Chapter 3

Molecular Cloning Using In Vivo DNA Assembly

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Abstract

Here we describe the in vivo DNA assembly approach, where molecular cloning procedures are performed using an *E. coli* recA-independent recombination pathway, which assembles linear fragments of DNA with short homologous termini. This pathway is present in all standard laboratory *E. coli* strains and, by bypassing the need for in vitro DNA assembly, allows simplified molecular cloning to be performed without the plasmid instability issues associated with specialized recombination-cloning bacterial strains. The methodology requires specific primer design and can perform all standard plasmid modifications (insertions, deletions, mutagenesis, and sub-cloning) in a rapid, simple, and cost-efficient manner, as it does not require commercial kits or specialized bacterial strains. Additionally, this approach can be used to perform complex procedures such as multiple modifications to a plasmid, as up to 6 linear fragments can be assembled in vivo by this recombination pathway. Procedures generally require less than 3 h, involving PCR amplification, DpnI digestion of template DNA, and transformation, upon which circular plasmids are assembled. In this chapter we describe the requirements, procedure, and potential pitfalls when using this technique, as well as protocol variations to overcome the most common issues.

Key words Molecular cloning, In vivo DNA assembly, recA-independent recombination, IVA cloning, Sub-cloning, Site-directed mutagenesis

1 Introduction

Molecular cloning is a cornerstone of biomedical research and has been continuously developed over recent decades to provide simpler and more efficient methodologies. Here we describe the use of the in vivo DNA assembly approach for molecular cloning, which relies on an endogenous *E. coli* DNA recombination pathway capable of joining linear DNA fragments with short homology regions at their termini [1]. This pathway is independent of recA and is present in all laboratory *E. coli* strains. The "recA-independent" recombination pathway has been exploited for molecular cloning, facilitating cloning procedures with minimal handling but having the advantages of recombination-based methodologies (scarless, sequence-independent, single-base precision, and directional) [1–

7]. Using in vivo DNA assembly, all standard plasmid DNA modifications can be performed, including sequence insertions, deletions, point-mutagenesis, and sub-cloning of large fragments between vectors. When employed as a cloning tool, linear DNA fragments are generated in vitro, primarily by PCR, with homologous termini of around 15–30 bp that direct plasmid assembly in vivo. After transformation, endogenous single-stranded exonucleases (ExoIII/V) [8] degrade the termini of these linear fragments to single-stranded DNA, which allows annealing between homologous fragments in vivo before DNA repair (LigA) assembles a circular plasmid [9, 10].

Cloning protocols generally comprise the following: (1) primer design, (2) PCR amplification, to introduce modifications and homologous sequences, (3) digestion of the parental DNA using the methylase-dependent restriction enzyme DpnI, and (4) transformation into standard laboratory *E. coli* before subsequent colony screening and selection. This protocol can be used to perform all types of plasmid modifications, from inserting and deleting sequences to site-directed mutagenesis and sub-cloning, each dictated by primer design. Furthermore, up to six DNA fragments can be assembled simultaneously, allowing complex cloning strategies to be achieved in a single step; however, method efficiency decreases as procedure complexity increases. Given the principal requirement for cloning using recombination is linear DNA fragments, in vivo DNA assembly can also be combined with restriction enzyme-linearized plasmids or synthesized linear double-stranded genes, which can overcome PCR amplification issues or further simplify procedures (Fig. 1). Given that there are no requirements for commercial kits or specialized bacteria, this approach is accessible to any molecular biology laboratory. Additionally, since the homology requirements are similar to other enzyme or recombination-based commercial approaches (e.g., Gibson assembly [11]), primers designed for in vivo assembly cloning can also be combined/used with such in vitro assembly methods as an alternative backup route. Here, we describe reagents and protocols to perform in vivo DNA assembly for plasmidic DNA cloning and modification as performed in our laboratory.

2 Materials

2.1 Polymerase Chain Reaction

1. Premixed PCR master solution, prepared as 23 μL premixed reactions, stored at −20 °C (*see* **Note 1**): 250 μM each dNTP nucleotide (dATP, dGTP, dCTP, and cCTP), 1 M betaine, 2,5% DMSO, Phusion Polymerase Buffer (1X), Phusion HF Polymerase (1 μL per 25 μL reaction) (*see* **Note 2**), and deionized H_2O (we generally use 200 μL thin wall PCR tubes).

2. PCR Thermocycler.
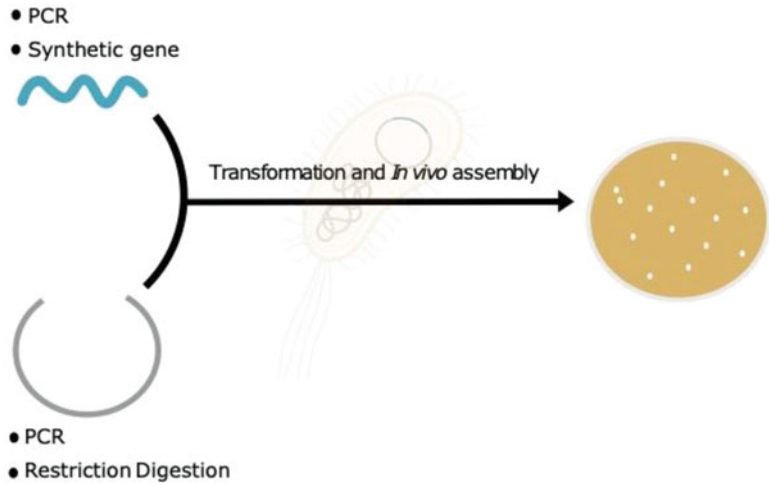
Fig. 1 General in vivo DNA assembly scheme. A DNA fragment of interest resulting from PCR amplification or gene synthesis can be assembled in vivo into a plasmid (linearized as a result of a PCR amplification or through restriction-enzyme digestion) after transformation in standard *E. coli*

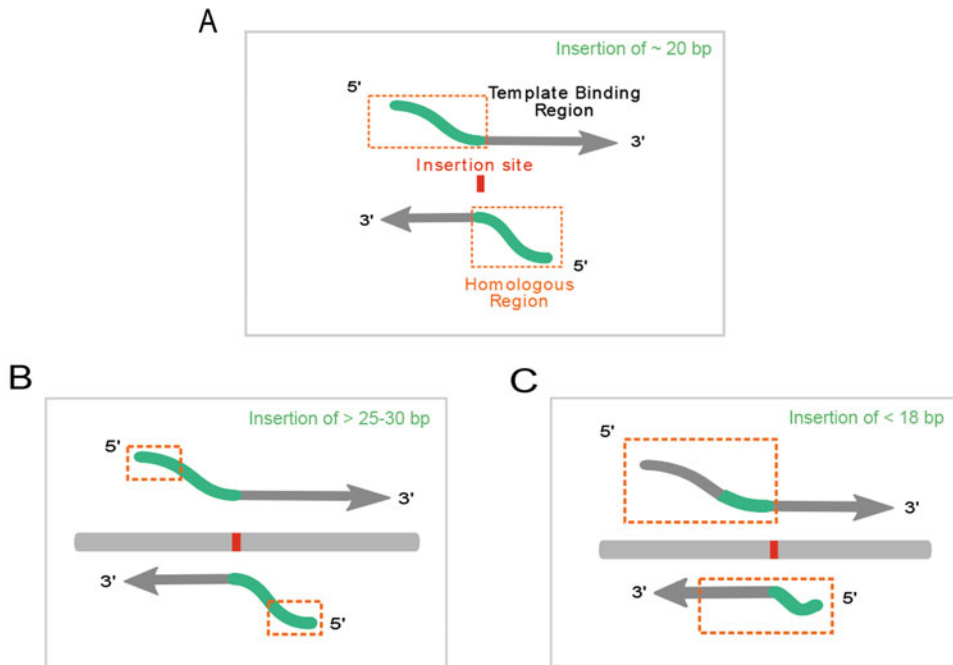|	
2.2 DNA Gel Electrophoresis and Eliminating Parental DNA	1. Agarose powder (*see* **Note 3**).
	2. DNA stain (e.g., SYBR™ Safe DNA Gel Stain (Thermo Fisher) or GreenSafe Premium (Nzytech)).
	3. 10X TB agarose electrophoresis buffer: 440 mM Tris, 440 mM Boric acid in water Weigh 54 g of TRIS and 27.5 g Boric acid, and dissolve by stirring in 1 L H_2O. We do not use EDTA in the TB buffer as compared to the commonly used TBE recipe (*see* **Note 4**).
	4. DNA Gel Loading dye.
	5. DNA Molecular Weight Marker Ladder.
	6. DNA Gel Electrophoresis Tank and power supply.
	7. UV/Blue light Transilluminator.
	8. DpnI (FastDigest, Thermofisher) (*see* **Note 5**).
2.3 Transformation	1. Chemically competent bacteria: XL-10 Gold® Ultracompetent cells (*see* **Note 6**).
	2. Water bath (set to 42 °C).
	3. Super Optimal Broth (SOB): 20 g bactotryptone, 5 g yeast extract, 2 mL of 5 M NaCl, 2.5 mL of 1 M KCl, 10 mL of 1 M $MgCl_2$, 10 mL of 1 M $MgSO_4$, and distilled H_2O to 1 L.
	4. Lysogeny Broth (LB): 7.5 g agar, 5 g tryptone, 5 g NaCl, 2.5 g yeast extract, and distilled H_2O to 500 mL.
	5. LB agar plates with antibiotics as appropriate.
	6. 37 °C incubator.

3 Methods

3.1 Primer Design

We recommend using software for visualizing both the original and target DNA sequences for the design of oligos, as well as software for the calculation of annealing temperatures (T_m). We use the freely accessible Snapgene Viewer program for primer sequence design and the OligoCalc webserver (http://biotools.nubic.northwestern.edu/OligoCalc.html) for annealing temperature calculation [12]. All T_m values reported in this chapter are calculated using this webserver. Accurate primer design is critical to the success of in vivo DNA assembly. Regardless of the modification to be made (insertion, deletion, mutagenesis, or sub-cloning), primers consist of two regions: a 3′ region that anneals to the template DNA (template binding region) and a 5′ homologous region that drives in vivo recombination. First, design the template binding region, which has the same requirements of standard PCR oligo design (at least 18–22 bp and T_m values of ~60 °C). The homologous region is included 5′ to this sequence, and it should be ≥15 bp and have a T_m ≥ 50 °C (*see* **Note 7**). As a rule of thumb, a homologous region of ~20–25 bp is sufficient to ensure efficient recombination (usually providing a T_m ≥ 50 °C), and lengths of up to 35 bp can been used to enhance efficiency when assembling ≥5 DNA fragments simultaneously. Specific primer design requirements for each DNA modification are as follows:

3.1.1 Insertion

We define an insertion as the introduction of a new segment of DNA that can be fully included within a single pair of PCR primers (independent of size) and are typically up to 200 bp. Using one pair of PCR primers, the whole vector is amplified during PCR, and recircularization occurs after transformation through a single recombination event. To insert a DNA fragment to a plasmid, design primers with the template binding regions binding astride the insertion site and add the homologous regions at the 5′ ends. If the length of the insertion is around ~20 bp, this new sequence can form the homologous region by inclusion at the 5′ end of both primers (Fig. 2a). If the insertion is significantly larger than a typical homologous region (>30 bp), the desired sequence should be divided in two, with each primer encoding half of the total insert. Insert coding regions on each primer must be designed to overlap (~20 bp) to act as the homologous region (Fig. 2b) (*see* **Note 8**). If the new sequence is significantly shorter than ~20 bp, the 5′ end of one primer will need to be extended to overlap with the annealing region of other primer in order to create a larger homologous region that guarantees an efficient recombination (Fig. 2c).

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Fig. 2 Primer design to perform insertions. Primers must have template binding regions (grey) that bind either side of the insertion site (red) and a homologous region at the 5′ end (orange box) that allows in vivo recombination. Based on the length of the desired insertion (depicted in green), the homologous regions for each of the primers could be: (**a**) the entire insert (when the insertion is ~20 bp), (**b**) a sub-region of the insert (when the insertion is >25–30 bp), or (**c**) extended beyond the insert sequence alone (<18 bp).

3.1.2 Deletion	A deletion occurs when a desired DNA sequence is removed from the template plasmid. Primers for a deletion are designed such that they have their template binding regions annealing either side of the deletion site, amplifying outwards (i.e., amplifying the entire plasmid aside from the fragment to remove). A 5′-DNA sequence is added to one of the primers, which is homologous to the 5′ end of the primer's pair (Fig. 3a) (*see* **Note 9**).
3.1.3 Mutagenesis	Mutagenesis involves the replacement of short DNA sequences, typically a single base pair or triplet codon. It can therefore be considered as a simultaneous deletion and insertion, with a corresponding primer design. Template binding regions of mutational primers bind astride the undesired sequence, with the novel sequence included in primer 5′ ends. The required homologous region should be added at the 5′ end of one of the primers, upstream of the codon to be replaced (Fig. 3b) (*see* **Note 10**).
3.1.4 Sub-cloning	Sub-cloning involves the incorporation of a larger DNA sequence into a target vector, which is too long to be achieved by "insertion" (see above). While previous modifications all involved recombination of termini from a single DNA fragment, sub-cloning requires

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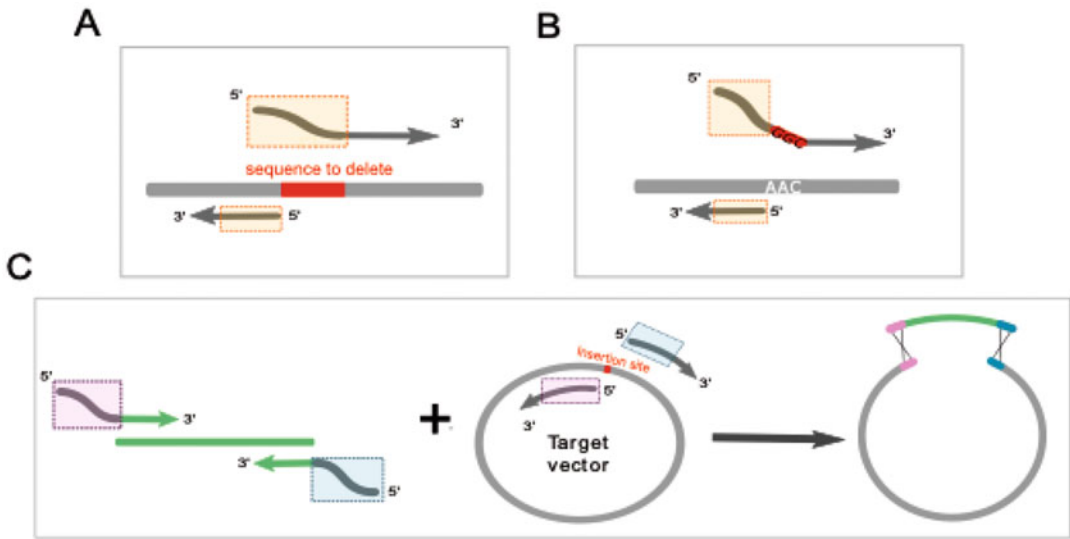
Fig. 3 Primer design to perform deletions, mutagenesis, and sub-cloning. (**a**) Deletions. Primers bind astride the sequence to remove, with homologous regions at termini encoded in one primer. (**b**) Mutagenesis. Primers flank the mutation site, similarly to deletions, with the new codon encoded in primer sequences. (**c**) Sub cloning. Four primers are needed to add a DNA fragment to a target vector. Insert amplification primers are designed to have specific homology to target vector termini (specific homologous regions for Fw (purple box) and Rv primer (blue box) match vector sequence)

assembly of two separate linear fragments, through recombination 166
of two distinct homologous overlaps (driven by unique sequences 167
at each site). To perform sub-cloning, both an "insert" and "target 168
vector" are amplified, requiring two pairs of primers (four primers 169
total). Assembly requires homologous regions at both fragment 170
join sites; therefore, at least one primer at each site must be 171
designed to include a region homologous to the other DNA frag- 172
ment (Fig. 3c) (*see* **Note 11**). The inclusion of homologous 173
sequences in either "insert" or "vector" fragments, etc., is of no 174
significance to successful cloning, the only requirement being pro- 175
duction of linear DNA fragments with specific DNA sequences 176
shared between termini, which will be subsequently assembled. 177

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3.2 Using PCR for DNA Modification and Amplification

In the vast majority of cloning approaches, PCR will be used to 179
amplify and/or modify the DNA sequences involved. Employing 180
restriction enzyme-dependent DNA linearization or synthetic 181
genes are possible alternatives, which will be addressed in Subhead- 182
ing 3.5. PCR-based cloning proceeds as follows: 183

1. Prepare PCR Master Mix (see Materials) and freeze aliquots for 184
   future use. Defrost on ice on the day of use. 185

2. If primers arrive desiccated, make up to 100 μM in deionized 186
   water. 187

3. Prepare a solution containing 5 µM (in deionized water) of each primer required for the cloning procedure in a single tube (for minimal handling) (*see* **Note 12**).

4. Dilute template DNA to 1 ng/µL in deionized water (*see* **Note 13**).

5. Add 1 µL of the 5 µM primer stock and 1 µL of 1 ng/µL template DNA stock to the 23 µL PCR reaction mix (*see* **Note 14**).

6. Run the PCR according to the following cycling parameters (shown as specified for the Phusion® HF polymerase—protocol should be adjusted according to manufacturer instructions for alternative polymerases):

   (a) Initial denaturation—2 min at 98 °C.

   (b) Denaturation—30 s at 98 °C.

   (c) Primer annealing—30 s at a T_m specific to primer template binding region (approx. 60 °C).

   (d) Extension—specific to sequence length (15–30 s/kb) at 72 °C.

   (e) Run a total of 18 cycles of **steps b–d** (*see* **Note 15**).

   (f) Final elongation—10 min at 72 °C.

7. Add DNA Gel Loading Dye to 5 µL of PCR samples and load on a 1% agarose gel to confirm amplification of correctly sized fragments (*see* **Note 16**).

8. Add 1µl of DpnI enzyme to the remaining unpurified reaction mix and incubate at 37 °C for 15 min to eliminate parental DNA.

3.3 Transformation and Colony Selection

1. Transform 3–4 µL of the reaction mix to 100 µL of competent cells (*see* **Note 18**), and spread on an agar plate containing appropriate antibiotics (corresponding to plasmid resistance) and incubate overnight at 37 °C.

2. Select and grow individual colonies further in LB containing antibiotics overnight.

3. Isolate plasmid DNA using standard DNA purification kits (such a Qiagen Miniprep kit), and confirm new clones through Sanger sequencing.

3.4 Performing Complex Procedures

Here, we have detailed how specific primers can be used to create a variety of individual plasmid modifications; however, multiple modifications are often required and are carried out sequentially, increasing the length of a cloning procedure. Using in vivo DNA assembly, up to six fragments have been assembled simultaneously [2, 5], where final assembly from individual fragments is driven by their unique homologous sequences. This means that multiple
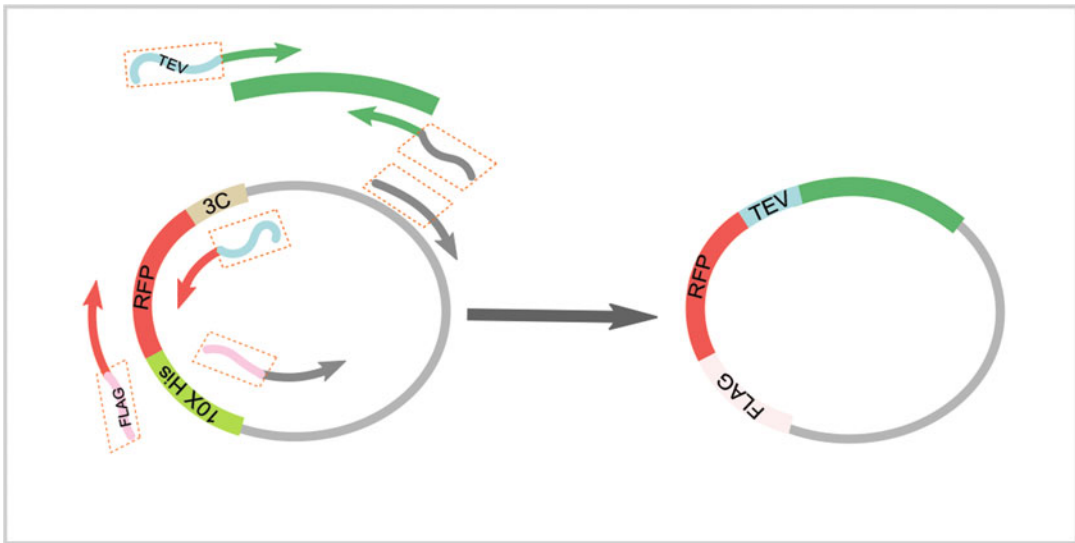
Fig. 4 Example of in vivo DNA assembly for complex cloning procedures. By combining three pairs of primers, a N-terminal HisTag (×10 HisTag, yellow) is exchanged for a FLAG tag (FLAG, pink) and a new coding sequence (green) is added downstream of the Red Fluorescent Protein gene (RFP, red), while replacing 3C cleavage site (3C, brown) for a TEV protease site (TEV, blue). These multiple modifications can be performed simultaneously using standard in vivo assembly primers

modifications can be made to a plasmid template in a single step. These modifications can be of the same or different types, for example, performing multiple simultaneous mutations, or insertion and deletion at two locations on a plasmid. An example of such versatility is demonstrated in Fig. 4, where an original plasmid containing a Red Fluorescent Protein (RFP) gene flanked by a N-terminal Histag and a C-terminal 3C cleavage site is modified in a single step to contain a N-terminal FLAG tag followed by the RFP, a TEV protease site, and a new coding region. These multiple modifications can be achieved simultaneously using a single PCR, using three pairs of primers (Fig. 4).

Experimentally, multiple modifications can be achieved using primers designed exactly as previously described, but simply used in combination to amplify multiple different fragments. Such procedures can be performed in a single PCR tube (with variations to the protocol found in **Notes 12** and **13**). If single-tube PCR poses efficiency issues, each individual fragment can be amplified in a separate tube, before combining all reactions after DpnI treatment and prior to transformation. In this case, individual PCRs are carried out by combining the Fw primer of one modification with the Rv primer of the nearest downstream modification (*see* **Note 17**; Fig. 4).

3.5 Alternative Routes for Linear Fragment Generation: Restriction Enzymes and Synthetic Genes

In vivo DNA assembly can assemble any linear DNA fragment that 252
contains homologous regions to another linear DNA. Although 253
the most common and practical route to obtaining such sequences 254
is PCR, alternative routes are possible. Restriction enzymes can be 255
used to linearize a vector at a desired site for modification, allowing 256
insertion of a fragment of interest without complete vector amplifi- 257
cation. The inserted fragment must be designed to contain termini 258
homologous to the vector sequence either side of the restriction 259
site, which typically involves PCR amplification of the insert. 260
Co-transformation of these fragments allows assembly of the 261
novel plasmid in vivo (*see* **Note 19**). 262

An increasingly useful and accessible route to obtain linear 263
DNA fragments is gene synthesis. Currently, linear double- 264
stranded synthetic DNAs (e.g., Gene Fragments or gBlocks^{TM}) 265
can be designed and purchased to be readily used in transformation 266
for in vivo DNA assembly. Novel sequences must simply be 267
designed to include termini homologous to the vector insertion 268
location (as previously described). This approach can be used for a 269
multitude of purposes: new genes, synthetic proteins, DNA frag- 270
ments that are not amenable for PCR amplification or simply, a 271
DNA fragment with many modifications that would be trouble- 272
some to obtain from the parental DNA. For use in in vivo DNA 273
assembly cloning, synthetic genes are co-transformed with a linear- 274
ized vector (linearized through PCR or restriction enzymes) (*see* 275
Note 20). 276

4 Notes

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1. Although higher volume PCR reactions will also work success- 278
fully (i.e., 50 μL), we find 25 μL suitable and cost-effective. We 279
generally prepare ~100 × 23 μL reactions with all components 280
(except for DNA template and primers) and store them at − 281
20 °C. We have stored PCR premixes for 1–1.5 years without 282
noticeable detrimental effects. 283

2. We generally use Phusion® HF Polymerase since it is the most 284
cost-effective currently polymerase accessible to us. There are 285
no essential requirements for polymerases used for PCR in 286
in vivo cloning; however, optimal cloning requires high effi- 287
ciency and low-error rate polymerases to produce sufficient 288
DNA and avoid point mutations when amplifying whole plas- 289
mids. For this reason, Taq/Pfu would be poor choices, while 290
Phusion or Q5® polymerase are the current best options. We 291
routinely use betaine and DMSO as default additives in our 292
PCR premixes to enhance DNA polymerization across high 293
GC-content regions. 294

3. Use low melting point agarose to make a 1% agarose gel. We use GreenSafe Premium (Nzytech), but any other DNA-binding dye should perform well enough for DNA visualization. 295 296 297 298

4. EDTA is used to eliminate residual DNA nucleases which are generally absent in our procedures. However, should these present an issue, EDTA can also be included. 299 300 301

5. We recommend FastDigest or equivalent so that DpnI digestion can proceed within 5–15 min of incubation. Standard enzymes will perform sufficiently but will increase protocol duration. 302 303 304 305

6. We use homemade competent cells generated by the Inoue et al. method [13] and stored as 100 μL aliquots. In general, procedures requiring single or double recombination events require competent cells of a competency at least 10^6–10^7 cfu/μg of DNA, while more complex procedures with three or more recombination events require competent cells of 10^7–10^9 cfu/μg. When performing multi-fragment assemblies, commercial competent cells can be used, and transformations typically use 2–3 μL DNA mixture in 30 μL cell suspension. 306 307 308 309 310 311 312 313 314 315

7. T_m values referred to here are calculated with the *Basic Melting Temperature Calculations* using the OligoCalc webserver. 316 317

8. Theoretically, this strategy could be used for the insertion of fragments up to ~440 bp (current technology allows primer synthesis up to 250 bp in length). However, for very large fragments a sub-cloning of small (~500 bp) linear double-stranded synthetic DNA would be more cost-effective (see sub-cloning section) than through expensive long primers. 318 319 320 321 322 323

9. To avoid primer-dimer formation (and poor PCR amplification), the T_m of template binding regions should be higher than that of homologous regions, ensuring maximal PCR efficiency. 324 325 326 327

10. Several variations in mutagenesis primer design can be used, with no difference in efficiency. Either a single primer or both primers may contain the new codon/base-pair, as long as this region is taken into account during homologous region calculations. 328 329 330 331 332

11. Although the homologous 5′ region can be added to either insert or vector primers, we prefer to add them onto the insert primers so the vector primers can be reused to clone different target genes into the same vector. Please note that fragments below 400 bp are not amenable for sub-cloning using in vivo DNA assembly. 333 334 335 336 337 338

12. For multiple modifications performed in a single-tube PCR, make a single stock with all required primers, each at 5 µM.

13. If more than one template DNA must be used, dilute each plasmid in the same tube to 1 ng/µL.

14. Adding 1 ng of template DNA ensures that parental DNA is eliminated during DpnI digestion; however, when PCR amplification becomes challenging, we use up to 5–10 ng of template DNA.

15. 18 PCR cycles provides sufficient PCR product while minimizing the chances of polymerization errors. However, we use up to 25 cycles when challenging cases are found.

16. Using 1 ng of template DNA and 18 PCR cycles should yield obvious bands after agarose electrophoresis. If no bands are found, it is better to optimize the PCR amplification than to proceed with in vivo assembly. In such cases, we recommend confirming primer sequences, testing PCR additives, and adjusting annealing temperature during cycling. If contaminant bands are found from PCR, the desired products can be isolated by gel extraction before proceeding with transformation (no DpnI treatment is required when gel extraction is performed).

17. When performing fragment amplification in separate PCRs, each product is mixed in equal ratios before by DpnI treatment and transformation as per standard procedures. The ratio of each fragment can be compensated if significant intensity differences are found after agarose electrophoresis.

18. This is for homemade competent cells with 10^6–10^7 cfu/µg of DNA. If commercial competent bacteria with 10^9 cfu/µg of DNA competency are used, 25 µL of cell suspension is sufficient.

19. Using in vivo DNA assembly with restriction enzymes is useful for cases where plasmids are not amenable to PCR amplification, for example strongly GC-rich promoters, or containing repetitive sequence elements. In these cases, purify the digested vector and co-transform 30–50 ng with 2 µL of the homology-containing insert product after PCR. Efficiency can be improved by using a restriction enzyme or combination of enzymes which do not leave small complementary "sticky-ends," to limit vector recircularization in vivo.

20. When using gene synthesis, solubilize synthesized DNA fragments in deionized water to obtain a 50 ng/µL stock solution and transform 1 µL (50 ng) along with the PCR amplified, DpnI-treated vector.

383 References

385 1. Watson JF, García-Nafría J (2019) In vivo
386   DNA assembly using common laboratory bac-
387   teria: a re-emerging tool to simplify molecular
388   cloning.   J   Biol   Chem   294:jbc.
389   REV119.009109

390 2. García-Nafría J, Watson JF, Greger IH (2016)
391   IVA cloning: a single-tube universal cloning
392   system exploiting bacterial In Vivo Assembly.
393   Sci Rep 6:27459

394 3. Beyer HM, Gonschorek P, Samodelov SL et al
395   (2015) AQUA cloning: a versatile and simple
396   enzyme-free cloning approach. PLoS One 10:
397   1–20

398 4. Jacobus AP, Gross J (2015) Optimal cloning of
399   PCR fragments by homologous recombination
400   in *Escherichia coli*. PLoS One 10:e0119221

401 5. Kostylev M, Otwell AE, Richardson RE et al
402   (2015) Cloning should be simple: *Escherichia
403   coli* DH5a-mediated assembly of multiple
404   DNA fragments with short end homologies.
405   PLoS One 10:1–15

406 6. Huang F, Spangler JR, Huang AY (2017) In
407   vivo cloning of up to 16 kb plasmids in *E. coli* is
408   as simple as PCR. PLoS One 12:1–21

409 7. Jones DH, Howard BH (1991) A rapid
410   method for recombination and site-specific

411 mutagenesis by placing homologous ends on
412 DNA using polymerase chain reaction. Bio-
413 techniques 10:62–66

414 8. Nozaki S, Niki H (2018) Exonuclease III
415   (XthA) enforces in vivo DNA cloning of *Escher-
416   ichia coli* to create cohesive ends. J Bacteriol
417   201:20181210

418 9. Conley EC, Saunders VA, Saunders JR (1986)
419   Deletion and rearrangement of plasmid DNA
420   during transformation of *Escherichia coli* with
421   linear plasmid molecules. Nucleic Acids Res 14:
422   8905–8917

423 10. Yang Y, Wang T, Yu Q et al (2021) The path-
424   way of recombining short homologous ends in
425   *Escherichia coli* revealed by the genetic study.
426   Mol Microbiol:1–14

427 11. Gibson DDG, Young L, Chuang RR-Y et al
428   (2009) Enzymatic assembly of DNA molecules
429   up to several hundred kilobases. Nat Methods
430   6:343–345

431 12. Kibbe WA (2007) OligoCalc: an online oligo-
432   nucleotide properties calculator. Nucleic Acids
433   Res 35:43–46

434 13. Inoue H, Nojima H, Okayama H (1990) High
435   efficiency transformation of *Escherichia coli*
436   with plasmids. Gene 96:23–28

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