Why Johnny can't clone: Common pitfalls and not so common solutions

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The demand for cloned genes has increased incessantly over the past 32 years, but some who need recombinant plasmids struggle to produce them. While the pitfalls of traditional ligation-dependent cloning are non-trivial, most can be avoided with sufficient effort and attention to detail. Here, the chemical properties of enzymes and reagents used to clone genes into plasmids are reviewed to draw attention to the most pertinent details. In particular, the virtues of agarose gel electrophoresis monitoring, the nature of the interactions between DNA and silica, and challenges associated with thermostable DNA polymerases, restriction endonucleases, and T4 DNA ligase are explored. Common pitfalls associated with *Escherichia coli* transformation and DNA modifying enzymes are also described. A thorough understanding of established methods is essential for troubleshooting, implementing alternative approaches, and inventing new techniques in response to changes in technology and demand.

The integration of DNA into a vector and subsequent use of the recombinant product to transform a microorganism (molecular cloning) seems deceptively straightforward. Cloning of DNA fragments into a bacterial plasmid was first reported in 1973 (1), while the cloning of PCR products was first reported in 1986 (2). Ongoing refinement of techniques, the development of alternative approaches, commercialization of cloning kits, and the emergence of specialized contract research organizations (outsourcing) has offered users a wide variety of options when it comes to molecular cloning experiments. In some ways, the production of plasmid constructs thus appears commodified to those who don't do the actual work.

The technical obstacles in molecular cloning are not all trivial, but the expertise and effort required to overcome them is underappreciated. Novices don't always have the wherewithal to understand or circumvent pitfalls, leading in some instances to shame or anxiety for those who struggle to clone genes and libraries. If a technique fails to produce a desired construct, researchers may resort to repetition of the experiment, generally with the same unfortunate results, or the ill-informed trial-and-error of alternative approaches. The aforementioned proliferation of new cloning techniques and kits is driven at least in part by reliability issues associated with those that already exist. This abundance of options

can be problematic though, as it also creates confusion for scientists because developers argue, often without evidence, that their method is faster, more convenient, more efficient (at least under optimal conditions), and cheaper than competing approaches. The fixed costs of research, particularly labor in high-income countries, far exceed the marginal variable costs of the reagents used in molecular cloning, so nothing is more costly than experimental failure and repetition. Therefore, the most effective way to improve cloning productivity is to utilize techniques that are easy to understand, monitor, and troubleshoot, so that repetition is minimized.

Cloning reactions are mediated by enzymes that did not evolve

for the sake of molecular biologists. Their activities *in vitro* are easiest to understand within the framework of classical biochemistry. Here, I review the catalytic and biophysical properties of the enzymes, substrates, and reagents used to clone DNA fragments and PCR products and connect these properties with common molecular biology techniques such as DNA purification and isolation. Recent reviews have been biased toward newer cloning approaches (3), so it would be easy for students to think paradoxically that established techniques don't work as well. Every reaction, both desired and dreaded, proceeds at some rate that is itself a function of particular concentrations and microscopic rate constants. As such, a mechanistic understanding of these reactions is essential, both to optimize existing protocols and develop new ones.

“Awesome” agarose

PCR products are traditionally cloned using primers that incorporate unique restriction sites near (but not at) the ends (4, 5). Restriction endonucleases are used to generate complementary “sticky” overhangs, or blunt ends, at the termini of the PCR product and the recipient plasmid. Digestion products are separated via agarose gel electrophoresis; desired restriction fragments are then extracted from the gel. The fusion of the two DNA fragments is catalyzed by T4 DNA ligase. *E. coli* cells are then transformed with the ligation product(s), and transformants are selected by virtue of antibiotic resistance markers encoded on the plasmid (2). This traditional approach is labor-intensive, but the pitfalls of every step are well-understood and, except for purity of the DNA and structural heterogeneity at the ends of the PCR product (6), easy to monitor using agarose gel

Figure 1. The workflow of traditional molecular cloning of a PCR product (green) into a plasmid (blue). Most of the common pitfalls (solid red) can be directly observed via agarose gel electrophoresis. The exceptions—the presence of impurities in purified DNA and incomplete digestion of restriction sites near the ends of the PCR product (red outline)—are usually detected indirectly by the failure of a downstream reaction.

electrophoresis (Figure 1). Plasmids and PCR products should thus be visualized before and after purification, as well as after restriction digestion, gel purification, and ligation so that the completion of each step in the cloning process can be confirmed before moving on to the next. Micro-volume UV spectroscopy has become popular in recent years (7), but these instru-

ments were not designed to differentiate between the substrates and products of DNA modifying reactions. Agarose gel electrophoresis enables recognition of problems as they arise; experienced cloners can save time by solving these problems or terminating experiments flawed beyond repair and starting over.

Alternatives to agarose gel electrophoresis have been devised

(8–10), but none are as widely used. In my lab, mini-gels are favored for the resolution of smaller fragments (<5 kb) since they offer convenience, speed, and greater sensitivity (compared with larger gels) at lower reagent cost (agarose, stain, and electrophoresis buffer, see Supplementary Material). Mini-gels poured on 75 × 52 mm glass slides can be produced in bulk and then stored (Figure 2). The shorter and thinner gels can be electrophoresed more quickly without over-heating than those that are more substantial (5 V/cm in a short gel box, such as the Owl C2-S). The resolution (physical separation of bands) is not as good as what can be obtained with larger gels (Figure 3), but it is adequate for shorter fragments used in routine cloning experiments. We use low molarity electrophoresis media (10 mM lithium borate, pH 8.2) for rapid resolution of small (<2 kb) bands (11); we use higher conductivity TAE buffer

(40 mM Tris base, 20 mM acetic acid, 1 mM EDTA) for larger bands because these do not resolve as well in our mini-gels in lower molarity media (5 mM lithium acetate, pH 7.2). Any contrivance that encourages cloners to run more gels (12–15) promotes continuous improvement of quality control standards.

“Stick-less” silica

DNA purification (isolation of plasmids from nucleic acids and other contaminants in bacterial cell extracts, PCR products from primers, template, and polymerase, restriction fragments from other fragments and agarose) can make or break any cloning project (Figure 1). The efficiency of any subsequent enzyme-catalyzed reaction is predicated upon the concentrations of DNA substrates and inhibitors, including undesired DNA (16,17). Most molecular biologists now rely upon DNA purification

kits based on silica, or less frequently ion exchange resins, as opposed to the phenol/chloroform extractions (18), alcohol precipitations (19), and cesium chloride gradient ultracentrifugations that were common in the past (20). Anion exchange chromatography is time-consuming, so it is generally employed only in those instances where the purity of plasmids isolated from cell extracts is essential, such as mammalian cell transfection. Silica spin columns are relatively inexpensive, convenient (disposable), and versatile, as they can be used for plasmid preparation, PCR product purification, or restriction fragment purification from agarose gels.

Purification of DNA from agarose gels is worth special consideration here since isolation of a desired restriction fragment prevents the generation of undesired ligation products. However, excision of a band from an agarose gel is technically demanding, and therefore recalcitrant to automation. Furthermore, exposure of DNA to UV light sharply reduces transformation activity, although the addition of guanosine to the running buffer does offer significant protection (21). Purification yields obtained from current gel purification techniques, described briefly below, are often modest, so most cloners digest microgram quantities of DNA (hundreds of billions of double-stranded DNA molecules) to generate a single final recombinant molecule. The benefits of gel purification, which include improved cloning efficiency and fewer undesired ligation products, nevertheless usually outweigh the aforementioned drawbacks and pitfalls, so every cloning expert masters at least one agarose extraction technique.

Not all researchers favor the same gel purification technique, which implies that none are infallible. In our lab, we use silica spin columns to purify restriction digested DNA from

Figure 2. Glass side agarose minigels. LE agarose (0.8%) is dissolved in Tris Acetate EDTA (TAE) buffer by boiling in a microwave oven. The agarose solution is cooled so that the bottle can be held without protection (50°C); 12 mL of the solution is poured onto the 75 × 52 mm glass slide and is retained on the slide by surface tension. The ruler and binder clips suspend the combs just above the slides. The 10-tooth, 1-mm-thick comb (MGC-1010; C.B.S Scientific) is used to prepare gels to assess the quality of PCRs, plasmid preparations, restriction digests, gel purifications, and ligations; each lane can hold up to 10 µl of sample, including loading dye. The single-tooth comb, composed of 2 halves of a 75 × 52 mm glass slide clipped together, is used to create gels from which desired bands will be excised and purified; the lone well can hold 100 µl of a restriction digest plus loading dye.

agarose but deviate from commercial protocols (as described below) to maximize both yield and purity. Other commonly used gel purification techniques include electroelution (from gel slices or by running bands into second wells) (22, 23), adsorption and elution from anion exchange paper (Whatman DE81 or Schleicher and Schuell NA-45), phenol/chloroform extraction of low melting temperature agarose, freeze-squeeze, and agarase digestion (24). It is likely that most cloners utilize silica spin columns for gel extraction not because they produce the highest yields or purity but rather because they are relatively easy to use. Residual agarose or the various solvents and salts introduced in different gel extraction protocols may inhibit the activity of T4 DNA ligase and, therefore, the overall cloning efficiency. These substances are difficult to detect, so they are generally regarded as “known unknowns” until efficient ligation is subsequently observed via agarose gel electrophoresis.

Our understanding of the chemical interactions between DNA and silica remains incomplete, but it is sufficient to understand and overcome common DNA purification pitfalls. In a model proposed by Melzak et al., binding is driven first by the electrostatic shielding of the negative charges of the phosphate backbone of DNA and silica, second by the disruption of ordered water molecules that solvate each of the binding partners by chaotropic salts and organic solvents (entropy), and third by the formation of intermolecular hydrogen bonds (25). Subsequent studies have elaborated specific aspects of the model, such as the roles of buffer, pH, and ionic strength in the absence of chaotropic salts (26). The equilibrium between free double-stranded DNA and silica-bound DNA is sensitive to the concentra-

tions of several reactants: protons (pH), salts (ionic strength), chaotrope (guanidine hydrochloride), organic solvent (alcohol), DNA, competing contaminants (including undesired nucleic acids), and silica (dimensions of column). The phosphodiester linkages in the DNA backbone and the silanol groups on the surface of silica are negatively charged at neutral and basic pH. The consequent electrostatic repulsion is overcome at acidic (low) pH and high ionic strength. We use sodium acetate (0.3 M final), pH 5.2; double-stranded DNA can denature or even hydrolyze at substantially lower pH values (Supplementary Material). Both DNA and silanol form hydrogen bonds with water, so release of this bound water by chaotropic salts, organic solvents, or the binding of

the two macromolecules is entropically favorable.

Since the enthalpy of the binding of DNA to silica is apparently modest, the reaction temperature should not have much effect upon the concentrations of free and silica-bound DNA at equilibrium. Linearized plasmid binds silica more readily than does the super-coiled form of the same DNA, consistent with the prediction that conformational constraint is entropically unfavorable and limits the formation of binding interactions (25). The ideal sample would contain the desired DNA (and no other nucleic acids) at an amount roughly equal to the binding capacity of the column in a minimum volume. A chaotropic salt would then be added to saturating concentrations at pH $\approx$ 5 prior to applying the sample on a silica matrix.

10. **lambda HindIII digest**
9. **insert + ligase**
8. **gel purified insert**
7. **vector + insert + ligase**
6. **vector + ligase**
5. **gel purified vector**
4. **donor plasmid digest**
3. **recipient plasmid digest**
2. **uncut donor plasmid**
1. **uncut recipient plasmid**

Figure 3. Plasmid preps, restriction digests and ligations assessed by agarose minigel electrophoresis. Plasmids from an arbitrarily chosen cloning experiment (*NcoI*-rpmG-rbs-*NdeI*-tagRFP-*HindIII*-pUC57-mini and 6his-galM-pUC57-mini) were prepared by alkali lysis, isopropanol precipitation, and silica purification. The purified plasmids were digested overnight with restriction enzymes *NcoI* and *SpeI*. The recipient vector and insert were separated from undesired bands in a preparative single well minigel (see Figure 2). The desired bands were excised with a razor blade and purified with silica spin columns as described in the text. The recipient vector and insert were ligated for 6 hours (cycled between 10 and 30°C, 30 s each) in a T4 DNA ligase-mediated reaction (1 Weiss unit per 20 μ l reaction, 5% polyethylene glycol 8000). A 0.8% LE agarose minigel was submerged in TAE buffer in a gel box (Figure 2). Samples were mixed with loading dye, transferred into the wells of the gel, and the DNA was separated for 15 min at 150 V. The recipient plasmid preparation was contaminated with chromosomal DNA (lane 1, bottom, high molecular weight bands) that subsequently reacted with the restriction enzymes (lane 3) and was mostly removed during gel purification (lane 5). The ligation of the vector to other copies of itself can be observed (compare lane 6 to lane 5). The ligation products were subsequently reacted with restriction enzyme *NdeI* (not shown), which recognizes only the stuffer fragment carried by the intact recipient plasmid and used to transform *E. coli* Mach1 (Table 1).

Recipes for buffers associated with commercially available kits can sometimes be discerned from patents (27). Ideal binding conditions can be approximated through the addition of 0.1 volumes of 3 M sodium acetate, pH 5.2, followed by an equal weight/volume quantity of crystalline guanidine hydrochloride, rather than the recommended larger volumes of proprietary buffers that come with most commercial kits.

We have found that plasmid preps can be optimized by harvesting and lysing higher than recommended volumes of *E. coli* cell culture using sequential addition of 3 solutions: 50 mM Tris-HCl, pH 7.6, 10 mM EDTA without RNase A; 1% sodium dodecylsulfate, 200 mM sodium hydroxide; 3 M sodium acetate, pH 5.2. The insoluble debris should be eliminated by centrifugation, and the nucleic acids should be precipitated with isopropanol to eliminate protein and small molecule contaminants. DNA and RNA should be resuspended in TE containing RNase A and analyzed by agarose gel electrophoresis, so that the quantity of treated extract (mixed with guanidine hydrochloride) applied to each spin column can be calibrated. For PCR product purification, the concentration of a 50% w/v solution of guanidine hydrochloride is roughly 5.5 M, which is sufficient to denature *Taq* DNA polymerase. Thermostable polymerases tend to co-purify with PCR products and interfere with subsequent enzyme-catalyzed cloning reactions (*vide infra*). For gel extraction of the desired restriction fragments, the agarose should be dissolved in a minimum volume prior to the addition of crystalline guanidine hydrochloride to achieve the maximum concentration of DNA and chaotrope in the minimum volume (Supplementary Material). In our experience, these measures improve yield and apparent purity of DNA.

“Problematic” polymerases

While thermostable DNA polymerases such as *Taq* make PCR possible, these enzymes complicate subsequent cloning steps. Newer DNA polymerases and mixtures of polymerases that are more accurate, more processive, and more thermostable than *Taq* DNA polymerase (28–35) might be better for PCR, but seem worse for subsequent cloning for reasons I will detail below. All DNA polymerases used in PCR are A-type (polA, DNA polymerase I) or B-type (polB, DNA polymerase II), both of which are composed of single polypeptide chains (36). C-type DNA polymerases, which serve as the main replicative enzyme *in vivo*, are composed of multiple subunits, making these enzymes more technically difficult to reconstitute *in vitro*. DNA polymerases used in PCR are not very processive (28), so some primers are not completely extended. These “ragged ends” are difficult to detect by agarose gel electrophoresis and are not recognized by restriction endonucleases (37).

There are two additional properties of DNA polymerases that are essential for PCR but detrimental to subsequent cloning steps. First, the dissociation constant of the *Taq*/DNA complex is ~20 nM at room temperature (38), comparable to the final concentrations of enzymes and amplification products, so the enzyme tends to co-purify with its amplification product. Most other commercially available DNA polymerases actually bind DNA even more tightly (39). Undesired polymerases can occlude the ends of the DNA from restriction enzymes or fill in overhangs created by them. Second, DNA polymerases engineered for PCR are more thermostable than *Taq* (36). Thermostability generally correlates with greater conformational stability in organic solvents (40–42) and chaotropic salts

(43), making it more difficult to purify PCR amplification products away from the polymerase. Guanidine hydrochloride concentrations achieved in commercial PCR purification kits are not sufficient (<4.5 M) to denature *Taq* (44–48). Low processivity, affinity for DNA ends, and resistance to chaotropic salts are intrinsic common properties of thermostable A- and B-type DNA polymerases, so the solution to cloning problems lies not in the choice of polymerases but rather with the purification protocol.

“Rambunctious” restriction endonucleases

Type II restriction endonucleases, which catalyze the hydrolysis of DNA sequences in or near specific recognition sites, evolved to protect bacteria from phages and other invaders. The K_M values of restriction enzyme/DNA substrate complexes are consequently quite low (high affinity; <2 nM for *EcoRV* in reactions with plasmid DNA), as are the corresponding k_{cat} values (slow; 1.7 min⁻¹) (49). Molecular biologists will at times try to maximize the concentration of DNA in restriction digests so that yields from subsequent gel purification steps will be adequate for ligation reactions. Unfortunately, restriction enzymes are effectively substrate inhibited in reactions with complex DNA, as sequences similar to the target restriction site function as enzyme inhibitors or alternative substrates (star activity) at sufficiently high concentrations (50). However, incomplete digests are costly because they ultimately lead to high background populations of colonies carrying parental plasmids from which the desired recombinant plasmids must be identified in high-throughput screens (51–57).

Restriction enzyme digests cannot be “forced” to completion

by simply adding saturating concentrations of restriction endonucleases since high concentrations of enzyme, substrate, salts, or glycerol can lead to star activity (50). The optimum strategy in these cases is to set up digestion reactions with moderate concentrations of enzyme and substrate DNA, each slightly higher than the K_M , and let those reactions proceed for many hours to completion. The concentration of *EcoRV* in a typical reaction (20 units/50 μ l) is 1.8 nM (specific activity = 8 units/ng, according to New England Biolabs; molecular weight = 28.3 kDa); the concentration of target restriction sites is 6 nM (1 μ g of 5 kb plasmid, containing one *EcoRV* site per molecule, in a 50 μ l reaction). Brief incubations might be tempting but are inadvisable, at least for digestions of cloning vectors, because reaction velocity decreases as a substrate is depleted (this is a characteristic of any post-steady-state, enzyme-catalyzed reaction). If overnight incubations of the restriction digest reaction fail to achieve complete DNA digestion, it is possible that the enzyme is denatured or inhibited by a contaminant in the DNA preparation (58). Such hypotheses can be tested through the use of a positive control template, such as commercially prepared bacteriophage λ DNA (e.g., New England Biolabs N3011S). At times, inhibitors can be controlled by dilution (the reaction of the same amounts of enzyme and substrate in larger volumes of buffer). It may subsequently become necessary to concentrate the digested DNA via silica spin column purification for loading into the well of a preparative agarose gel. Any background population of parental plasmids can be further reduced after the ligation by using a second restriction endonuclease that recognizes the parental plasmid but not the desired recombinant one (59, 60) (Figure 3).

“Lamentable” ligases

T4 DNA ligase evolved to seal nicks in double-stranded DNA. The effective K_M of this enzyme in complex with annealed sticky-ended DNA (600 nM) or blunt-ended DNA (50 μ M) (61) is nearly 2 orders of magnitude higher (much lower affinity) than that of an analogous type II restriction endonuclease/double-stranded DNA complex. The rates of ligation reactions, as well as the relative proportions of desired and undesired ligation products, are a function of the concentration of reactants, catalyst, and crowding agents and, to a lesser extent, time and temperature (Supplementary Material). Manufacturers recommend only 40 nM T4 DNA ligase (0.1 Weiss units = 6.7 cohesive end units; specific activity = 2000 Weiss units/mg; molecular weight = 60 kDa) and 1 nM DNA substrate (20 fmoles/20 μ l) be used in a ligation reaction, but higher concentrations should increase reaction rates. Reaction temperatures are usually some compromise between the annealing temperature of the overhangs and the optimal temperature for T4 DNA ligase activity; reaction rates can actually be increased by cycling between these temperatures (62). The margin for error here is relatively large since the consequence of suboptimal ligation reactions (ie. fewer transformants) is far less costly than that of incomplete restriction digests (ie. higher proportion of undesired ligation products).

Low concentrations of DNA substrates ($\ll K_M$) will favor intramolecular ligation, at least in the case of double-stranded molecules with compatible overhangs at each end. Increased concentrations of DNA will also favor intermolecular ligations, but it should be noted that too high concentrations lead to long concatemer formation (assemblies of multiple vector and insert

monomers) that don't transform *E. coli* efficiently (63,64). Optimal concentrations of vector and DNA insert in a ligation reaction (those producing the most desired vector-insert recombinant product) is dependent upon the nature of the insert and vector overhangs (ie. blunt, short overhangs, long overhangs) and the length of DNA since the ends of shorter strands are more likely to react with each other (65). It is not practically possible to set up ligation reactions with DNA substrate concentrations that approach or exceed the K_M of the enzyme/substrate complex. However, the inclusion of a crowding agent such as polyethylene glycol (5%, 8000 MW) can optimize the ratio of inter- and intra-molecular ligations (66–70).

Ligation reactions fail for numerous reasons, but failure is most commonly the result of problems that occur prior to the addition of T4 DNA ligase: non-uniform DNA ends produced from incomplete DNA polymerase extensions, incomplete restriction digests, ligase inhibitors, or the fill-in of overhangs catalyzed by thermostable DNA polymerases that co-purified with the PCR product. In some cases, ligation reactions fail because DNA ends fail to anneal frequently enough, possibly because the effective concentration is too low. Alternatively, ATP concentrations in the reaction buffer can be too low (perhaps due to hydrolysis after extended incubations at room temperature) or the enzyme can denature. [T4 DNA ligase is fairly stable at ambient temperatures (71), though not when diluted in water instead of a proper buffer.] When there is a question, the integrity of the enzyme and buffer can be tested using a positive control; for instance, the commercially available λ *HindIII* digest (e.g., N3012S, New England Biolabs), which is more often employed as a molecular weight standard for agarose gel electrophoresis (Figure 3), can serve as a control ligation.

The progress of ligation reactions, and indirectly the uniformity of the overhangs at the ends of DNA fragments, can be monitored using agarose gel electrophoresis. In these cases, the mixture of ligation products (i.e., insert/insert, vector/vector, insert/vector, etc.) will typically migrate as a smear at a higher molecular weight range than the no ligase control reaction (Figure 3); very high molecular weight concatemers, which don't transform *E. coli*, will in many instances fail to migrate out of the wells (not shown). Confirmation of the desired ligation product can also be tested via PCR using insert-specific and vector-specific primers; the product can then be sequenced to confirm that the insert and vector recombined as expected. Finally, it should be noted that although the ligation of a plasmid molecule to another copy of itself produces a long inverted repeat that cannot replicate efficiently within *E. coli* (20), recombination or rearrangement events in vivo can lead to the creation of undesired plasmids that contribute to the background colonies observed in colonies after *E. coli* are transformed with ligation reactions (Table 1).

"Incompetent" cells

The efficiency by which *E. coli* cells are transformed with any ligation reaction is a critical determinant of the success of a cloning project. As already noted, enzyme-catalyzed reactions don't always reach equilibrium in vitro. However, highly efficient transformations can compensate for molecular cloning inefficiencies at any previous step(s). The two most common transformation approaches are heat shock of chemically treated cells and electroporation, although several other quicker but less efficient methods that been developed (72–78). Electroporation is the most efficient of these techniques, and, more importantly, in

Table 1. Transformation of *E. coli* with ligation reactions^a

Ligation reactants	amp ^r cfu/ 0.1 mL ^b
vector only	0
vector + ligase	35 ^c
vector + insert + ligase	281
insert only	0
insert + ligase	0

^aThe experiment is described in the Figure 3 legend.

^bThe transformation efficiency of the *E. coli* Mach1 cells was $2 \times 10^7/\mu\text{g}$ of closed, supercoiled recombinant plasmid.

^cBackground colonies probably carry plasmids with undesired inserts derived from chromosomal DNA in the plasmid preparation or vector-vector dimers that have rearranged to eliminate long inverted repeats.

electroporation the number of transformed cells is proportional to the quantity of DNA up to microgram quantities (79,80). In our lab, electroporation transformation efficiencies are not as strain dependent or plasmid size-dependent as when we use the heat shock approach (81,82). These considerations make electroporation the clear method of choice for difficult cloning projects, such as the construction of libraries. Many users rely on electroporation for all of their *E. coli* transformation experiments.

Chemical transformation is not as efficient as electroporation, particularly for wild strains, and is inhibited by an excess of DNA (>10 ng per 200 μL competent cells). Nevertheless, chemical transformation is very commonly employed by molecular biologists because it is far more convenient than electroporation (81, 83–85). Some strains remain chemically competent for many months when stored at -80°C , making the technique less laborious. In addition, chemical transformation is not sensitive to salts in ligase buffers. In our lab, we routinely add 1 ng DNA in heat-inactivated (65°C for 10 minutes) ligase buffer to 25 μL chemically competent cells in a thermocycler initially set to 4°C ; the instrument is subsequently programmed to continue to cold incubation for 30 min, followed by a 42°C heat shock

for 40 s and subsequent reduction of temperature. SOC medium (2% tryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM MgCl_2 , 10 mM MgSO_4 , and 20 mM glucose) (100 μL) is added to each transformation, and the cells are incubated for 1 h at 37°C prior to spreading on an agar plate supplemented with the appropriate antibiotic.

Mid-log phase cells for use in both protocols are prepared in a similar way—both electrocompetent (86) and chemically competent cells (81) that have been agitated in rich media at 18°C transform more efficiently than cells propagated at 37°C . However, chemically competent cells are less concentrated (12.5-fold more concentrated than mid-log cultures) than electrocompetent cells (>500-fold more concentrated than mid-log cultures). Researchers should also note that the efficiency of chemically competent cells increases with storage at -80°C (81), while fresh electrocompetent cells transform more efficiently than do frozen electrocompetent cells (87). Since electroporation is highly sensitive to the salts normally found in ligase buffers, thus necessitating measures to purify the ligated DNA (88–90), many molecular biologists continue to rely upon heat shock of chemically treated cells in routine cloning projects.

New techniques, new pitfalls

Development of new cloning techniques continues regularly, driven in part by dissatisfaction with existing methods. A comprehensive review of such "alternative" cloning techniques is beyond the scope of this article, but I would encourage readers to consider using these techniques with the same understanding and application of nucleic acid biochemistry presented here. Many developers, particularly those

who sell commercial kits, claim that their techniques are faster, cheaper, and more efficient, but experienced scientists are skeptical of those who fail to test or disclose the limitations of their methods. No DNA purification technique or enzyme-catalyzed reaction is 100% efficient or specific, particularly under suboptimal conditions, so the techniques that are easiest to monitor and troubleshoot tend to be most reliable.

TA-cloning is based upon the propensity of *Taq* DNA polymerase to add a single adenosine overhang to the 3' end of an amplified target. The amplification product can be ligated into a linearized plasmid with complementary 3' thymidine extension (91–95). This technique depends to a large degree on the efficiencies of the polymerase-catalyzed end-modifying reactions and subsequent ligation reaction. Inserts generally end up ligating in both orientations. Yet another class of cloning reactions employs recombinases, such as topoisomerase or λ integrase, to fuse targets to an acceptor plasmid. In these reactions, a target gene must be flanked on both sides by regions that are identical to those in the acceptor plasmid. The PCR primers, therefore, contain extensions ranging from 1 to 50 bases in length, depending on the particular variation (96–98). These methods rely upon the efficiency of the end-modifying enzyme, which is difficult to monitor, and in some cases upon proprietary components (pretreated vector, enzyme mixes, or specific competent *E. coli* cells).

Ligation independent cloning (LIC) is a technique based on single-stranded complementary extensions ranging from 10 to 15 bases in length on both the vector and insert; an annealing reaction creates a doubly-nicked, but stable, plasmid that can be directly transformed into *E. coli* (99,100). Single-stranded DNA overhangs are created through the

3'–5' exonuclease activity of DNA polymerase (if the sequence at the ends of both the insert and linearized plasmid lack one of the four nucleotides) (100,101), a nicking endonuclease (102), uracil DNA glycosylase (103,104), or rare earth metals (105) (if oligonucleotide primers used to amplify the insert and vector contain uracil at certain positions). LIC reactions, like TA cloning, are difficult to monitor. Furthermore, all approaches to LIC require the design of insert and the vector sequences according to strict criteria, the efficient creation of single-stranded DNA overhangs prior to cell transformation, and the efficient repair of gaps and nicks in vivo. Ligations and recombination events within a cell are generally facile but difficult to optimize (106,107), as are in vitro ligations of three or more DNA fragments (108–110). The point of this partial recitation is not to deny the utility of alternative cloning techniques, but rather to encourage readers to fully understand a protocol before employing it.

In the end, there continues to be a strong demand for plasmid constructs, and this will likely continue. Gene synthesis will never obviate molecular cloning because its products, like those of PCR, are usually ligated into at least one plasmid (though not necessarily by the end user). In the past, the invention of new molecular cloning techniques was motivated by the discovery of new DNA modifying enzymes. I expect that in the near future, advances in DNA purification chemistry and laboratory automation will prove more consequential to the evolution of cloning technologies. Syntheses of oligonucleotides, oligopeptides, and genes are now automated, as are many analytical techniques. Molecular cloning, however, is still mostly done by hand, likely because the pitfalls described above are too wide and too deep. The development

of new methods to purify DNA that achieve higher yields and greater purity at lower overall cost, could potentially improve the efficiencies of downstream enzyme-catalyzed reactions such that automation will become more feasible. The demand for recombinant plasmids could then be met by machines in consolidated facilities analogous to those that custom synthesize oligonucleotides.

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Competing interests

The author declares no competing interests.

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