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Efficient cloning of linear DNA inserts (ECOLI) into plasmids using site-directed mutagenesis

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This study introduces a novel cost-effective technique for cloning of linear DNA plasmid inserts, aiming to address the associated expenses linked with popular in vitro DNA assembly methods. Specifically, we introduce ECOLI (Efficient Cloning Of Linear Inserts), a method utilizing a PCR product-based site-directed mutagenesis. In comparison to other established in vitro DNA assembly methods, our approach is without the need for costly synthesis or specialized kits for recombination or restriction sites. ECOLI offers a fast, efficient, and economical alternative for cloning inserts up to several hundred nucleotides into plasmid constructs, thus enhancing cloning accessibility and efficiency. This method can enhance molecular biology research, as we briefly demonstrated on the *Dishevelled* gene from the WNT signaling pathway.

Keywords In vitro DNA assembly, DNA cloning, Mutagenesis, PCR, Plasmid-based cloning, Site-directed mutagenesis, *Dishevelled*

Abbreviations

CM	Conditioned medium
CPEC	Circular polymerase extension cloning
DEP	Dishevelled, Egl-10, and Pleckstrin domain
DMEM	Dulbecco's modified eagle medium
Dvl/DVL	Dishevelled/DISHEVELLED
ECFP	Enhanced cyan fluorescent protein
EFC	Enzyme-free cloning
ECOLI	Efficient cloning of linear inserts
FZD	FRIZZLED
HEK293	Human embryonic kidney 293
KO	knock out
LB	Lysogeny Broth
LGK	Porcupine Inhibitor for WNT Secretion
OE	Overexpression
RE	Restriction enzymes
RSpo	R-Spondin
TKO	Triple knock-out
WNT	Wingless/integrated
WB	Western Blot

In the last fifty years, the advent of DNA cloning has significantly advanced our understanding of the life sciences[1–3]. The utilization of plasmid DNA, particularly in applications such as DNA transfection[4–6], mRNA synthesis[7] and mRNA microinjection[8], together with the CRISPR/Cas9 method[9,10], has proven invaluable for studying intricate biological processes. Various cloning approaches, including gBlock synthesis (Integrated DNA Technologies, USA), Hot Fusion cloning[11], In-Fusion cloning (Takara Bio, Japan), Gibson assembly (Telesis Bio, USA), or Gateway cloning (Thermo Fisher Scientific, USA) have been employed for creating and subcloning DNA inserts (Suppl. Table 1). Despite their effectiveness, these methods can be associated with certain drawbacks, such as high financial investments for the cloning services, the necessity of specialized and often expensive kits and constructs, or reliance on specific restriction sites that may not be compatible with the target plasmid.

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To address these limitations, our study introduces a novel in vitro DNA assembly method that is both cost-effective and straightforward, enabling the creation and subcloning of new plasmids without the need for specialized kits, tools or restriction sites. Traditionally, in vitro DNA assembly methods have been categorized into three main classes: (i) Restriction Enzyme-Mediated Methods such as gBlocks or Golden gate, (ii) Recombination-Based Assembly Methods, such as Gibson assembly and CEPC, and (iii) Enzyme-Independent DNA Assembly such as EFC (for more information about these methods, we refer a reader to the great review in[12]). However, our ECOLI technique represents a novel approach, as it is restriction enzyme-free, not based on recombination, and does require enzymes like polymerases. Such a method has not been previously published.

Specifically, the ECOLI technique involves the design of two primers, PCR amplification, and a PCR product clean-up, together with a standard site-directed mutagenesis reaction, all accomplished without the need for uncommon laboratory kits (Suppl. Fig. 1). Using our method, researchers can successfully generate their desired plasmids with inserts of interest, complete with expression and validation, within just few days, if also including primer synthesis.

In this study, we demonstrate the effectiveness of the ECOLI method by cloning linear inserts into plasmid constructs containing the gene encoding the Dishevelled (Dvl/DVL) protein from the Wnt signaling pathway[13–15]. We confirmed the functionality of these constructs through standard readouts, including immunofluorescence assays showing membrane recruitment and dual luciferase assays. In conclusion, the ECOLI approach offers affordable plasmid DNA insertion without specialized kits, providing thus a novel efficient alternative to current cloning techniques.

Materials and methods

Primer design

The mutations, i.e., insertions were conducted in the pDONR221 donor Gateway (Thermo Fisher Scientific, USA) with the human *Dishevelled3* gene (*hDVL3*, aa 1–716) (DNASU, plasmid no. HsCD00040582) and the pcDNA3-Flag plasmid with the gene encoding mouse *Dishevelled1* (*mDvl1*, aa 1–695)[16], lacking its C-terminal DEP domain (aa Δ425–499) (see Data availability section). DNA insertions of genes encoding the ECFP tag and the DEP domain were performed to demonstrate the efficiency of the ECOLI process. Two primers, forward and reverse, were designed for each mutation, and the primer sequences are provided in Table 1 below. Primer design principles are explained in the results section and illustrated in Fig. 1.

PCR reaction

The PCR mixture, totaling 50 µL, comprised 10 µL of 5X SuperFi ™ Buffer (Invitrogen, #12351010), along with 1 µL of 10 mM dNTP mix (0.2 mM each), 2.5 µL of 10 µM forward and reverse primers (final concentration 0.5 µM), and 100 ng of template DNA. Amplification was conducted using 1 U of Platinum™ SuperFi™ DNA Polymerase (Invitrogen). The process commenced with an initial denaturation step at 98 °C 30 s, followed by 35 cycles utilizing the following parameters: denaturation at 98 °C for 10 s, annealing at 68 °C for 10 s, and extension at 72 °C for 1 min. Subsequently, a final extension step at 72 °C for 5 min was performed, after which the mixture was maintained at 4 °C until further procedures. For this PCR reaction, we recommend using any proof-reading polymerase.

DNA isolation of the PCR product was achieved using the GeneJet Gel Extraction Kit (Thermo Scientific), and the integrity of the isolated DNA was assessed through standard 1.5% agarose gel electrophoresis and the NanoDrop 2000 spectrophotometer (ThermoFisher Scientific, USA). An example about the quality and characteristics of the cleaned PCR product shown on the hDVL3 DEP domain is provided in Suppl. Fig. 2.

Site-directed mutagenesis reaction

Mutagenesis reactions were performed either using the QuikChange II XL Site-Directed Mutagenesis Kit following the manufacturer's instructions (Agilent Technologies, #200521), or it can be performed by a simple substitution of these chemical reagents. Briefly, the PCR mixture for site-directed mutagenesis procedure, totaling 50 µL, comprised 5 µL of 10X reaction polymerase buffer (Agilent Technologies, #200516), along with 1 µL of dNTP, 250 ng of cleaned product from the previous PCR reaction, 3 µL of QuikSolution (or pure DMSO), and 100 ng of template DNA. Amplification was conducted using 2.5 µL of PfuTurbo DNA polymerase (Agilent Technologies, #200516). The process commenced with an initial denaturation step at 95 °C for 1 min, followed by 18 cycles utilizing the following parameters: denaturation at 95 °C for 50 s, annealing at 60 °C for 50 s, and extension at 68 °C for 7–8 min (i.e., 1 min/kb of plasmid length). Subsequently, a final extension step at 68 °C for 7–8 min

Purpose of the primer	Name	Primer sequence (5′–3′)
Insertion of the ECFP tag into the hDVL3 pDONR221 plasmid	ECFP fwd	gtacaaaaaagcaggctccaccATGGTGAGCAAGGGCGAGGAG
	ECFP rev	cgtctcctgcccatccaagtgGTAGATGATCTTGGTCTCGCCCAT
Insertion of the hDVL3 DEP domain into the Flag-mDvl1(ΔDEP) plasmid	DEP fwd	tgaggaggcaccgctgactgtgaagAGTGACATGGCTGCCATCGTAAAAG
	DEP rev	tgaggttcagggatgcgaggttactGTCACCGAAGATGTAGTAGCACTGC

Table 1. Primers used in ECOLI. The flanking regions are underlined. Inserted nucleotide regions coding the start and the end of the ECFP tag or DEP domain, respectively, are in uppercase.

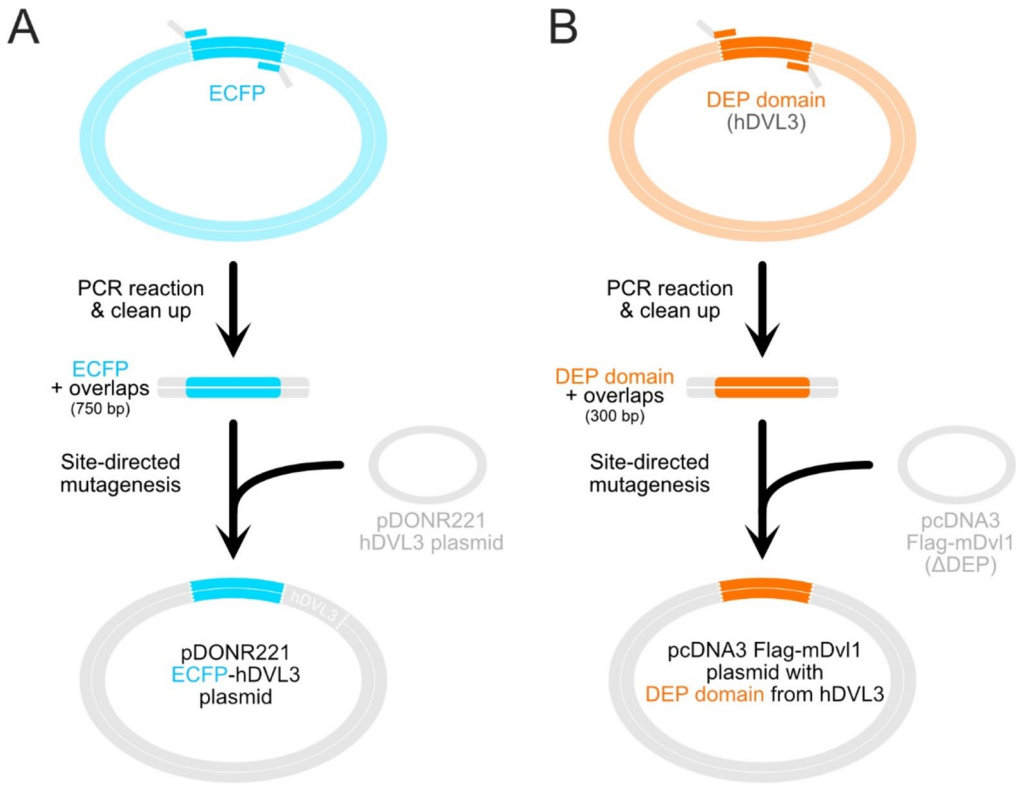

Fig. 1. The general scheme of ECOLI—Efficient cloning of linear inserts, as shown on the example of inserting the ECFP tag-encoding sequence into the pDONR221 hDVL3 construct (on the left) and inserting the DEP domain-encoding sequence from hDVL3 into the Flag-mDVL1 (ΔDEP) plasmid construct (on the right).

was executed. Following the reaction, the mixture was incubated at 37 °C with 1 µL of DpnI methylase (Agilent Technology or NEB, # R0176) supported by the buffer, in order to eliminate the maternal plasmid DNA construct.

Transformation and DNA minipreparation

Following the site-directed mutagenesis procedure, the entire reaction mixture was utilized for the transformation of *Escherichia coli* XL10-Gold ultracompetent cells (Agilent Technologies). In summary, 45 µL of the *E. coli* XL10-Gold ultracompetent cells were thawed on ice and incubated with 2 µL of β-mercaptoethanol for 10 min. The mixture was combined with 2–4 µL of Dpn I-treated DNA (with a competency of approximately 5×10^9 colony forming units per µg of plasmid DNA) and incubated on ice for 30 min. Subsequently, the cells were subjected to a heat shock at 42 °C for 30 s, followed by immediate chilling on ice for 2 min. The cells were regenerated through incubation at 37 °C for 1 h in a horizontal bacterial shaker with 250 rpm upon the addition of 250 µL of pre-warmed SOC media (15544-034, Invitrogen); alternatively, a standard LB (lysogeny broth) medium can be also used as an alternative. The whole mixture was then spread onto LB-agar plates with appropriate antibiotics and incubated overnight at 37 °C. The following morning, several colonies from each agar plate were selected and inoculated into 5 mL of standard LB medium supplemented with antibiotics. The cultures were then allowed to grow for 14–16 h at 37 °C with 250 rpm in a horizontal bacterial shaker. Subsequently, we performed the plasmid DNA minipreparation procedures using the NucleoBond Xtra kits (Macherey–Nagel, Germany). All obtained plasmids were then sequenced by the commercial provider (Eurofins Genomics, Ebersberg, Germany), according to its instructions.

Restriction digestion screening

To test the positive clones, 1 µg of the isolated pDONR221 hDVL3 both maternal and ECFP plasmids underwent cleavage using BtgZI (NEB, #R0703) and AsiSI (NEB, #R0630S) enzymes for 2 h, first with 2 µL of AsiSI at 37 °C, then with 2 µL of BtgZI at 60 °C. AsiSI cleaved both the original and mutated plasmids at one site, whereas the BtgZI cleaved only one site in the *ECFP* gene, leading to the excision of the single DNA part about 2000 bp. The cleavage reactions were conducted in appropriately buffered environments. Subsequently, the results were analyzed using 1.5% agarose gel electrophoresis, where the entire cleavage cocktail was loaded, and a gel was stained by ethidium bromide (#sc-203735, Santa Cruz) and captured using the GeneSys gel imaging and analysis system (Syngene, UK). The design of the mutations affecting the aforementioned restriction sites is illustrated in Suppl. Fig. 3A.

Western blot analysis and sample preparation

The T-REx HEK293 *DVL1-2–3* TKO cells[16] were grown at 37 °C and 5% (vol/vol) CO_2 in Dulbecco's modified Eagle's medium (DMEM; Gibco, #41966-029), 10% (vol/vol) fetal bovine serum (Gibco, #10270), 2 mM L-glutamine (Life Technologies, #25030024), and 1% (vol/vol) antibiotics (penicillin/streptomycin; Hyclone-Biotech, #SV30010). Cells were seeded (60,000 per well) directly on 24-well plates and next day transfected with 10 ng of each corresponding plasmid for 6 h using the PEI (1:6 ratio, 15 min incubation) in order to deliver the plasmid DNA into cells. Consequently, DMEM was removed after 24 h, and 100 µL of 2 × standard Laemmli buffer (1% SDS, 10% glycerol, 50 nM Tris pH 7,6, 1% β-mercaptoethanol, 0.04% bromphenol blue) was added in order to lyse the cells. Finally, the samples were collected into 1 mL Eppendorf tubes and boiled at 90 °C for 10 min.

Equal amounts of total protein were subjected to 10% SDS PAGE, electro-transferred onto PVDF membranes (Immobilon), immunodetected using appropriate primary and secondary antibodies, and the membranes were visualized using the chemiluminescence documentation system FusionSL (Vilber-Lourmat, Collégien, France). The antibodies used in this study were anti-FLAG M2 (concentration 1:1000; Sigma-Aldrich, #F1804), β-actin (1:1000; Cell Signaling Technology, #4970), pLRP6 (1:000, Santa Cruz, #15399), followed by the anti-mouse HRP-conjugated secondary (1:5000; Sigma-Aldrich, #A9044) and anti-rabbit HRP-conjugated secondary (1:5000; Sigma-Aldrich, #A0545), all mixed and used in 5% dry milk buffered with standard TBS-T solution.

Dual luciferase assay

For the Dual Luciferase TopFlash/Renilla Reporter Assay, the T-REx HEK293 wt and DVL1-2–3 TKO cells[16] were seeded directly into 24-well plates at a density of 60,000 cells per well and subsequently treated with the 1 µM Porcupine inhibitor LGK974 (#974–02; Stem RD). After 24 h, the cells were transfected with 0.1 µg of the Super8X TopFlash construct, 0.1 µg of the Renilla luciferase construct, and 0.01 µg of corresponding plasmids per well for 6 h according to Paclikova et al.[16]. After transfection cells were treated for at least 14 h by 50ul of R-Spondin1 condition medium (CM) and by 200ul control or Wnt3a CM per well in total medium volume 500 ul (for more information, see[16]). The assay was performed using the Promega Dual Luciferase assay kit (Promega, #E1910) following the manufacturer's instructions. Luminescence was measured using a Hidex Bioscan Plate Chameleon Luminometer. Results are presented as the ratio of TopFlash to Renilla signal (TopFlash fold induction), normalized to a specific control for each experiment (normalized TopFlash fold induction). Data were analysed using MS Excel 2007 and GraphPad Prism 6, and results are shown as means ± S.D. (n = 3 experiments) and statistical significance was confirmed by paired Student's t-test.

Immunofluorescence

For immunofluorescence, the DVL1-2–3 TKO T-REx HEK293 wt cells were seeded at a density of 40,000 cells per well onto gelatin-coated coverslips in 24-well plates and transfected with 20 ng of each corresponding DVL plasmid, including 50 ng of V5-FZD5[17] and equalled to total 400 ng DNA per well using the pcDNA3.1 control plasmid. After 24 h, cells were fixed with fresh 4% paraformaldehyde, permeabilized using 0.5% Triton X-100, and blocked with PBS/BSA/Triton/Azide buffer (PBTA) [3% (wt/vol) BSA, 0.25% Triton X-100, 0.01% NaN3] for 1 h. They were then incubated overnight at 4 °C with primary antibodies using anti-V5 (#R96025; Life Technologies) and anti-FLAG M2 (#F1804; Sigma) diluted 1:500 in PBTA. The following day, coverslips were washed with PBS and incubated with secondary antibodies conjugated to Alexa Fluor 488 (Invitrogen; #A11001) and/or Alexa Fluor 568; (Invitrogen; #A-11004), both diluted 1:500. After additional 3–4 × PBS washes, the cells were stained with DAPI (1:5000) and the coverslips were mounted onto microscopic slides. Cells were visualized using a Leica SP8 fluorescent microscope with a × 40 objective. The images were processed using LAS X software (Leica).

Results

This paper aims to demonstrate the effectiveness of a novel cloning method through various examples of DNA plasmid constructs encoding Dishevelled (Dvl/DVL) proteins from the WNT signaling pathway, illustrated by two specific DNA inserts. In the initial scenario, we endeavored to subclone the gene encoding the ECFP tag (714 bp; ≈ 750 bp with overlaps—see below) into the pDONR221 vector containing the human *DVL3* gene (DNASU, plasmid no. HsCD00040582). In the second scenario, we subcloned the gene encoding the DEP domain from the human DVL3 protein (261 bp, ≈ 300 bp with overlaps; aa 410–495 in hDVL3) into the N-terminally Flag-tagged mouse Dvl1 construct lacking the DEP domain (aa Δ413–501), creating thus a "chimera" construct of mDvl1/hDVL3 (Fig. 1). These examples serve as illustrations, suggesting the versatility of this technique for various cloning purposes.

Regarding the process, we first designed a pair of primers for each reaction, essentially merging flanking regions from one construct with another. Specifically, each forward primer consisted of a 5'-end flanking region from the future insertion site in the receiving plasmid and a 3'-end amplifying the insert itself, each with approximately 15–20 nucleotide overlaps. For the reverse primer, the same procedure was performed but then, the reverse complement variant of the reverse primer was used in order to gather the double stranded DNA product. Utilizing the Multiple Primer Analyzer online tool (ThermoFisher Scientific, USA), we ensured the absence of secondary loop formations in both primers, which was in our cases negligible. Following primer validation and commercial synthesis (Sigma Aldrich, USA), PCR reactions were conducted according to the manufacture's instructions, with parameters calculated using the NEB Tm calculator online tool (see Materials and methods for more information). The integrity and yield of PCR products were assessed via agarose gel electrophoresis (Fig. 2), with slight by-products observed but removable through gel excision. The final PCR product size was slightly increased due to the necessity of incorporating 15–20 nt overlaps on both ends. Subsequently, purified DNA concentrations were measured using a NanoDrop 1.0 device, with satisfactory yields (Suppl. Fig. 2).

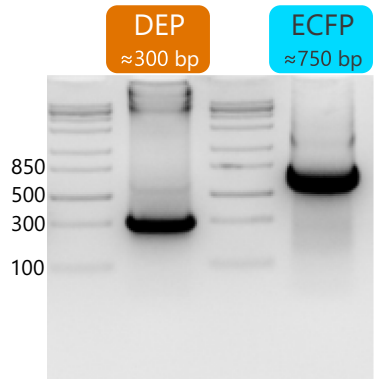

Fig. 2. Analysis of ECOLI PCR products using agarose gel electrophoresis. The PCR product (5 µL) was resolved by agarose gel electrophoresis (1.5% gel) and stained with ethidium bromide. Some slight non-specific products are also visible. The PCR product size was slightly enlarged due to the inclusion of 15–20 nt overlaps on both ends. Marker: ZipRuler Express DNA Ladder 1 (SM1373; Thermo-Scientific).

For site-directed mutagenesis, we calculated the required primer amounts (250 ng total equaled approximately 2.5 µL volume) and initiated the reaction, optimizing the extension time to 7–8 min to facilitate the incorporation of linear inserts into the target plasmid. Classic DNA transformation via heat shock with *E. coli* XL10-Gold ultra-competent cells was then performed, followed by efficiency assessment through colony enumeration, yielding tens of colonies per plate. We employed various methods to identify positive colonies, including restriction analysis with digestive enzymes, and comparison to maternal plasmid DNA, as shown on the ECFP tag (Suppl. Fig. 3B) and Sanger sequencing (Suppl. Fig. 4). The restriction analysis was designed in the way to find and cut one size within inserted ECFP gene (Suppl. Fig. 3A). Finally, we assessed the protein expression of the newly cloned plasmid construct, as shown on the inserted DEP domain (Fig. 3; for full uncropped WB images, see Suppl. Fig. 8).

In both cloning scenarios, our ECOLI approach demonstrated an efficiency of approximately 60–85%, with 5 out of 7 clones positive in the first scenario with ECFP and 2 out of 3 clones positive in the second scenario with the mDvl1/hDVL3 chimera. Consequently, we assert the effective subcloning of both linear DNA inserts into plasmids of interest, within a timeframe of less than two weeks, including commercial primer design, considering both efficiency and cost factors.

Next, we validated the functionality of the DVL3/Dvl1 DEP chimera construct by assessing its ability to bind to the cell membrane, in comparison to a construct lacking the DEP domain. We utilized an established assay to assess the FZD5-dependent binding of Dvl/DVL[18]. Our results demonstrated that the DVL3/Dvl1 chimera effectively binds to the membrane in the presence of FZD5, in stark contrast to the Dvl1 construct lacking the DEP domain (Fig. 4, bottom, for all channels, see Suppl. Fig. 5). In the absence of FZD, both constructs formed puncta (Fig. 4, top, for all channels, see Suppl. Fig. 5), a characteristic feature of Dvl/DVL[18]. This finding further supports the functionality of the DVL3/Dvl1 DEP chimera construct and validates the efficiency of the ECOLI approach.

Finally, we assessed the impact of the DVL3/Dvl1 chimera construct on WNT/β-catenin pathway activity using a dual luciferase assay, as described by Korinek et al.[19]. Our results revealed that the chimera construct activated the WNT/β-catenin pathway more effectively than either Dvl1 wt or Dvl3 wt alone (Fig. 5). Notably, this increased activity was not attributable to increased protein levels, as both Dvl1 and Dvl3 constructs exhibited similar (in case of Dvl1 wt), or even higher (Dvl3 wt) protein levels (see Suppl. Fig. 7, uncropped membranes

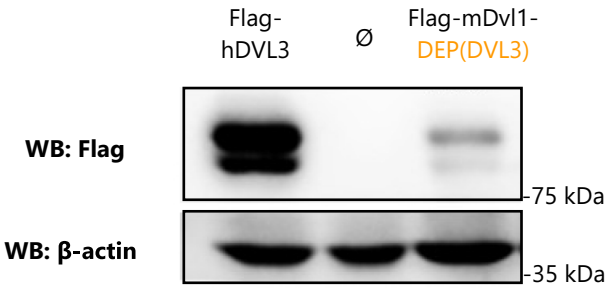

Fig. 3. The protein expression of a construct with the inserted sequence encoding DEP domain from hDVL3 (aa 422–496) into the Flag-mDVL1 (ΔDEP; aa Δ425–499) shown by the Western blot technique. The full-length Flag-hDVL3 (aa 1–716) represents the positive control for protein expression, and β-actin is the loading control. The full uncropped WB blots are shown as Supplementary Fig. 8.

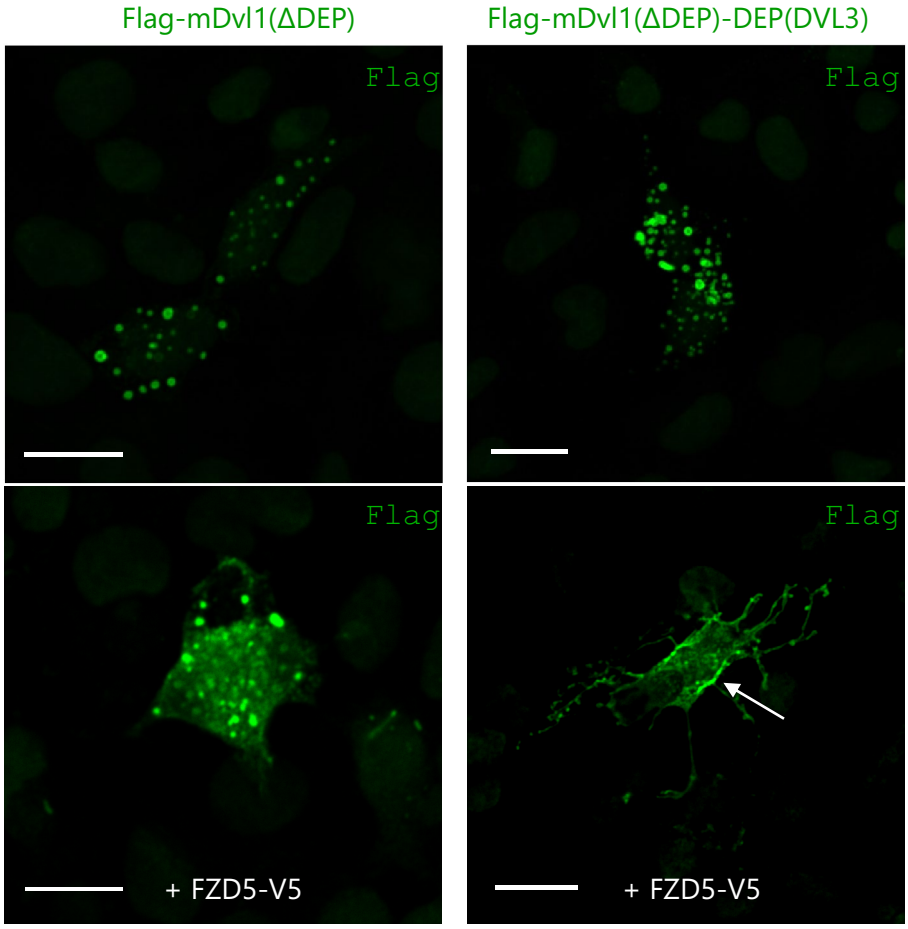

Fig. 4. Immunofluorescence of HEK293 cells shows that the DVL3/Dvl1 DEP chimera construct binds strongly to the cell membrane in the presence of FZD5-V5 (highlighted by a white arrow), unlike the mDvl1 DEP domain-deleted construct, confirming thus its functionality. Without FZDs, both constructs form puncta. All channels are shown as Supplementary Fig. 5. Scale bar is 10 μm.

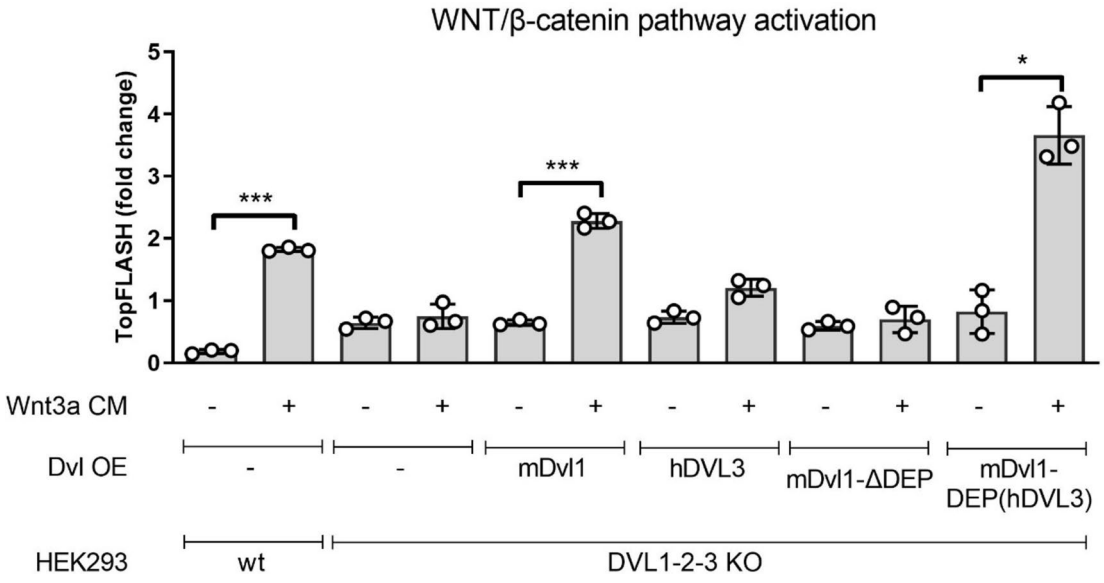

Fig. 5. The overexpressed (OE) Dvl1/DVL3 DEP chimera construct activates the WNT/β-catenin pathway more effectively than both OE Dvl1 and Dvl3 alone, as demonstrated by the dual luciferase assay measuring the WNT/β-catenin pathway activity using the Wnt3a conditioned medium (CM) in HEK293 wt and *DVL1-2-3* knock-out (KO) cells. For WB lysates and protein levels, see Supplementary Figs. 7 and 9.

in Suppl. Fig. 9). To further investigate this phenomenon with increased activity, we aligned the DEP domains sequences from mDvl1 and hDVL3 (Suppl. Fig. 6A) and identified non-conserved residues that may be responsible for these functional differences. These residues were mapped onto the mDvl1 DEP domain structure as potential candidate amino acids responsible for increased activity (Suppl. Fig. 6B).

These findings underscore the critical role of this specific region in hDVL3 for WNT/β-catenin pathway signaling and affirm the utility of the ECOLI approach for cloning relevant candidates for subsequent proteomic studies.

Discussion

In this study, we introduced a novel method for cloning DNA inserts of up to approximately 1,000 base pairs into plasmids, utilizing resources commonly found in laboratories without the need for specialized kits or restriction sites. Our ECOLI method stands out in the realm of in vitro DNA assembly due to its unique combination of features: it is restriction enzyme-free, not based on recombination, and relies on enzymes like polymerases. To our knowledge, such a method has not been previously published, positioning it as a new category in in vitro DNA assembly techniques.

While our method shares similarities with Circular Polymerase Extension Cloning (CPEC)[12,20], it differs notably in the use of a linearized vector, which simplifies the process and eliminates the need for circularization steps. The simplicity and accessibility of our approach make it theoretically feasible for implementation in any lab around the world. However, there are certain challenges that could impede the process. One potential challenge is the formation of secondary structures by the PCR product, which could reduce reaction efficiency. Similarly, inserts with high GC content may pose difficulties during annealing, crucial for successful reactions. Although we did not encounter these issues in our cases, the addition of DMSO to reactions, adding more DNA template, or adjusting annealing temperatures could potentially mitigate these challenges[21].

For PCR reactions, we utilized Platinum SuperFI DNA polymerase, which combines a DNA-binding domain with proofreading DNA polymerase activity. This fusion enhances processivity and reduces extension speed, thus saving time without compromising accuracy[22]. While our method is compatible with various polymerases commonly used for PCR, proofreading polymerases are preferred to minimize the risk of random mutations, particularly in exponential PCR, where mutations can be amplified in subsequent cycles. Though we did not detect proofreading errors in our sequences, as confirmed by Sanger sequencing, vigilance for this potential issue is always warranted.

In terms of time efficiency, our method offers a significant advantage, completing the procedure in just two weeks including commercial primer design with minimal personnel intervention. If the circumstances are good, then the synthesis of primers from external companies such as Sigma Aldrich, which takes usually 1–2 weeks and are not considered in time course here, may be the "most time-consuming step" of ECOLI.

Using ECOLI, we successfully cloned and analyzed genes encoding the Dishevelled protein (DVL), discovering a key function of the DEP domain from human DVL3 in WNT signaling transduction. Notably, when mapping the residues that differ between mouse Dvl1 and human DVL3, which could potentially explain differences in signal transduction (Suppl. Fig. 6), we found that these residues are predominantly located in structured regions such as helices, rather than unstructured loops. While this observation is intriguing, it requires further investigation and falls outside the primary focus of this study.

Our demonstration of the ECOLI method in proteomic analyses is just one example of its potential applications. The method's simplicity makes it versatile for inserting DNA sequences of up to 1,000 base pairs, with potential utility across a broad range of biological contexts, including bacterial and viral studies. While we cannot predict all future applications, we anticipate that time will reveal the full scope of this method's utility. In summary, while other methods for in vitro DNA insertions into plasmids and assembly exist, many are costlier and may require specialized enzymes or kits. In contrast, our method is theoretically straightforward and universally applicable in laboratories worldwide.

Conclusions

In conclusion, our study presents a robust method for efficiently cloning DNA inserts into plasmids, showcasing its effectiveness through successful experimentation and thorough discussion of potential challenges and advantages. With its simplicity, accessibility, and time efficiency, this approach offers a valuable alternative to existing methods, underscoring its potential as a widely used tool in molecular biology research. The ECOLI approach allows for insertions of up to 1,000 base pairs and perhaps more; nevertheless, its capacity for insertions of more than 1,000 base pairs is likely but this has not yet been tested in our experiments.

Data availability

"The protein sequences derived from Sanger sequencing for both the inserted DEP domain and ECFP gene in the appropriate plasmids (Suppl. Fig. 4) can be readily accessed in the GenBank (BankIt) database under the accession codes below. Except for the pDONR221 hDVL3 plasmid, which is commercially available (DNASU, plasmid no. HsCD00040582), all plasmids used in this study have been uploaded to the GenBank (BankIt) database under the accession codes #PQ197126 [pcDNA3-Flag-mDvl1-delta_DEP (ΔDEP; Δ413–501)], #PQ197127 [pcDNA3-Flag-mDvl1-DEP_hDVL3 (DEP 410–495 from hDVL3)], and #PQ197128 [pDONR221 ECFP-hDVL3 (1–716)]."

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References

1. Cohen, S. N. DNA cloning: A personal view after 40 years. *Proc. Natl. Acad. Sci. USA* **110**(39), 15521–15529 (2013).
2. McKinnell, R. G. & Di Berardino, M. A. The biology of cloning: History and rationale. *BioScience* **49**(11), 875–885 (1999).
3. Alberts, B. *et al.* Isolating, cloning, and sequencing DNA. In *Molecular Biology of the Cell* 4th edn (Garland Science, 2002).
4. Chong, Z. X., Yeap, S. K. & Ho, W. Y. Transfection types, methods and strategies: A technical review. *PeerJ* **9**, e11165 (2021).
5. Kim, T. K. & Eberwine, J. H. Mammalian cell transfection: The present and the future. *Anal. Bioanal. Chem.* **397**(8), 3173–3178 (2010).
6. Stepanenko, A. A. & Heng, H. H. Transient and stable vector transfection: Pitfalls, off-target effects, artifacts. *Mutat. Res. Rev. Mutat. Res.* **773**, 91–103 (2017).
7. Kwon, H. *et al.* Emergence of synthetic mRNA: In vitro synthesis of mRNA and its applications in regenerative medicine. *Biomaterials* **156**, 172–193 (2018).
8. Sive, H. L., Grainger, R. M. & Harland, R. M. Microinjection of Xenopus oocytes. *Cold Spring Harb. Protoc.* **2010**(12), 5536 (2010).
9. Lino, C. A. *et al.* Delivering CRISPR: A review of the challenges and approaches. *Drug Deliv.* **25**(1), 1234–1257 (2018).
10. Jinek, M. *et al.* A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity. *Science* **337**(6096), 816–821 (2012).
11. Fu, C. *et al.* Hot Fusion: An efficient method to clone multiple DNA fragments as well as inverted repeats without ligase. *PLoS ONE* **9**(12), e115318 (2014).
12. Wang, W., Zheng, G. & Lu, Y. Recent advances in strategies for the cloning of natural product biosynthetic gene clusters. *Front. Bioeng. Biotechnol.* **9**, 692797 (2021).
13. Sharma, M. *et al.* Dishevelled: A masterful conductor of complex Wnt signals. *Cell Signal.* **47**, 52–64 (2018).
14. Gao, C. & Chen, Y. G. Dishevelled: The hub of Wnt signaling. *Cell Signal.* **22**(5), 717–727 (2010).
15. Mlodzik, M. The dishevelled protein family: Still rather a mystery after over 20 years of molecular studies. *Curr. Top. Dev. Biol.* **117**, 75-91. https://doi.org/10.1016/bs.ctdb.2015.11.027 (2016).
16. Paclikova, P. *et al.* Roles of individual human Dishevelled paralogs in the Wnt signalling pathways. *Cell Signal.* **85**, 110058 (2021).
17. Strakova, K. *et al.* Dishevelled enables casein kinase 1-mediated phosphorylation of Frizzled 6 required for cell membrane localization. *J. Biol. Chem.* **293**(48), 18477–18493 (2018).
18. Bernatik, O. *et al.* Functional analysis of dishevelled-3 phosphorylation identifies distinct mechanisms driven by casein kinase 1ϵ and frizzled5. *J. Biol. Chem.* **289**(34), 23520–23533 (2014).
19. Korinek, V. *et al.* Constitutive transcriptional activation by a beta-catenin-Tcf complex in APC-/- colon carcinoma. *Science* **275**(5307), 1784–1787 (1997).
20. Quan, J. & Tian, J. Circular polymerase extension cloning for high-throughput cloning of complex and combinatorial DNA libraries. *Nat. Protoc.* **6**(2), 242–251 (2011).
21. Obradovic, J. *et al.* Optimization of PCR conditions for amplification of GC-Rich EGFR promoter sequence. *J. Clin. Lab. Anal.* **27**(6), 487–493 (2013).
22. Bebenek, A. & Ziuzia-Graczyk, I. Fidelity of DNA replication-a matter of proofreading. *Curr. Genet.* **64**(5), 985–996 (2018).

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Author contributions

P.P. and J.H. performed experiments and analysed the data. J.H. drafted the manuscript and performed a literature search. P.P. and J.H. contributed to the methodology, and P.P. edited the manuscript. J.H. had the main ideas, made all the Figures, critically revised the manuscript, and supervised the overall work.

Competing interests

The authors declare no competing interests.

Additional information

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