the biological processes regulated by the substrate. The most comprehensive DUBs interaction study were carried out a decade ago, in which 774 high-confidence interacting proteins of 75 DUBs were identified using mass spectrometry and analyzed using a quantitative scoring system called CompPASS (23). Their method has successfully identified six high confidence interactors for OTUB1 and one for OTUB2 (23). As the newest member of OTU DUBs family, OTULIN was not included in that study and probably has the least information regarding its interaction partner. It is thus of prime importance to study the OTULIN PPI to unveil more information

regarding its roles especially of which related to diseases. As for OTUB1 that has been linked to cancer, having more information regarding its interactors will increase understanding of this protein behavior in cancer systems biology point of view. In contrast, OTUB2 does not seem to play any role in cancer despite being the closest homologue to OTUB1. Structural comparison revealed that OTUB2 N-terminal domain is architecturally simpler and shorter than OTUB1, which may contribute to their functional differences (24). A comprehensive PPI analysis will confirm that OTUB2 is indeed interacts with a different spectrum of substrates.

For the purpose of screening OTULIN-, OTUB1- and OTUB2-interacting proteins, an efficient and high-quality cDNA library is necessary. Therefore, in this study, we aim to establish a high quality cDNA library synthesized from HEK293 cells and subsequently clone our cDNA library into Y2H prey vector. Simultaneously, Y2H bait vectors containing the genes of interest *OTULIN*, *OTUB1* and *OTUB2* will also be constructed for further study involving Y2H screen to validate the quality of cDNA library and also to find novel interactors for OTULIN, OTUB1 and OTUB2. Following this, we anticipate that the PPI network for OTULIN, OTUB1 and OTUB2 could be established and contribute towards completing human interactome map.

MATERIALS AND METHODS

Amplification of OTULIN, OTUB1 and OTUB2 to generate entry clones

The genes were PCR-amplified from gBlocks® gene fragments encoding *OTULIN*, *OTUB1* and *OTUB2* (IDT, USA) using specifically designed primers (Table I). The PCR reaction were set up in 50 µl reaction mixture containing 1 µl of 10-100 ng templates, 1 µl forward and reverse primers, respectively, 22 µl ddH₂O and 25 µl Taq 2X master mix (NEB, USA). The amplification program consisted of an initial denaturation step at 95 °C for 30 s, followed by 30 cycles of 30 s denaturing (95 °C), 1 min annealing (51 °C for *OTULIN*, 54.4 °C

Table I: Primer sequences

Primers	Sequence 5' → 3'
OTLN_F	ATGAGTCGGGGGACTATGC
OTLN_R	TCATAGACTGGTCTCCTCACAC
OTB1_F	ATGGCGGCGGAGGAAC
OTB1_R	CTATTTGTAGAGGATATCGTAGTGTCCAGG
OTB2_F	ATGAGTGAACATCTTTCAACC
OTB2_R	TCAATGTTTATCGGCTGCATAAAG
pDEST32_F	AACCGAAGTGCGCCAAGTGCTCG
pDEST32_R	AGCCGACAACCTTGATTGGAGAC
M13F	GTAACACGACGGCCAGT
M13R-pUC	CAGGAAACAGCTATGAC
pDEST22_F	TATAACGCGTTTGGAACTACT
pDEST22_R	AGCCGACAACCTTGATTGGAGAC

for *OTUB1* and 46.2 °C for *OTUB2*), 1 min elongation (68 °C), and a final extension at 68 °C for 30 min. The amplicons with 3'-end dA overhangs were confirmed by 1% (w/v) agarose gel before cloning them into the pCR™8/GW/TOPO® vector (Invitrogen, USA) to create entry clones pENTR-OTULIN, pENTR-OTUB1 and pENTR-OTUB2. Upon transformation into NEB® 5α competent *E. coli*, positive colonies were selected on LB agar plate containing 100 µg/ml spectinomycin and subjected to colony PCR for insert validation.

Transferring baits from entry clones into Y2H DB-destination vector

Inserts in entry clones were transferred to destination vectors pDEST™32 for expression in yeast system using Gateway® LR Clonase® II enzyme (Invitrogen, USA). Upon transformation, positive colonies were selected on LB agar plate containing 10 µg/ml gentamicin and validated by colony PCR using vector-specific primers (Table 1) and DNA sequencing. Sequence-verified pDEST32-OTULIN, pDEST32-OTUB1 and pDEST32-OTUB2 were then purified, quantitated and stored in -20 °C for future use as Y2H bait vectors.

HEK293 cell culture

Human embryonic kidney (HEK293) cells (ATCC, USA) were cultured in a humidified 5% (w/v) CO₂ atmosphere at 37 °C in Dulbecco's Modified Eagle's Medium (DMEM) (Gibco, USA) supplemented with 10% (v/v) fetal bovine serum (FBS) (Gibco, USA), 1% (v/v) L-glutamine (Gibco, USA) and 1% (v/v) penicillin-streptomycin (Gibco, USA). Cells were maintained in culture flask until ~80% confluence. For subculturing, cells were washed with phosphate buffered saline (PBS) (Invitrogen, USA) and trypsinized with 2 ml TrypLE Express Enzyme (1X) (Gibco, USA).

Total RNA extraction

HEK293 cells were seeded in a 6-well plate and incubated at 37 °C in 5% (w/v) CO₂ until approximately 80% confluence before proceeding to RNA extraction. Total RNA was extracted using TRIzol (Invitrogen, USA). Briefly, the cells were lysed by pipetting up and down several times in 700 µl TRIzol. Protein and DNA were separated from the lysate by adding 150 µl chloroform and centrifugating at 10,000 rpm for 5 min at 4 °C. Post-centrifugation, the upper aqueous phase containing RNA was collected by precipitation with 500 µl of 100% isopropyl alcohol. The RNA was then re-suspended in 20 µl of RNase-free water. The integrity of total RNA was analyzed by 1% (w/v) agarose gel electrophoresis run at 70 V for an hour. The RNA concentration and purity were determined by NanoDrop 2000/2000c Spectrophotometer (Thermo Fisher Scientific, USA) at 260 and 280 nm, respectively.

cDNA synthesis

Poly(A)+ mRNA was purified from 0.25 mg total RNA using Oligotex® mRNA mini kit (Qiagen, Germany)

according to the manufacturer's protocol. The cDNA library was constructed using CloneMiner™ II cDNA Library Construction Kit (Invitrogen). Firstly, priming reaction mixture was prepared by incubating 0.4 µg poly(A)+ mRNA with 0.5 µl Biotin-*attB2*-Oligo(dT) primer (30 pmol/µl) at 70 °C for 7 min. First strand reaction was synthesized by adding 2 µl 5X First Strand Buffer, 1 µl 0.1 M dithiothreitol (DTT) and 0.5 µl of 10 mM (each) dNTPs into the priming reaction. After 2 min incubation at 45 °C, 1 µl of SuperScriptR® III reverse transcriptase (200 U/µL) was added and the mixture was incubated in stepwise increments as follows: 45 °C (20 min), 50 °C (20 min), and 55 °C (20 min). To generate second strand reaction, the first strand reaction mixture was incubated at 16 °C for 2 h with the following reagents: 45.5 µl diethyl pyrocarbonate (DEPC)-treated water, 15 µl 5X Second Strand Buffer, 1.5 µl 10 mM (each) dNTPs, 0.5 µl *E. coli* DNA ligase (10 U/µl), 2 µl *E. coli* DNA polymerase I (10 U/µl) and 0.5 µl *E. coli* RNase H (2 U/µl). The reaction was then converted into blunt-ended double strand cDNA using 1 µl T4 DNA polymerase (5 U/µl). The cDNA was collected by centrifugation following phenol;chloroform;isoamyl alcohol (25:24:1) (NEB, USA) treatment. An *attB1* adapter was then ligated to the 5' end of cDNA by incubating the cDNA with 5 µl 5X Adapter Buffer, 2 µl *attB1* adapter (1 µg/µl), 4 µl 0.1 M DTT and 3 µl T4 DNA ligase (1 U/µl) at 16 °C for 16-24 h. Column chromatography was used to size-fractionate the cDNA and the representative cDNA library was collected from the column using TEN buffer, washed with 70% (v/v) ethanol and eluted in TE buffer. NanoDrop Spectrophotometer was used to determine the cDNA yield.

Construction and qualification of entry cDNA library

About 63.6 ng/µl of *attB*-flanked cDNA was cloned into 150 ng *attP*-containing donor vector (pDONR™222) by overnight incubation with Gateway® BP Clonase® II enzyme (Invitrogen, USA) to generate cDNA library entry clone pENTR-cDNA. The BP reactions were washed with 70% (v/v) ethanol and resuspended in TE buffer prior to transformation. Six tubes of 1.5 µl cDNA library was transformed individually into 50 µl ElectroMAX™ DH10B™ T1 phage resistant cells (Invitrogen, USA) using Gene Pulser Xcell Electroporation System (Bio Rad, USA) at 1.8 kV, 200 Ω resistance and 25 µF capacity. The transformed cells were pooled and subjected to plating assay performed in 10⁻¹, 10⁻², 10⁻³ and 10⁻⁴ serial dilution on kanamycin (50 µg/mL) added LB agar media. Titer for each plate was calculated using:

$$\text{cfu/ml} = \frac{\text{colonies on plate} \times \text{dilution factor}}{\text{volume plated (ml)}}$$

and the total number of colony-forming unit (cfu) was determined using:

$$\text{total CFU} = \text{average titer (cfu/ml)} \times \text{total volume of cDNA library (ml)}$$

For verification of cDNA inserts, 51 single clones were

randomly picked and subjected to colony PCR using vector-specific primers (Table I). The reaction was electrophoresed on 1% agarose gel at 100 V for 60 min to determine the number of clones containing inserts, percentage of recombinants, average insert size and insert size range.

Shuttling entry cDNA library into Y2H AD-destination vector

The pENTR-cDNA was purified before proceeding to LR recombination reaction to transfer the cDNA library into Gateway® destination vector pDEST™22. Positive colonies on 100 µg/ml ampicillin were randomly selected and subjected to colony PCR using vector-specific primers (Table I) and agarose gel electrophoresis. Upon confirmation and analysis of inserts, the pDEST22-cDNA was purified, quantitated and stored in -20 °C for future use as Y2H prey vector.

RESULTS

Y2H bait vector containing OTULIN, OTUB1 and OTUB2

Fig. 1A shows that the genes of interest *OTULIN*, *OTUB1* and *OTUB2* were successfully amplified from gBlocks® gene fragment templates at correct size, which are 1059 bp for *OTULIN*, 816 bp for *OTUB1* and 705 bp for *OTUB2*. Entry clones were established by cloning the PCR products directly into donor vector pCR™8/GW/TOPO® vector. During verification by colony PCR, insert specific primers (OTB1_F and OTB2_F) were used to validate the pENTR-OTUB1 and pENTR-OTUB2 while the pENTR-OTULIN were amplified using M13F vector specific primer, giving 316 extra bases to the band (Fig. 1B). The Y2H bait vector were constructed by transferring the entry clones into pDEST32 destination vector by LR recombination cloning and confirmed by colony PCR (Fig. 1C). pDEST32-OTULIN, pDEST32-OTUB1 and pDEST32-OTUB2 bands observed in Fig. 1C were 579 bp larger than expected size due to plasmid sites flanking the inserts. The bands were further confirmed by DNA sequencing. Analysis of sequencing

chromatogram by Chromas software (Technelysium Pty Ltd, Australia) shows clean nucleotide peaks with minimal baseline noise (Supplementary Fig. S1-S3). Nucleotide sequence identity was confirmed using the nucleotide sequence search in NCBI GenBank database using the Basic Local Alignment Search Tool (BLAST) program available at <http://www.ncbi.nlm.nih.gov/blast> (Supplementary Fig. S4).

cDNA library analysis

Gel electrophoresis analysis of total RNA integrity extracted with the TRIzol reagent from HEK293 cells shows two bands corresponding to ribosomal 28S and 18S RNA, and no visible degradation (Fig. 2). The A260/280 ratio of total RNA was 1.99 with a concentration of 1090.5 ng/µl. Purification of the total RNA produced 18.6 ng/µl poly A+ mRNA with A260/280 ratio of 2.06. Using 0.4 µg of the high quality poly A+ mRNA, 4 µl cDNA library with concentration 63.6 ng/µl was successfully produced.

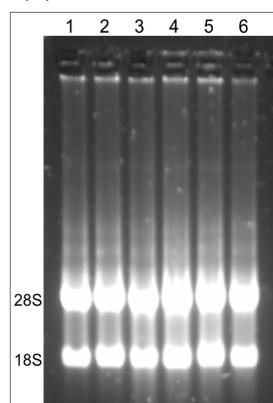


Fig. 2: Agarose gel electrophoresis of total RNA from HEK293 cells. Lane 1-6, total RNA.

cDNA library in Y2H prey vector

A cDNA library was first constructed as entry clones in pDONR222 vector. Based on the number of colony on 50 µg/ml kanamycin selection plates, this cDNA library was estimated to consist of approximately 8.13×10^6 cfu. To identify the length of the inserts

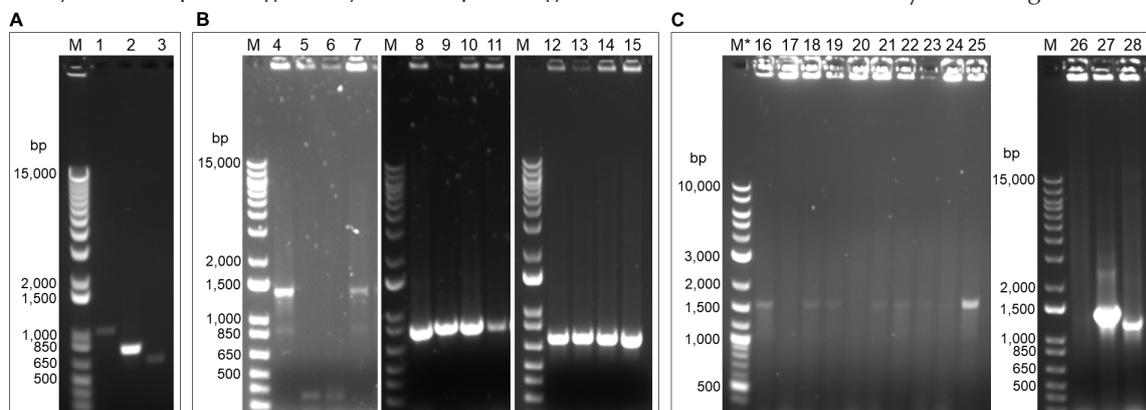


Fig. 1: Agarose gel electrophoresis of OTULIN (1059 bp), OTUB1 (816 bp) and OTUB2 (705 bp). (A) PCR amplification from gBlock gene fragments. Lane 1, OTULIN; Lane 2, OTUB1; Lane 3, OTUB2; M, TrackIt 1 kb Plus DNA Ladder (Thermo Fisher Scientific, USA). (B) Colony PCR of entry clones. Lane 4-7, pENTR-OTULIN; Lane 8-11, pENTR-OTUB1; Lane 12-15, pENTR-OTUB2; M, TrackIt 1 kb Plus DNA Ladder (Thermo Fisher Scientific, USA). (C) Colony PCR of destination clones. Lane 16-25, pDEST32-OTULIN; Lane 27, pDEST32-OTUB1; Lane 28, pDEST32-OTUB2; M*, Quick-Load Purple 1 kb Plus DNA Ladder (NEB, USA); M, TrackIt 1 kb Plus DNA Ladder (Thermo Fisher Scientific, USA).

and the recombination rate of the cDNA library, 51 positive clones were randomly selected and analyzed by colony PCR. Results showed that most of the inserts ranged from 0.3 to 2.5 kb, with average insert size of 0.98 kb (Fig. 3A). Randomly picked clones show that the recombination rate was 84.31%. The entry library carrying cDNA inserts were then shuttled into pDEST22 prey vector by LR reaction. This cDNA library consisted of 5.2×10^6 clones. 53 positive clones were randomly selected and identified by colony PCR. Results showed that the recombinant rate was 83% and most of the inserts ranged from 0.45 to 3.4 kb, with average insert size of 1.15 kb (Fig. 3B).

DISCUSSION

In studying PPI by Y2H method, correct sequence of DNA insert in bait and prey vectors is crucial to ensure precise mRNA transcription in the yeast nucleus. An error-free DNA sequence served as a key foundation required by a protein to ultimately adopt the correct three-dimensional conformation that enable the protein to bind with its partner and perform biological functions. Based on our sequencing result, *OTULIN* and *OTUB2* inserts in pDEST32 were 100% match with *OTULIN* and *OTUB2* sequence in NCBI database (Supplementary Fig. S4). Since sequencing quality normally reduced at approximately 900-1000 bp, 1059 bp *OTULIN* insert had to be confirmed by two-way sequencing by both forward and reverse primers (Supplementary Fig. S1). Meanwhile, it was 99% match for pDEST32-*OTUB1* due to an unidentified nucleotide at 1199 bp (Supplementary Fig. S4). Despite that, visual inspection on the sequencing chromatogram confirmed that it was indeed adenine, the correct nucleotide (Supplementary Fig. S2). Functionally, *OTULIN*, *OTUB1* and *OTUB2* are cysteine protease DUBs that rely on a catalytic triad of cysteine, histidine and aspartate to cleave the isopeptide bond within ubiquitin polymer (25). Mutation study was frequently done to determine the role of particular DUBs in certain cellular pathways, usually by substituting the

DUBs' active site with other residue. This will typically inactivate the DUBs and consequently, changing substrate ubiquitination as well as downstream signaling pathways (26). In this study, we confirmed that our DUBs sequence are correct and anticipated that the expressed DUBs in Y2H host cells will be in their active conformation.

cDNA library is equally important in PPI research as a high quality library will increase the possibility to obtain positive interactions and subsequently facilitates the construction of comprehensive PPI network of our protein of interest. In this study, our Y2H cDNA library was constructed using RNA isolated from HEK293, a transformed cell line originated from human embryonic kidney, which is the most used cells after HeLa in cell biology studies (27). Two independent mass spectrometry studies of human proteome from a panel of most common cell lines indicates that HEK293 yielded notably more protein identification compared to the other cell lines (28,29). Both experiments were performed in at least two technical replicates hence HEK293 higher number of proteins is unlikely to have been caused by experimental bias. Based on this, we presumed that our Y2H cDNA library prepared from HEK293 cells will be representing a high number of cDNA. This study also found out that there was a high degree of overall similarity of the proteomes of the diverse cell lines (29), suggesting that it may not be necessary to choose specific cell lines to investigate cellular processes that occur in their tissue of origin. Most importantly, a notable DUBs interaction study is also reported to adopt HEK293 as model system (23). In that study, 75 hemagglutinin-tagged DUBs were stably expressed in HEK293 as bait to pull out the interacting proteins prior to mass spectrometry and bioinformatics analysis. HEK293 is considered a suitable system based on expression analysis that show nearly all DUBs are endogenously expressed in HEK293 (23), which perhaps suggesting a high deubiquitination event in this cells. By using the same cell line for constructing Y2H cDNA

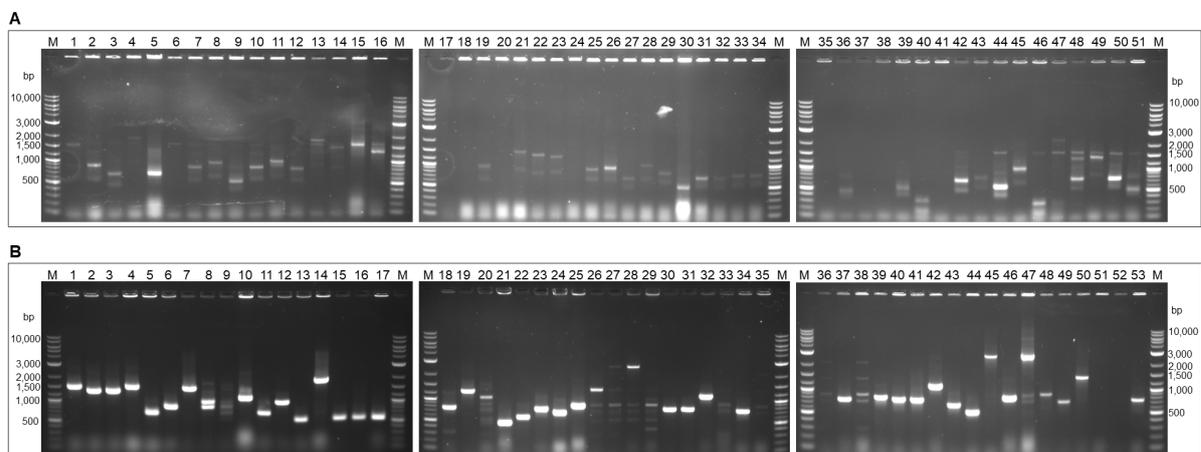


Fig. 3: Agarose gel electrophoresis of cDNA library. (A) Colony PCR of entry clones. Lane 1-51, randomly picked colonies from pENTR-cDNA; M, Quick-Load Purple 1 kb Plus DNA Ladder (NEB, USA). **(B) Colony PCR of destination clones.** Lane 1-53, randomly picked colonies from pDEST22-cDNA; M, Quick-Load Purple 1 kb Plus DNA Ladder (NEB, USA).

library, we are hoping that our future Y2H study could provide a fair comparison between the use of Y2H and mass spectrometry techniques in identifying DUBs-associated proteins.

During cDNA synthesis, total RNA was extracted using TRIzol method and produced 1090.5 ng/ μ l total RNA. Following this, purification of the total RNA produced 18.6 ng/ μ l mRNA, which is approximately 1.7% from the total RNA. At least 80% of the total RNA in mammalian cells is rRNA and approximately 15% is tRNA, thus protein-coding mRNA constitutes only around 1-5% from the total RNA (30). Based on this, we could assume that our mRNA purification is good. The 260/280 ratio at 2.06 indicated that the mRNA has high purity and quality to construct the cDNA library (31). The cDNA library was size fractionated by column chromatography to remove excess of primers, adapters and other low molecular weight DNA. The result shows average insert size is of 1.15 kb, suggesting no excess of extremely small or large fragments.

Construction of cDNA library using conventional cloning method that relies on DNA restriction and ligation enzymes has several major drawbacks (32). The most alarming is the possibility of losing whole or small cDNA fragments, especially mRNAs longer than 2 kb, and the occurrence of methylation that could affect gene expression and inhibit certain restriction enzymes (33). Therefore, in this work, a highly versatile Gateway cloning technology was adopted for constructing a full-length cDNA library to ensure the construction of cDNA libraries with high yields of representatives. Gateway cloning is considered the most effective and easy to use for its straightforward site-specific recombination instead of conventional restriction endonucleases and ligase (34). This technology provides a powerful versatile system for transferring DNA segments between vectors. It consists of a BP reaction that transfers any open reading frames (ORFs) or expression clones flanked by *attB* sites into a donor vector containing *attP* to produce an entry clone containing the gene of interest. Meanwhile, LR reaction directly transfers any entry clone containing the gene of interest flanked by Gateway *attL* recombination sites into a destination vector containing Gateway *attR* sites, creating an expression clone. To prove the efficiency of Gateway cloning, we examine the results of transferring our entry library from entry vector pDONR222 to a downstream Y2H library destination vector pDEST22. In Table II, the total clones and average clone length are not strikingly different before and after recombinant transfer, suggesting that shuttling of the libraries by Gateway recombination were efficient. Our Y2H cDNA library contained 5.2×10^6 cfu with an average insert size of 0.45 to 3.4 kb, which signified this library as of reasonably good quality and quantity.

To validate the quality of cDNA library, Y2H screen should be performed to ensure that the interacting

Table II: Comparison of cDNA library in entry and destination vectors

	Entry Library (pDONR222)	Y2H Prey Library (pDEST22)
Total clones (cfu)	8.13×10^6	5.2×10^6
Minimum length (kb)	0.3	0.45
Maximum length (kb)	2.5	3.4
Average length (kb)	0.98	1.15

proteins could be effectively isolated. To perform this task, a protein with well-described interacting partners would be the best candidate. Since OTUB1 was observed to be associated with a number of proteins (35), it is expected that a set of known OTUB1 interactors could be isolated from our cDNA library and thus, validate the quality of our library.

CONCLUSION

In this study, we confirmed that our Y2H bait vectors contain inserts with correct sequence. Based on the number of clones and average insert size, we also conclude that our cDNA prey library is in good quality and should represent human cDNA well. To validate the bait and cDNA prey library clones, transfection of the clones into competent yeast *Saccharomyces cerevisiae* strain MaV203 has been performed and Y2H experiment to screen novel OTULIN-, OTUB1- and OTUB2-interacting protein from human cDNA library is underway. Our ultimate aim is to establish PPI network for OTULIN, OTUB1 and OTUB2 that could contribute towards the completion of human interactome map.

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