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A single step three-strain *in vivo* Gateway reaction

Aaron Nicholas Gillman [a,b,*], Alexandra Helleux [a], Sören Abel [a,b,c,d]

[a] Department of Pharmacy, Faculty of Health Sciences, UiT - The Arctic University of Norway, 9037 Tromsø, Norway
[b] Department of Veterinary and Biomedical Sciences, The Pennsylvania State University, University Park, PA 16802, USA
[c] Centre for Molecular Medicine Norway, Nordic EMBL Partnership, 0318 Oslo, Norway
[d] Huck Institutes of the Life Sciences, The Pennsylvania State University, University Park, PA 16802, USA

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ABSTRACT

We developed a simplified, highly efficient Gateway reaction that recombines target DNA to expression (destination) plasmids *in vivo* and subsequently conjugates the final vector into a recipient strain, all in a single step. This recipient strain does not need to contain any selective marker and can be freely chosen as long as it is sensitive to *ccdB* counterselection and can be targeted by the RP4α conjugation system. Our protocol is simple, robust, and cost effective. It works in 96-well plate format and performs across a range of temperatures. We designed modular, minimal destination vectors containing a modified Gateway insert to ease vector design by providing locations for insertion of tags, promoters, or conjugations. To demonstrate the utility of our system, we created destination vectors with split adenylate cyclase tags for bacterial two-hybrid (B2H) studies and screened a library of diguanylate cyclases for protein-protein interactions in a single step.

1. Introduction

Gateway cloning technology was developed by Invitrogen in the 1990s to enable fast and reliable (sub-) cloning by site directed recombination (Hartley et al., 2000). Gateway technology relies on the specific recombination of *att* sites on the DNA catalyzed by BP or LR Clonase enzymes, which enable translocation of genetic elements between DNA strands based on conserved sequences (Liang et al., 2013). Most applications of this technology rely on the use of purified enzymes. House et al., described a set of engineered bacterial strains that eliminate the need for purified enzymes and are able to recombine DNA between plasmids by an elegant combination of bacterial conjugation, *in vivo* recombination and selectable as well as counterselectable markers (House et al., 2004). In this work, we have created a simplified and versatile *in vivo* Gateway system that can produce the recombined destination vector in a recipient strain in a single step following library construction. We have reduced the number of bacterial strains necessary to perform the recombination and, importantly, use auxotrophic markers that enable us (largely) free choice of the final strain from which the recombined vector can be recovered.

To expand on current *in vivo* Gateway technologies, we developed a 3-strain *in vivo* Gateway system consisting of a donor strain, worker strain, and helper strain to perform the Gateway reaction and subsequent transfer of the recombined plasmid into a recipient strain in a single-step reaction (Fig. 1). The reaction consists of the donor strain transferring the donor vector into the worker strain, which contains the destination vector and the excision and integrase enzymes expressed by an inducible promoter. The LR clonase reaction occurs within the worker strain and the final plasmid containing the gene within the destination vector is transferred into the recipient strain. The recipient strain with the final vector is selected for by loss of *ccdB*, the plasmid selectable marker, and the recipient strain selectable marker or positive selection against the reaction strains. To aid in selection, we developed the system within the diaminopimelic acid dependent *Escherichia coli* MFD*pir* strain, allowing positive selection and thus removing the requirement of a unique selection marker in the recipient strain. This includes an *E. coli* MFD*pir* CcdB-resistant strain. We tested the system utilizing the CcdB resistant *E. coli* strain DB3.1 as the worker strain, which significantly increased reaction efficiency, but requires a unique selectable marker in the recipient strain.

In designing the system, we constructed modular minimal destination vectors that allow easy insertion of Gateway constructs. The minimal destination vectors were created with an RP4 origin of transfer and compatible origins of replication (p15a or pBM1) with antibiotic

* Corresponding author at: Department of Pharmacy, Faculty of Health Sciences, UiT - The Arctic University of Norway, 9037 Tromsø, Norway.
E-mail address: Gillman.aaron.n@gmail.com (A.N. Gillman).

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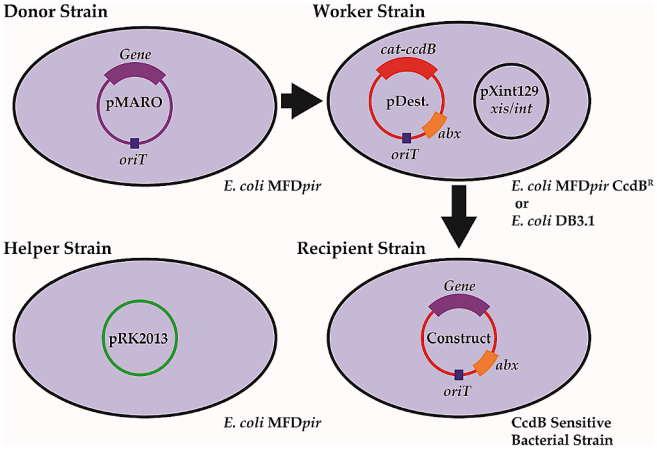


Fig. 1. Schematic of *in vivo* Gateway reaction. The donor vector pMARO containing the gene or DNA of interest is transferred *via* conjugation to the working strain containing the customizable destination vector pDest (pANG01-03 or pAH01-08) that contains a unique antibiotic marker (*abx*). The helper strain with the pRK2013 plasmid aids in conjugation between strains. Gateway excision (*xis*) and integrase (*int*) enzymes expressed by pXint129 in the working strain and facilitate transfer of the gene from the donor vector into the destination vector. At the same time, the *cat-ccdB* cassette is transferred to the donor vector. The final construct enters the recipient strain by conjugation. After selection on media without diaminopimelic acid and with antibiotics, the auxotroph *E. coli* MFD*pir* strains die and only the recipient strain containing the destination plasmid and its antibiotic resistance gene but lacking the *ccdB* gene can survive. If using *E. coli* DB3.1 as a worker strain a unique counterselectable marker is required within the recipient strain.

resistance markers encoding ampicillin (*bla*), gentamycin (*aacC1*), or tetracycline (*tetA, tetR*) resistance to create pANG01, pANG02, and pANG03, respectively. Each minimal destination vector includes a modified Gateway cassette consisting of the site-specific recombination sites (*attR*1, *attR*2), a chloramphenicol resistance gene (*cat*) and a counterselectable marker (*ccdB*). The cassette is flanked by unique restriction sites (*Pme*I-*attR1*-*cat*-*ccdB*-*attR2*-*Pac*I) to allow for easy insertion of C- and N-terminal tags. Our destination vectors were paired with the donor vector pMARO, a pMK2010 derived plasmid, containing the same RP4 origin of transfer (*oriT*), kanamycin resistance (*aph*(3′)) and the pBR332 origin of replication (House et al., 2004). To facilitate the Gateway reaction, we utilized the vector pXint129 encoding the excision (*xis*) and integrase (*int*) genes, kanamycin resistance, and no origin of transfer (Platt et al., 2000). The plasmid pRK2013 contains the conjugation machinery to facilitate the transfer of plasmids containing the RP4 *oriT* (Flgurski and Helinski, 1979).

The system developed offers several improvements to the Gateway system. Our scaled down reaction is performable in micro centrifuge tubes or in a 96-well plate over a range of temperatures and conditions without requiring a unique selectable marker in the recipient strain. The modular nature of our designed vectors allows for the easy incorporation of tags, promoters, or conjugations to the gene of interest. The reaction also allows for selection of the final vector construct in the recipient strain of choice, assuming it is CcdB sensitive, in a single step. We demonstrate the utility of this system by designing a bacterial 2-hybrid system and screen several genes using sequential *in vivo* Gateway reactions to incorporate multiple vectors into a reporter strain to screen for protein-protein interactions.

2. Material and methods

2.1. Vector construction

The minimal destination vectors were constructed by PCR amplification then Gibson assembly of PCR fragments (Gibson et al., 2009). pANG01 (GenBank OK571346) was constructed with the *bla* from pGP704, $oriT_{RP4}$ from pG704, and the $oriV_{p15a}$ from pBAD33 (Guzman et al., 1995; Miller and Mekalanos, 1988). pANG02 (GenBank OK571347) was constructed with the *aacC* from a pGP704-gent^R construct, $oriT_{RP4}$ from pG704, and $oriV_{pBM1}$ from pET28b (Sigma, Cat. 69865). The vectors were constructed to introduce unique *Ecor*V, *Spe*I, and *Xho*I sites between fragments (Table S2, Supp. Methods). pANG03 (GenBank OK571348) was constructed by replacing the *bla* resistance gene from pANG01 with *tetA-tetR* from pRK415 (Keen et al., 1988). The Gateway sequence was synthesized based off the commercial vector pDONR201 (Invitrogen) and inserted into the *Ecor*V sites of minimal vectors by Gibson assembly to include unique *Pme*I and *Pac*I restriction sites flanking the *att* sites (Section 3, Supp. Methods). Bacterial 2-hybrid T25 and T18 along with the lactose promoter (*plac*) and terminator sequences were incorporated in the *Pme*I and *Pac*I sites following restriction enzyme digestion then Gibson assembly using the commercial vectors pKT25, pKNT25, pUT18, and pUT18C (Euromedex, EUP-25N/25C/18N/18C) to construct the plasmids pAH01-08 (Table S3, Supp. Methods). The donor vector pMARO (GenBank OK571349) was constructed from pMK2010 by insertion of the *tetA-tetR* cassette flanked by *Ecor*V sites between the *attP* sites by the BP clonase reaction. This allows for creation of entry vectors either by restriction digest with *Ecor*V to linearize the vector then using blunt end ligation or Gibson Assembly to insert the gene of interest, or by BP clonase reaction of pMK2010. The *tetA-tetR* cassette is also cut by *Ecor*V, which reduces background and allowing ligation by Gibson Assembly without subsequent purification steps. Sequences are listed in supplementary methods.

2.2. Strains

The *E. coli* strain MFD*pir* was obtained from Andrea Moell (Boston, MA) and *E. coli* DB3.1 obtained commercially (Invitrogen, 11782-018) (Ferrieres et al., 2010). In this study, a λ-*pir* variation of DB3.1 *E. coli* was used to complement *E. coli* MFD*pir* so that all strains could support plasmid containing R6K origins of replication if required. Use of λ-*pir* variants of *E. coli* DB3.1 or MFD should not be required for plasmids not dependent on λ-*pir*. MFD*pir* was made CcdB resistant by transformation with the *ccdB* containing pANG01 and confirmed by curing and retransformation with other destination vectors. Rifampicin resistant recipient strains *E. coli* DH5α (NEB, C2987) and *E. coli* BTH101 (Euromedex, EUB001) were made by serial plating on LB agar containing rifampicin (50 μg/ml) until resistant colonies arose.

2.3. In vivo gateway reaction

All strains are grown separately with corresponding antibiotics resistance encoded on the plasmid(s) in LB media (Sigma, L3022 or L2897) with the donor strain carrying pMARO using kanamycin (50 μg/ml), the worker strain carrying the destination vector using carbenicillin (50 μg/ml), gentamycin (20 μg/ml), or tetracycline (12 μg/ml) in combination with kanamycin (50 μg/ml), and the helper strain carrying pRK2013 using kanamycin (50 μg/ml). When grown, *E. coli* MFD*pir* is supplemented with diaminopimelic acid (57 μg/ml). The three *in vivo* Gateway strains: donor, worker, and helper along with the recipient strain are grown overnight in LB broth containing the corresponding antibiotics. Cultures are pelleted and decanted, washed twice and suspended in equal volumes of phosphate buffered saline (PBS). Equal volumes of each culture (125 μl for micro centrifuge tubes, 50 μl for 96-well plates) are combined and concentrated 10-fold by pelleting and resuspension in PBS. The concentrated suspension is placed as a single droplet onto a 0.45 μm MCE membrane (Millipore, HAWP04700) on top of LB agar containing diaminopimelic acid (57 μg/ml) and 1 mM isopropyl β-d-1-thiogalactopyranoside (IPTG) and incubated at 37 °C for 20–24 h with the filter side facing up. Following incubation, the filter is placed in a 12 ml conical test tube containing 1 ml PBS and vortexed for 30 s to suspend the pellet. The suspension is serial diluted in PBS and

plated for single colonies on LB agar containing the destination vector antibiotic without diaminopimelic acid and antibiotics selecting only for the recipient strain are incorporated (*e.g.* rifampicin) if using *E. coli* DB3.1 as the worker strain. Single colonies typically appear with a 1:100 dilution with DB3.1, and with a 1:10 dilution using an MFD*pir* worker strain.

2.4. Bacterial 2-hybrid

For bacterial 2-hybrid assays, the adenylate cyclase deficient reporter *E. coli* strain BTH101 (F-, *cya-99*, *araD139, galE15, galK16, rpsL1 (Str r)*, *hsdR2, mcrA1, mcrB1, Rif r*) was used as the recipient strain for the *in vivo* Gateway reaction. A paired set of bacterial 2-hybrid vectors pAH01-04 derived from pANG01 backbone or pAH05-08 derived from pANG02 backbone were used for screening. *In vivo* Gateway reactions were performed in two steps, incorporating each pair of plasmids and genes sequentially. For heterodimer screening *E. coli* BTH101 pAH04-*VC2697* was first constructed and used as a recipient strain for all tested genes cloned into pAH06. After construction, interactions were screened by growing the BTH101 pAH04-*VC2697* and pAH06-*Gene* combinations in LB containing carbenicillin (50 μg/ml), gentamicin (20 μg/ml), and 1 mM IPTG overnight at 37 °C with shaking. Overnight cultures were then spot plated (3 μl) on MacConkey agar (Sigma, M7408) supplemented with 2% maltose, carbenicillin (50 μg/ml), gentamicin (20 μg/ml), and 1 mM IPTG and grown at 30 °C for 24–48 h.

3. Results and discussion

To test the maximum reaction efficiency of the *in vivo* Gateway system, we incorporated the tetracycline resistance cassette (*tetA-tetR*) into pMK2010, creating pMARO. By selecting for the recipient strain utilizing the destination vector resistance (carbenicillin) and tetracycline, we select for both the loss of *ccdB* and the complete insert transferring correctly. This is expected to reduce the background of incorrect inserts or loss of CcdB activity and allow us to better estimate the maximum efficiency of the reaction.

The *in vivo* Gateway reaction was tested using scalable benchtop conditions. The donor and helper strains are replaceable with *E. coli* MFD*pir* and still maintain high reaction efficiency while utilizing *E. coli* DB3.1 as a worker strain (Fig. 2A). Replacing the worker strain with the CcdB resistant *E. coli* MFD*pir* still functions, albeit significantly reducing reaction efficiency (45 Colony Forming Units (CFU)/Reaction, 22–68 95% CI). *E. coli* MFD*pir* is diaminopimelic acid dependent by deletion of *dapA*. Using only the auxotrophic *E. coli* MFD*pir* strains removes the need for negative selection against the donor, worker, and helper strains in the recipient strain. Decreasing the temperature reduces the reaction efficiency (Fig. 2B), but still produces reasonable colony counts across 20–37 °C, allowing for temperature sensitive plasmids or strains as destination vectors or recipient strains. We tested the effect of bacterial density during the Gateway reaction. We concentrated increasing volumes of the same overnight cultures, re-suspended cells in the same small volume and placed on the filter covered agar plate. Unexpectedly, we observed decreased reaction efficiency with increased cell volume, *i.e.* increased numbers of cell and increased bacterial densities on the filter (Fig. 2C). Additionally, when the reaction was performed in 96-well plates with 50 μl volumes, we did not observe a significant difference between 125 μl culture volumes in micro centrifuge tubes (Fig. 2D). Overall, the reaction performed equally well in micro centrifuge tubes or 96 well plates, allowing for scalable benchtop reactions.

Transfer of the tetracycline resistance cassette into a CcdB sensitive strain determined the maximum reaction yield, with selection for both the gene to transfer intact and in the correct backbone. However, when the insert is not selectable, colonies could result from other ways that disrupt *ccdB* function (*e.g.* spontaneous non-sense mutations). To address this possibility, three non-selectable cyclic-di-guanosine monophosphate (*c*-di-GMP) producing genes from *Vibrio cholerae* were chosen

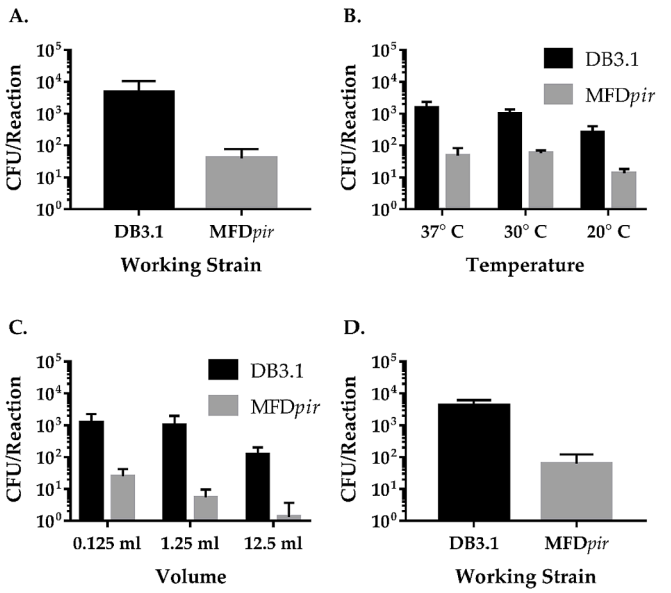
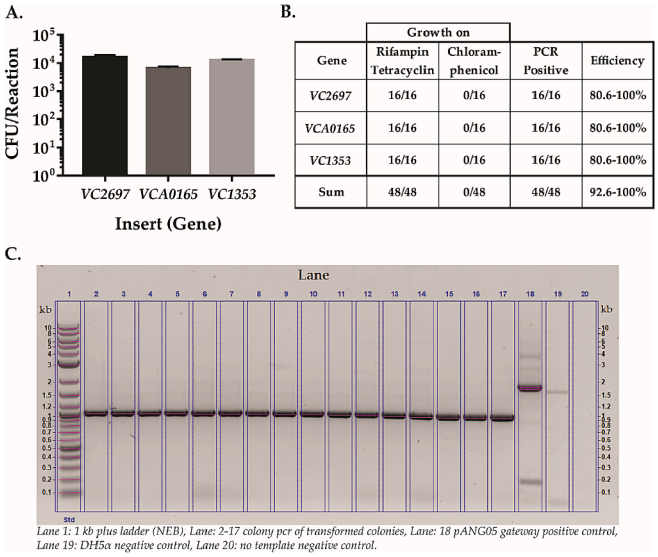


Fig. 2. *In vivo* Gateway reaction efficiency. *In vivo* Gateway reaction measuring the efficiency of transfer of the tetracycline-resistance cassette from pMARO to the carbenicillin-resistant destination plasmid pANG01 and transfer to the final recipient strain *E. coli* DH5α. (A) Total CFU of recipient cells containing the recombined final plasmids per reaction with DB3.1 or *E. coli* MFD*pir* as the worker strain. The reactions were performed in microcentrifuge tubes with 0.125 ml culture volume. The effect of (B) temperature and (C) amount of overnight culture on reaction efficiency. (D) Efficiency of reaction in 96-well plate with 50 μl culture volumes. Recipient cells with recombined plasmids were quantified after selection on tetracycline and carbenicillin containing media. Volumes represent amount of each culture component before concentration. All data shows mean ± std. with $n \geq 3$.

to test the reaction in the destination vector pANG03. The plate counts for the recipient strain following the *in vivo* Gateway reaction with the DB3.1 worker strain were similar for the selectable and non-selectable inserts (Fig. 3A, Range 6.4×10^3–1.6×10^4). From each reaction, 16 colonies were picked and patched onto LB agar containing rifampicin (50 μg/ml) and tetracycline (12 μg/ml) for growth to confirm presence of the destination vector. Lack of growth on LB agar containing chloramphenicol (20 μg/ml) confirmed loss of Gateway cassette (Fig. 3B). Colony PCR (Supp. Methods) was used to determine the insert size, and in all cases (48/48) was determined to be the correct size (Fig. 3B, C). Overall the reaction efficacy was determined to be 92.6–100% (95% CI, Wilson's Method), which means only a handful of colonies need to be screened to find a positive clone.

To test the applicability of the *in vivo* Gateway system, we developed bacterial 2-hybrid destination vectors. The bacterial 2-hybrid system relies on the dimerization of proteins tagged with the T18 and T25 fragments of the *Bordetella pertussis* adenylate cyclase to restore cAMP production in *cya* deficient strains (Karimova et al., 1998). Reconstitution of cAMP production activates catabolic operons restoring maltose and lactose fermentation, which is measurable by acidification of MacConkey media or by blue-white screening in X-gal containing media (Karimova et al., 1998). The minimal vectors pANG01–02 (Fig. 4A) were used to generate N-terminal and C-terminal constructs containing the T18 and T25 fragments (Fig. 4B). The general design of the bacterial 2-hybrid Gateway insert is the T18 or T25 fragment inserted in frame with the *att* sites and gene with the start and stop codon removed and expression under control of *plac*. Short sequences (3–6 amino acids) were added to the opposite terminal of the T18 or T25 tag to incorporate start or stop codons in frame with the *att* sites. The bacterial 2-hybrid Gateway fragments were inserted at the *Ecor*V site of pANG01-02 creating paired C- and N-terminal constructs of the T18 and T25 fragments (Fig. 4C). Following construction of the destination vectors,

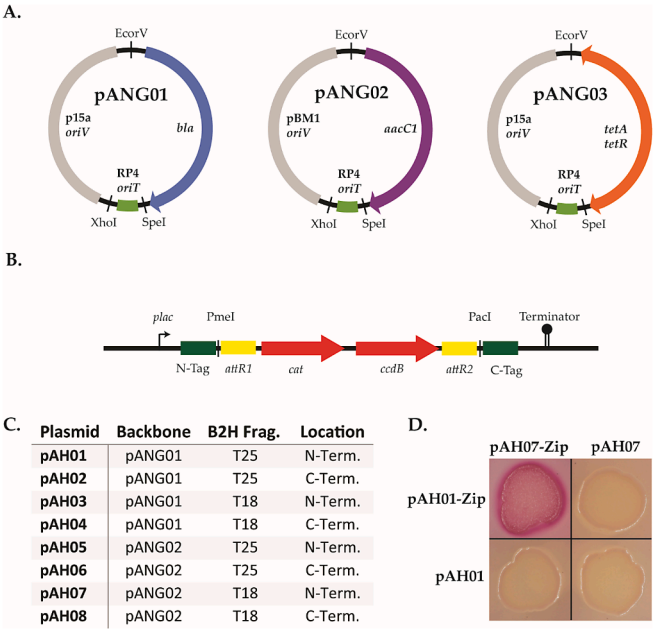


Gene	Growth on Rifampin Tetracyclin	Growth on Chloram-phenicol	PCR Positive	Efficiency
VC2697	16/16	0/16	16/16	80.6-100%
VCA0165	16/16	0/16	16/16	80.6-100%
VC1353	16/16	0/16	16/16	80.6-100%
Sum	48/48	0/48	48/48	92.6-100%

Fig. 3. Reaction efficiency across genes. (A) Total final recombinants per reaction with working strain *E. coli* DB3.1 for test genes from *Vibrio cholerae* showing mean ± std. with $n = 3$. (B) Results of pick and patch for growth on recipient strain selection (rifampicin) and plasmid backbone selection (tetracycline), and loss of Gateway cassette selection (chloramphenicol). PCR positive denotes pick and patch colonies positive for correct sized insert. Efficiency is 95% confidence interval (Wilson's method). (C) Representative image of results for gene *VC2697* showing inverted color image with overlay of 1% TAE gel for colony PCR results for the insert of gene *VC2697* (Lanes 2–17, 1108 bp expected) and non-transformed destination plasmid (Lane 18, 1756 bp expected). Lane 1 contains 1 kb plus ladder (NEB) with lane 19 containing DH5α colony PCR negative control and lane 20 with no template.

leucine zipper positive controls were cloned into the destination vectors in sequential 2-step *in vivo* Gateway reaction into the recipient strain *E. coli* BTH101. Leucine zippers self-dimerize with a non-covalent bond to bring together T18 and T25 fragments to restore cAMP production (Karimova et al., 1998). The 2-step *in vivo* Gateway constructs the first recombined destination vector into an empty recipient strain, and then uses the created strain with plasmid as the recipient strain for creation of a complimentary plasmid (Supp. Methods). All paired T18 and T25 tagged leucine zipper positive controls were constructed with corresponding negative controls: pAH01/07, pAH02/08, pAH03/05, and pAH04/06. All leucine zipper pairs showed positive colorimetric results and developed red colonies on MacCokeny reporter agar and no color development when tested against empty backbones (Fig. 4D).

After development of the bacterial 2-hybrid destination vectors, we screened a library of diguanylate cyclases, a class of proteins known to dimerize, for self- and hetero-interactions (Abel et al., 2011). Typically, diguanylate cyclases have N-terminal interaction domains and GGDEF output domains that harbor the enzymatic activity. We chose the destination vector pair pAH04/06 to fuse the T18/25 fragment to the C-terminus of the diguanylate cyclases, *i.e.* the enzymatic domains least likely to impair the dimerization (Chan et al., 2004). We first screened genes in pAH04/06 for self-interaction using 2-step *in vivo* Gateway and identified *VC2697* dimerization on MacConkey indicator media (Fig. 5A). Once *VC2697* was identified to self-dimerize, it was tested for interaction with a selection of similarly structured GGDEF containing diguanylate cyclases (Fig. 5B). Using a single step *in vivo* Gateway reaction, 10 genes were simultaneously transformed into pAH06 with *E. coli* BTH101 containing pAH04-*VC2697* as the recipient strain. Colonies from the reaction were screened on MacConkey indicator media and *VC1376* was found to specifically interact with *VC2697* (Fig. 5B–D).



Plasmid	Backbone	B2H Frag.	Location
pAH01	pANG01	T25	N-Term.
pAH02	pANG01	T25	C-Term.
pAH03	pANG01	T18	N-Term.
pAH04	pANG01	T18	C-Term.
pAH05	pANG02	T25	N-Term.
pAH06	pANG02	T25	C-Term.
pAH07	pANG02	T18	N-Term.
pAH08	pANG02	T18	C-Term.

Fig. 4. Incorporation of bacterial 2-hybrid into the *in vivo* Gateway system. (A) Shows the general plasmid backbone used for construction of the destination vectors and unique restriction sites. (B) The general map of the Gateway system incorporated into the *Ecor*V site. (C) Plasmid list of bacterial 2-hybrid Gateway vectors tested in pairs with the same terminal location of corresponding T25 and T18 tags. (D) Representative image of *E. coli* strain BTH101 grown on indicator media with insertion of leucine zipper by *in vivo* Gateway into N-terminal bacterial 2-hybrid vectors. Development of a red color indicates acidification of the media *via* maltose formation from restoration of adenylate cyclase activity. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

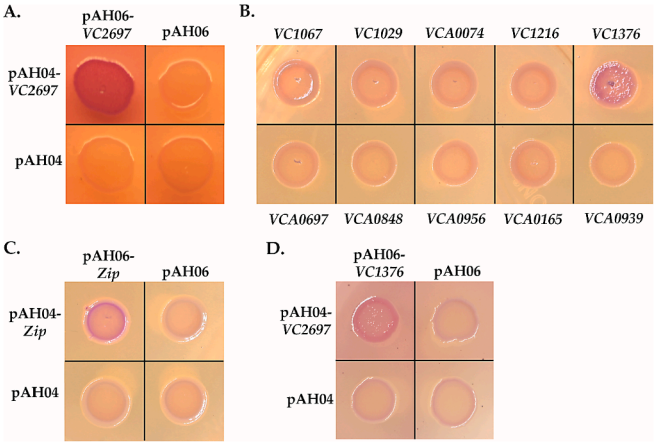


Fig. 5. Applying *in vivo* Gateway bacterial 2-hybrid system experimentally. (A) Screening for self-interaction utilizing 2-step *in vivo* Gateway reaction to create pAH04/pAH06 combinations of paired genes. Image shows positive results for self-interaction of VC2697. Grid denotes combinations of plasmids. (B) Screening of pAH04-*VC2697* against indicated gene in pAH06. A positive interaction with VC1376 is observed. (C) Positive and negative controls for pAH04 and pAH06 containing leucine zipper with grid denoting combinations of plasmids. (D) Conformation of VC2697 and VC1376 specific interaction with grid denoting combinations of plasmids. All data shows growth of plasmids in *E. coli* BTH101 on MacConkey indicator media. Development of a red color indicates acidification of the media *via* maltose formation from restoration of adenylate cyclase activity. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

4. Conclusions

In this study, we developed a simplified *in vivo* Gateway system with advantages over commercially available BP/LR Clonase reaction kits. Our method removes the requirement of the LR clonase and provides cheaper alternatives for the BP clonase reaction, *e.g.* blunt end ligation or Gibson assembly. In large-scale assays, purified enzymes can be responsible for a large portion of costs and our system only requires bacterial growth media and antibiotics to perform the Gateway reaction following library construction. Our system also provides a modular framework for construction of destination vectors. Each vector is constructed so that the origin of replication or resistance marker can easily be swapped out. Additionally, our modified Gateway cassette provides unique restriction sites directly flanking the *attR1* and *attR2* sequences, allowing for easy insertion of tags, promoters, or conjugations to the gene of interest. Like other tools, this system is not a replacement but rather an alternative approach with benefits. Where our system could provide the most benefit is in large-scale screenings such as bacterial 2-hybrid or when cloning genes into a library of destination vectors.

In summary, we developed a simplified *in vivo* Gateway system with modular customizable destination vectors. The system was developed for a range of laboratory conditions, and optimized for individual or scalable 96-well plate reactions. We have demonstrate the application of our system by incorporating the bacterial 2-hybrid into Gateway and screening a library of genes against a test gene in a single-step reaction. Combined, our system offers a new tool for the Gateway system to stream line cloning and transformation, and save on reagent costs.

Plasmid availability

The plasmids pANG01 (AddGene, 178083), pANG02 (AddGene, 178084), and pANG03 (AddGene, 178085), pMARO (AddGene, 178086) and pXint129 (AddGene, 178208) were deposited by us with AddGene. The plasmid pXint129 was deposited by us on behalf of Dr. Gregory J. Philips with his permission. The plasmid pRK2013 is commercially available from ATCC (Cat. 37159). The plasmids used to construct pAH01-08 are available as a commercial product "BATCH System Kit: Bacterial Adenylate Cyclase Two-Hybrid System Kit" from Euromedex (Cat. EUK001) with the primers listed in supplementary methods(Table S3).

Declaration of interests

The authors have no finical interest or conflicts of interest to declare.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.plasmid.2021.102608.

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