

General co-expression vectors for the overexpression of heterodimeric protein complexes in *Escherichia coli*

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Abstract

We have designed and constructed a novel pair of bacterial co-expression vectors to facilitate the production of substantial amounts of recombinant multiprotein complexes for biochemical, biophysical, and structural studies. pOKD4 (kanamycin-resistant) and pOKD5 (ampicillin-resistant) are derivatives of pACYC177 cloning and pET26b expression vectors. As a result, pOKD4 and pOKD5 are T7-based expression plasmids containing the p15A origin of replication. This feature permits either pOKD4 or pOKD5 to co-exist in the same bacterial cell with most *Escherichia coli* expression vectors including the popular pET expression vectors. The pOKD4 and pOKD5 vectors have been engineered to possess exactly the same multiple cloning sites as pET26b thus allowing for the relatively easy shuttling of genes to and fro. The efficacy and versatility of this novel pair of co-expression vectors was successfully applied to the production of significant amounts of active DFF40/DFF45 heterodimeric protein complex in *E. coli* for detailed biochemical and structural studies.

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The co-expression of two cistrons in *Escherichia coli* may be achieved via either the use of a single bicistronic construct or two separate plasmids. A bicistronic construct is a single plasmid bearing two over-expressible cistrons, commonly, but not always, arranged in tandem. A bicistronic construct has the following advantages over the use of two separate plasmids. First, it is the method of choice in cases where strict control of stoichiometry is necessary to form a functional protein unit. Second, it requires the use of only one antibiotic-resistance marker for plasmid selection.

However, to construct a bicistronic expression vector, at least two or three cloning cycles are needed. In

addition, if one wants to vary, e.g., 10 constructs for each of the two co-expressed components, a total of $10 \times 10 = 100$ bicistronic constructs will have to be made. On the other hand, a total of only $10 + 10 = 20$ constructs will need to be made if two separate plasmids are used. Therefore, the relative ease with which two separate plasmids can be assembled makes an attractive alternative to bicistronic expression.

Ideally, for two plasmid-mediated co-expression, one needs to use replicon-compatible plasmids, defined as vectors that can co-exist in the same *E. coli* host cell by virtue of the mutual compatibility of their origins of replication. For example, the vector pFL260 carries the ColE1 replicon [1], which permits it to co-exist with the p15A replicon-carrying vector pFL261 in the same *E. coli* host cell [2]. In addition, an ideal replicon-compatible overexpression system requires that each plasmid carries the gene encoding a different antibiotic resistance, e.g., β -lactamase to confer ampicillin resistance and chloramphenicol acetyltransferase to confer chloramphenicol

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resistance. While it is possible to maintain two incompatible plasmids with different antibiotic markers in *E. coli* by growing the transformed cells in the presence of high concentrations of antibiotics [3–5], this approach has severe drawbacks such as rendering the growth media un conducive for viable cell growth, significant differences in plasmid copy number, and poor yields of recombinant heterodimeric protein [2,3].

There are successful accounts [2–4,6–18] of co-expression experiments in *E. coli* showing that this organism can be manipulated to assemble a functional heterodimeric protein unit in sufficient amounts for structural and biochemical studies. However, co-expression vectors with desirable cloning and expression properties are not generally available.

We have been interested in X-ray crystallographic studies of the DFF40/DFF45 complex, which is the inhibited form of nuclease DFF40. At the terminal stages of apoptosis, caspase-3 cleaves DFF45, thus releasing DFF40 to relocate into the nucleus and degrade chromosomal DNA [19]. The DFF40/DFF45 complex is a system in which co-expression is mandatory because DFF45 is a specific chaperone and inhibitor of DFF40. In the absence of co-expressed DFF45, DFF40 will not fold properly. To pursue structural studies of the DFF40/DFF45 complex, we designed and constructed general expression vectors that are compatible with most commercial bacterial expression vectors for co-expression. These vectors, pOKD4 and pOKD5, are driven by the powerful T7 promoters, contain multiple cloning sites, and have either kanamycin or ampicillin resistance, respectively.

Materials and methods

Escherichia coli host strains, plasmid vectors, and cDNAs

Escherichia coli strains DH5 α and BL21-SI (Gibco Life Sciences, USA) were used for plasmid amplification and expression, respectively. The pACYC177 vector was obtained from New England Biolabs (Beverly, MA, USA). It possesses the p15A origin of replication and carries the gene for aminoglycoside 3'-phosphotransferase (kanamycin-resistance) and for β -lactamase (ampicillin-resistance). It is a low copy number (~15/cell) plasmid. The pET21d, pET24d, and pET26b vectors were obtained from Novagen (Madison, WI, USA). Each is a medium copy number (~30/cell) plasmid that carries the ColE1-based origin of replication. The pET21d vector is ampicillin resistant whereas pET24d and pET26b are kanamycin resistant. All the restriction endonucleases and DNA modifying enzymes were obtained from New England Biolabs. Unless otherwise stated all chemical reagents were obtained from Sigma (Milwaukee, WI, USA). The oligonucleotides used in

this study were purchased from Invitrogen Life Technologies (Carlsbad, CA, USA). The restriction sites that were incorporated into the primers are underlined. All recombinant DNA manipulations were confirmed by DNA sequencing at the Cornell Bioresource Center in Ithaca, New York.

Construction of pOKD1 vector

The pACYC177 vector was digested with *Bam*HI and *Sma*I restriction enzymes to remove the 1094 bp *Bam*HI–*Sma*I fragment containing the N-terminal half of the kanamycin-resistance gene. Similarly, pET26b was digested with *Bgl*II and *Sma*I. The resulting 1461 bp *Bgl*II–*Sma*I fragment was gel purified and ligated to the pACYC177 *Bam*HI–*Sma*I backbone fragment to create pOKD1 (Fig. 1).

Construction of pOKD4 vector

To generate pOKD4, pOKD1 was digested with *Ac*I (located at 3882 and 315 bp of pACYC177) and ligated to delete the middle segment (373 bp) of the β -lactamase gene resulting in the 3934 bp pOKD3 vector (Fig. 2). A superfluous *Xho*I site (located at 1951 of pACYC177) at the 3'-end of the aminoglycoside 3'-phosphotransferase gene was mutated to *Bgl*II to create pOKD4 (3934 bp) with primers:

forward—5'-P-GAGATCTGCCGCGATTAAATTCC
AACATGG-3 and
backward—5'-P-AAGACGTTTCCCGTGAATATGG
C-3'.

Construction of pOKD5 vector

The *Xho*I restriction site in pOKD1 (located at 1951 of pACYC177) was silently mutated [20] to introduce a second *Sma*I site (first *Sma*I site located at 2225 of pACYC177) with the following primers:

forward—5'-P-GCCCGGGGCCGCGATTAAATTCC
AACATGG-3 and
backward—5'-P-AAGACGTTTCCCGTTGAATATG
GC-3'.

The mutant plasmid (Fig. 2) was digested with *Sma*I to delete 274 bp from the 3'-end of the kanamycin gene and ligated to produce pOKD2 (4033 bp). Another *Sma*I site (located at 2471 of pACYC177) within the kanamycin gene, upstream of the first *Sma*I site (located at 2225 of pACYC177) was introduced by silent mutagenesis using primers:

forward—5'-P-TCCCGGGTTGCCATTCTCACCGG
ATTCAGTCG-3 and

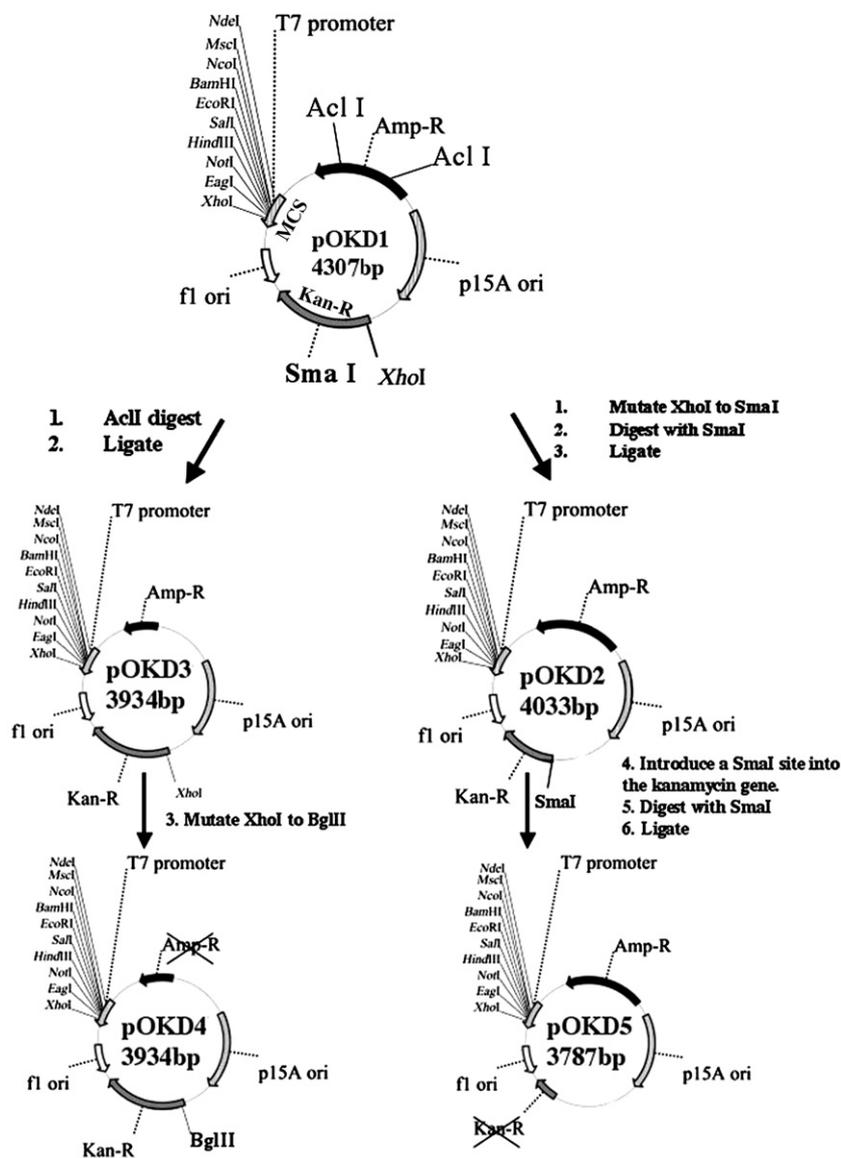


Fig. 2. Construction of pOKD4 (kanamycin-resistant) and pOKD5 (ampicillin-resistant) co-expression vectors. The pOKD1 vector was digested with *AcII* and religated to create pOKD3. This operation excised a critical, middle segment of 373 bp from the β -lactamase gene resulting in the destruction of the gene for ampicillin resistance. To generate pOKD4, a superfluous *XhoI* site in pOKD3 was silently mutated to *BglII*. The construction of pOKD5 out of pOKD1 entailed the silent mutation of an *XhoI* restriction site to introduce a *SmaI* site with the kanamycin-resistance gene. Subsequent digestion of the mutant pOKD1 plasmid with *SmaI* and religation excised 274 bp from pOKD1 to produce pOKD2. Since this operation failed to disable the kanamycin-resistance gene, another *SmaI* site was introduced into the kanamycin gene. Subsequent digestion with *SmaI* and religation led to the successful creation of pOKD5.

forward—5'-GCGGCGGGCGCATATGCTCCAGAAG
CCCAAG-3' and

backward—5'-CCGCTCGAGTCACTGGCGTTTCG
CA CAGG-3'.

Similarly, the DFF45 gene was amplified and sub cloned into pET21d and pET24d vectors as an *NcoI*–*XhoI* fragment using the following primers:

forward—5'-CATGCCATGGAGGTGACCGGGGA
CGCCGGG-3' and

backward—5'-CCGCTCGAGTGTGGGATCCTTCT
GGCTCG-3'.

Co-expression of DFF40/DFF45 using pOKD4 or pOKD5

The pair of recombinant constructs, pET21d containing DFF45 and pOKD4 containing DFF40 (or pET24d containing DFF45 and pOKD5 containing DFF40), were co-transformed into competent *E. coli* BL21-SI and plated onto LBON agar plates supplemented with ampicillin (100 μ g/ml) and kanamycin (35 μ g/ml). Transformants containing both plasmids were grown in LBON broth in the presence of ampicillin and kanamycin to an OD₆₀₀ of 0.9 at 30 °C. Protein expression was induced by the addition of a sterile solution of NaCl to a final

concentration 0.3 M and growth was continued for 25 min at 30 °C. A solution of rifampicin [21] was added to a final concentration of 200 µg/ml and growth was continued for 4 h.

The cells were harvested, resuspended in Buffer A containing phosphate-buffered saline (PBS), 20 mM imidazole, and 0.01% α -monothioglycerol (MTG) at pH 8.0, sonicated, and clarified by centrifugation. The lysate was loaded over a 5 ml HiTrap Chelating column (Pharmacia) charged with CoCl_2 and pre-equilibrated with Buffer A. The column was washed with Buffer A and eluted with a linear gradient of 0–500 mM imidazole in Buffer A. Fractions containing DFF40/DFF45 complex were pooled, concentrated, and applied to a Pharmacia Superdex-200 gel filtration column pre-equilibrated in GF buffer (20 mM Hepes–NaOH, pH 7.4, 200 mM KCl, 2.5 mM MgCl_2 , 1 mM EDTA, and 1% MTG). Homogeneous DFF40/DFF45 complex (15–20 mg) was pooled and concentrated.

Assay for DFF40 nuclease activity

Purified DFF40/DFF45 complex (10 µg) was pre-incubated with 100 ng of Granzyme B, obtained from Calbiochem (San Diego, CA, USA) at 37 °C for 20 min in a final volume of 20 µl in a buffer containing 10 mM Hepes, pH 7.4, 5 mM MgCl_2 , 50 mM NaCl, 1 mg/ml BSA, and 5 mM EGTA. Linearized plasmid DNA (3 µg for reaction 1–3 and 6 µg for control reaction 4) was added to 5 µg of the activated DFF40/DFF45 complex mixture and incubation was continued for 30 min at 37 °C. The reaction was analyzed on a 2% agarose gel for 30 min at 150 V.

Results

The criteria for an ideal *E. coli* co-expression vector include the possession of a strong promoter, a rich multiple cloning site, and a suitable antibiotic resistance. To achieve this goal, we constructed two vectors, pOKD4 with kanamycin resistance and pOKD5 with ampicillin resistance, to be used for co-expression with commercial vectors bearing either ampicillin or kanamycin resistance, respectively. Several successive steps were employed.

Introduction of the T7 promoter and a multiple cloning site into pACYC177: pOKD1

The pACYC177 cloning vector carrying the p15A replicon was used as the initial vector for the construction of general vectors for co-expression [22,23]. Because most commercial bacterial expression vectors contain ColE1-like replicon [24], derivatives of pACYC177 could co-exist in the same *E. coli* host cell

with most expression vectors, including the popular pET expression vectors. Earlier, recombinant DNA technology was used to transform pACYC177 into an expression vector equipped with the powerful T7 promoter expression system [3,25]. Here, we introduced both the T7 element and a multiple cloning site into pACYC177 by replacing its 1094 bp *Bam*HI–*Sma*I fragment with the 1461 bp *Bgl*II–*Sma*I fragment from the pET26b vector to create pOKD1 of 4307 bp. The ligation between the compatible sticky ends of *Bam*HI and *Bgl*II sites in the two fragments obliterated both the *Bam*HI and *Bgl*II sites, allowing the retention of a unique *Bam*HI restriction site from pET26b in MCS of pOKD1.

pOKD1 is resistant to both ampicillin and kanamycin. The presence of the β -lactamase gene in the pACYC177 template conferred ampicillin resistance on pOKD1. The ligation between the *Sma*I sites within the kanamycin-resistance gene of the fragments restored a full-length kanamycin-resistance gene in pOKD1. These manipulations resulted in a preliminary vector that can be used for co-expression, but further changes were required to render it as general a vector as possible.

Removal of ampicillin resistance and mutation of *Xho*I in pOKD1 to create pOKD4 with kanamycin resistance only and a MCS

To remove ampicillin resistance, pOKD1 was digested with *Ac*II (located at 3882 and 315 bp of pACYC177) and ligated to delete the middle segment (373 bp) of the β -lactamase gene resulting in a 3934 bp plasmid vector called pOKD3. Transformation and testing for antibiotic resistance showed that pOKD3 no longer retained ampicillin resistance.

To retain the uniqueness of all restriction sites within the MCS, a superfluous *Xho*I site (located at 1951 of pACYC177) at the 3'-end of the aminoglycoside 3'-phosphotransferase (kanamycin-resistance) was silently mutated to *Bgl*II to create pOKD4 (3934 bp). This vector is now the final version of our co-expression vector with kanamycin resistance.

Removal of kanamycin resistance in pOKD1 to create pOKD5 with ampicillin resistance only

To achieve the goal of constructing pOKD5, two features had to be altered in pOKD1: the removal of the *Xho*I site within the kanamycin-resistance gene and disruption of kanamycin resistance. To achieve both goals, we silently mutated the *Xho*I site in the kanamycin-resistance gene (located at 1951 of pACYC177) into a second *Sma*I site (first *Sma*I site located at 2225 of pACYC177). The mutant plasmid was digested with *Sma*I and ligated to produce pOKD2 (4033 bp). While the *Xho*I site was successfully removed in pOKD2, when this plasmid was transformed into competent DH5 α and tested for antibi-

otic resistance, the transformed cells survived on both ampicillin and kanamycin agar plates despite the deletion of 274 bp from the 3'-end of the kanamycin gene.

A further attempt was made to disable the kanamycin gene by the introduction of another *Sma*I site (located at 2471 of pACYC177) upstream of the first *Sma*I site (located at 2225 of pACYC177) within the kanamycin gene. This was achieved by silent mutagenesis, digested and ligated to create pOKD5 (3787 bp). Transformation of pOKD5 into DH5 α and subsequent testing for antibiotic resistance showed that the kanamycin gene had been successively disabled in pOKD5.

Application to the expression and purification of DFF40/DFF45 complex

The pOKD vectors were designed to take advantage of the T7 expression system which relies on the powerful

T7 promoter, high selectivity and activity of T7 RNA polymerase in *E. coli* [26]. Both pairwise combinations of expression constructs (pET24d/DFF45 plus pOKD5/DFF40) and (pET21d/DFF45 plus pOKD4/DFF40) directed protein expression in *E. coli* BL21-SI upon the addition of sodium chloride solution (Fig. 3A).

Although a comparative examination of induced and uninduced samples does not show a high level of overexpression, significant amount of the DFF40/DFF45 complex could be purified using cobalt-affinity column to allow routine yields in milligram quantities. In general, a wet mass of 3 g of *E. coli* cell pellet from 1 L culture yielded a milligram of pure DFF40/DFF45 complex after cobalt-affinity chromatography and gel filtration purification.

The DFF40/DFF45 complex obtained via co-expression is capable of degrading DNA, a test for functional activity, we subjected the DFF40/DFF45 complex to an in vitro activity assay in which Granzyme B [27] was used to cleave DFF45 thus releasing DFF40 to degrade the linearized plasmid DNA substrate (Fig. 3B).

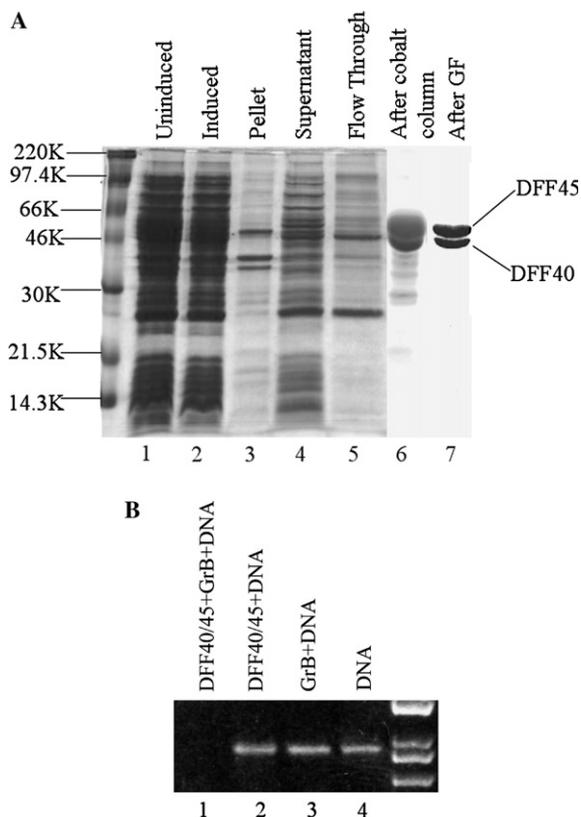


Fig. 3. Expression and activity of the DFF40/DFF45 complex. (A) Coomassie-stained 18% SDS-PAGE gel showing the co-expression of DFF40/DFF45 complex using pET24d/DFF45 plus pOKD5/DFF40 pair of vectors. The left lane contains high molecular weight (HMW) markers (Bio-Rad). Lane 1, total cell extract before induction. Lanes 2–6, total cell extract after induction, pellet, supernatant, cobalt-affinity flow through, and elution. Lane 7: a gel filtration fraction from Pharmacia Superdex-200. Similar results were obtained for the pET21d/DFF45 plus pOKD4/DFF40 pair of vectors (data not shown). (B) Nuclease activity assay shown by degradation of linearized plasmid DNA. Lane 1 contains DFF40/DFF45, Granzyme B (GrB), and DNA. Lane 2 contains DFF40/DFF45 and DNA. Lane 3 contains GrB and DNA. Lane 4 is DNA control.

Discussion

The formation of multiprotein complexes is integral to many biochemical and cellular processes. Signal transduction, cell cycle, and transcriptional regulatory pathways are a few examples where protein–protein complex formation is vital. To obtain a thorough biochemical and functional understanding of these large multiprotein complexes, it is important to elucidate their internal molecular organization in atomic detail. Although the number of detailed three-dimensional structures of proteins deposited in the PDB continues to increase inexorably [28], a comparative analysis shows that there is a dearth of atomic structures of multiprotein complexes, in spite of the increasing interest in the crystal structures of protein–protein complex structures [29–32]. This is in part due to the difficulties involved in generating significant amounts of multiprotein complexes for detailed biochemical, biophysical, and structural analysis.

Traditionally, the most popular method for generating multiprotein complexes involves in vitro reconstitution of individually expressed and purified proteins. While it works for some systems [33–36], it is not feasible for others [37]. Another in vitro reconstitution method is to express each individual component separately, mix and lyse the cell pellet, and copurify the resulting complex. In the case of the DFF40/DFF45 complex, DFF40 becomes poorly soluble and misfolded in the absence of its chaperone and inhibitor (DFF45) and therefore is impossible to obtain by either method.

The formation of multiprotein complexes via in vivo reconstitution by co-expression of individual components

within the same cell provides an attractive alternative to in vitro reconstitution. In the case of DFF40 and DFF45 where one of the individual components of the complex (DFF40) misfolds when expressed individually, co-expression provides a mechanism to generate recombinant DFF40/DFF45 heterodimeric complex by allowing DFF45 to specifically chaperone the folding of DFF40. As a general advantage, the cellular environment has molecular chaperones that may shepherd the folding of the co-expressed polypeptide into a complex by preventing aggregation of nascent polypeptides [38,39]. Even in cases where individual subunits are readily overexpressed and properly folded significant non-specific aggregation may exist within each protein to inhibit heterodimeric interactions, which may be overcome by co-expression. In addition co-expression often offers simplicity of purification and improved efficiency of assembly compared to in vitro reconstitution.

Although there are successful accounts of co-expression experiments in *E. coli*, we are yet to have the co-expression equivalent of versatile, general-purpose, monocistronic expression vectors like the pET (Novagen), pGEX (Pharmacia), pQE (Qiagen), and other popular *E. coli* expression vectors. The development of an efficient and versatile bacterial co-expression system can play an integral role in generating significant quantities of multiprotein complexes in the age of structural genomics [40,41].

Ideally, a useful and versatile, general-purpose bacterial co-expression system should consist of a pair of vectors that have compatible origins of replication to prevent segregation and ultimate loss of one plasmid in culture [24]. For the purposes of selection, it is important that each vector possesses a different antibiotic-resistance gene. Also, the co-expression system should be based on a strong promoter such as the T7 promoter [26]. To facilitate cloning procedures, both vectors should have a wide array of unique restriction sites in their multiple cloning sites (MCS). Both vectors should have similar copy numbers so that the level of expression could be stoichiometrically balanced.

Of the handful of reports [25,37,42–45] describing general-purpose vectors for bacterial co-expression, the pOKD system remains one of the most versatile and superior for the following reasons:

- (a) *Escherichia coli* host: an *E. coli* expression system was chosen over other systems because of the ease of genetic manipulation, efficient transformation, and fast rate of growth, ease of scaling up, e.g., for fermentation and comparatively low cost. Comparatively, a bacterial expression host system is the most convenient, affordable, and popular recombinant expression system [46].
- (b) T7 expression system: the pOKD vectors were designed to take advantage of the T7 expression

system which relies on the powerful T7 promoter and the high processivity and activity of T7 RNA polymerase in *E. coli*

- (c) Wide applicability: this pair of co-expression vectors with unique antibiotic-resistance genes allows for the use of the pOKD vectors in conjunction with a wide variety of other popular expression vectors such as the pET, pGEX, and pQE *E. coli* expression vectors.
- (d) Versatility: the presence of a multiple cloning site (MCS) containing a wide array of unique restriction enzymes in the pOKD vectors allows for the relatively easy shuffling of genes to and fro without tedious re-cloning steps.
- (e) Size: the relatively small sizes (<4kb) of the pOKD vectors confer increased plasmid stability.

The technique described here for constructing the pOKD vectors is general enough to allow for the conversion of other pET vectors (Novagen) such as pET28a to an efficient co-expression vector [25]. This points to the versatility and general applicability of this method that could potentially allow for the generation of a pOKD line of co-expression vectors to complement the pET, pGEX, pQE, and other popular *E. coli* expression vectors. This would in turn pave the way for the routine production of significant amounts of recombinant multiprotein complexes for structural studies.

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