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IVA Prime: automated primer design for *in vivo* assembly cloning

Radu Cristian Leonte[1], Patrick David Fischer[1], Beatriz Herguedas[2], Javier García-Nafría[2,*], Sebastian Hiller[1,*]

[1]Biozentrum, University of Basel, Spitalstrasse 41, 4056, Basel, Switzerland
[2]Institute for Biocomputation and Physics of Complex Systems (BIFI) and Laboratory of Advanced Microscopy (LMA), University of Zaragoza, Zaragoza, 50018, Spain

[*]To whom correspondence should be addressed. Email: sebastian.hiller@unibas.ch
Correspondence may also be addressed to Javier García-Nafría. Email: jgarcianafria@unizar.es

Abstract

Molecular cloning through *in vivo* assembly (IVA) is an efficient homology-based approach that can achieve complex cloning operations in a single step, bypassing the need for enzymes and reducing hands-on time and costs. However, primer design remains the most demanding task, requiring substantial expertise and a source of human error. Here, we introduce "IVA Prime," an intuitive online tool for automated primer design for IVA cloning, which reduces the workload and avoids errors. We describe how IVA Prime generates optimized primers for all cloning operations, i.e. mutagenesis, insertions, deletions, and subcloning. IVA Prime provides an intuitive interface and robust algorithms, thus mitigating the complexities associated with primer design into a single mouse click. A set of thoroughly tested default parameters allows both inexperienced users and experts to generate ready-to-order primer sequences for a standardized cloning protocol with high success rate. We describe the method and demonstrate its efficiency and reliability with example applications. IVA Prime is freely available at https://www.ivaprime.com.

Graphical abstract

Introduction

Molecular cloning techniques are a fundamental tool in biological research. Early methods were introduced in the 1970s and have evolved ever since [1–4]. Initially, methodology relied on the use of restriction enzymes and ligases [4], which involved the digestion of DNA fragments that could be later assembled through compatible ends using enzymatic ligation. Alternative approaches soon appeared such as polymerase chain reaction (PCR)-only [5–7], ligation-independent cloning [8], recombination-based methods [9–13], and multi-enzyme assembly methods [3, 14, 15]. However, each of these new methods came with their own demands, such as requiring either the use of specific enzymes, specific bacterial strains, or multiple DNA purification steps.

Although the ability of bacteria to assemble linear pieces of DNA with flanking homologous sequences was reported three

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decades ago [16], it is only in recent years that it has been expanded and fully exploited. *In vivo* assembly (IVA) cloning leverages endogenous recA-independent homologous recombination in *Escherichia coli* to facilitate enzyme-free molecular cloning in a directional and seamless manner [17–19]. IVA cloning enables simple and complex cloning operations (site-directed mutagenesis, insertions, deletions, and subcloning) to be carried out in a single PCR reaction with minimal hands-on time [17]. However, a bottleneck of the technique is the time investment and expertise required for primer design.

To address this issue, here we introduce IVA Prime, an online tool to automate primer design for IVA cloning. IVA Prime streamlines primer design on the plasmid map from a user-friendly interface directly into primer ordering formats. Thus, by alleviating the burden of primer design, IVA Prime improves the accessibility of IVA cloning for scientists across different fields. We describe the method and provide a set of example applications.

Materials and methods

IVA Prime development and deployment

Online deployment

IVA Prime was built as a purely JavaScript web application. The application is wrapped in the Django web framework and containerized in a Docker image for deployment to Google Cloud Platform (GCP). The latest version of IVA Prime is deployed automatically to GCP from the master branch of the GitHub repository.

Local deployment

IVA Prime can be locally deployed via Git and Docker with minimal setup. The application runs in the background and is accessible through any modern web browser. Detailed installation and usage instructions are provided in the GitHub repository.

Calculation of melting temperatures

In its default operation, the melting temperatures for the homologous regions (HRs) and template-binding regions (TBRs) are calculated using the following equation:

$$T_m\ (^\circ\mathrm{C}) = 64.9 + 41 \cdot f_{GC} - \frac{672.4}{L}, \qquad (1)$$

where f_{GC} is the fractional GC (guanine and cytosine) content and L is the length of the sequence. This basic equation is based on the original equation by Marmur and Doty [20] and includes a baseline salt correction for 180 mM Na^+ according to Schildkraut and Lifson [21], and a length dependence term, first introduced by Britten *et al.* [22]. Despite its simplicity, this equation has proven very robust for IVA cloning [17–19], and Equation (1) is therefore the default to calculate melting temperatures in IVA Prime. In special cases, a more realistic calculation of the melting temperature may be desired, and IVA Prime allows the user to replace Equation (1) by the melting point equation of SantaLucia *et al.* [23] for the calculation of the TBRs. The approach by SantaLucia *et al.* includes specific stacking energies between adjacent base pairs, as well as correction terms for salt and Dimethyl sulfoxide (DMSO). It can for instance be used to vary PCR buffer and program, in case the standard conditions do not yield satisfactory results for a given plasmid. In contrast, the HR melting temperature plays a role solely during the recombination events in the bacterial host. Its calculation is fixed to Equation (1), because IVA cloning has been optimized to its use [18].

Codon optimization

Codon optimization in IVA Prime is based on weighted codon randomization, which has proven effective in increasing yields and avoiding repetitive sequences [24–26]. The weights for the codons are calculated by exponentiating each frequency by the inverse square root of the standard deviation of all the frequencies corresponding to that amino acid (AA). This exaggerates the weights for organisms where specific codons are preferred but does not affect cases where many codons are equally likely. The codon usage tables used in the optimization are taken from the CoCoPUTs database [27].

Primer design for recombination efficiency experiments

Primers were designed for disrupting or repairing the lacZα gene in the pUC19 plasmid so as to induce a phenotype change (Supplementary Table S1). For single point mutations, the nonsense mutation of Pro6 to a stop codon was introduced. For short and long insertions, 8 and 32 bp of the lacZα N-terminus were deleted, respectively, then reintroduced using insertion primers. For subcloning, lacZα from a pUC19 vector without the lac promoter, was subcloned into an pUC19 plasmid where lacZα was deleted. For deletions, the lac promoter upstream of lacZα was targeted. For subcloning with short and long insertions, 8 and 32 bp, respectively, from the beginning of the gene were deleted in the plasmid lacking the lac promoter, then reintroduced during subcloning at the 5′ end.

Molecular cloning

PCR reaction mix

Corresponding forward and reverse primers were mixed at a concentration of 2.5 μM each. The PCR reaction mix is then assembled as such: 1 μl primer pair mix, 1 μl diluted template plasmid (1 ng/μl), 12.5 μl Phusion High-Fidelity PCR Master Mix with HF Buffer (Thermo Fisher Scientific), and 10.5 μl ddH_2O. For subcloning, the PCR reaction mix is as follows: 1 μl insert primer pair mix 1, 1 μl vector primer pair mix 2, 1 μl diluted template plasmid (1 ng/μl), 1 μl diluted source plasmid (1 ng/μl), 12.5 μl Phusion Master Mix, and 8.5 μl ddH_2O.

PCR program

The PCR program used was as follows: 98°C for 30 s (initial denaturation), 98°C for 10 s (denaturation), 60°C for 30 s (annealing), 72°C for 2 min (extension), go to step two 29 times, and 72°C for 5 min (final extension).

Transformation

The PCR product was digested with 0.5 μl DpnI (New England Biolabs) for 30 min at 37°C. Briefly, 2 μl of the PCR product were mixed with 48 μl KCM (500 mM KCl, 150 mM $CaCl_2$, and 250 mM $MgCl_2$) and added to 50 μl of TSS-competent *E. coli* DH5α. The bacteria–plasmid mixture was incubated on ice for 30 min, then 900 μl of super optimal broth with catabolite repression (SOC) medium was added and cells were incubated at 37°C for 1 h. For non-subcloning

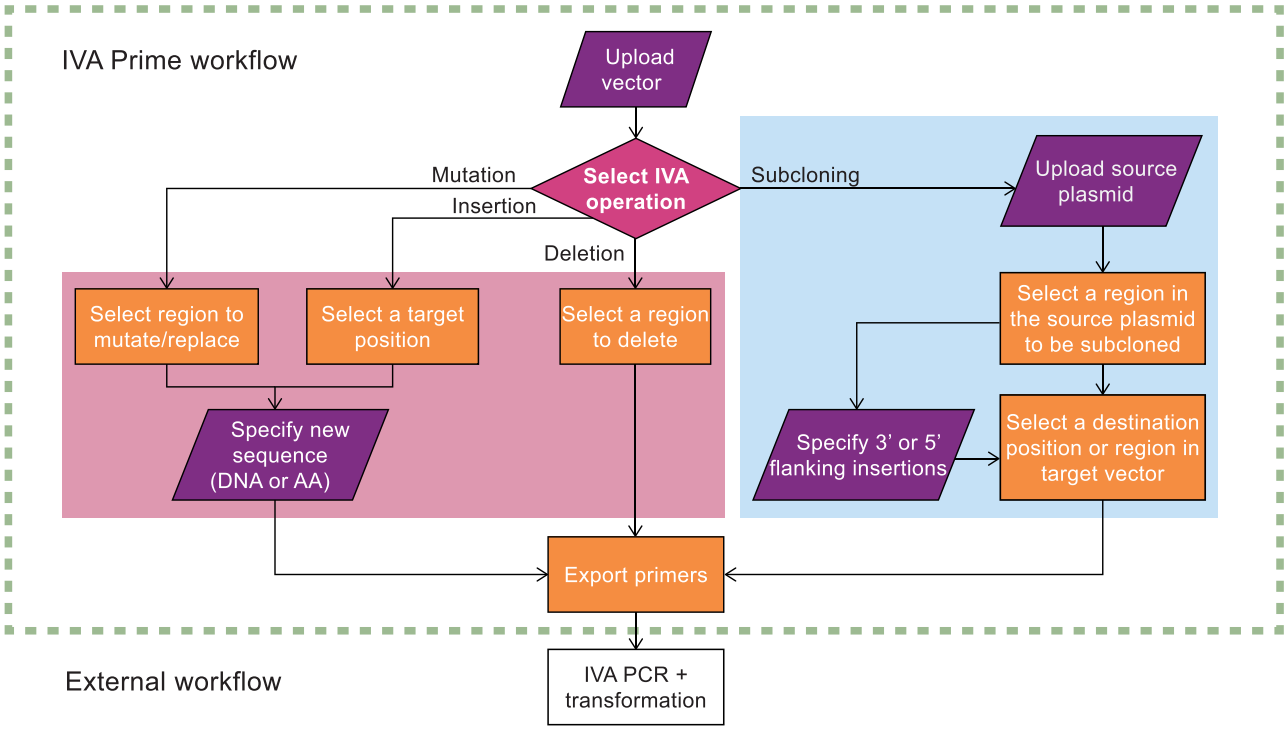

Figure 1. Flow chart illustrating the IVA Prime workflow. The IVA Prime workflow is framed in a dashed green line; subsequent experimental work is denoted outside of the frame. Operations on a single plasmid (mutations, insertions, and deletions) are shown in the pink box, operations involving genetic transfer between two plasmids (subcloning with or without insertions) are shown in the blue box.

operations, 100 µl were plated on LB-agar plates containing 100 µg/ml ampicillin, 0.2 mg/ml X-Gal (Sigma-Aldrich), and 1 mM Isopropyl-thiogalactopyranoside (IPTG; LuBio Science) and incubated at 37°C overnight. For subcloning operations, the entire 1 ml of bacteria suspension was centrifuged at 3000 × *g* for 3 min at room temperature, the supernatant was discarded, and the cell pellet was gently resuspended in 50 µl of SOC media and plated completely.

Results

IVA Prime workflow

IVA Prime is a pipeline to design DNA primers for IVA cloning. It is implemented as a web application that connects the user via an interface to a primer-generating algorithm and can be accessed at https://www.ivaprime.com. The workflow of IVA prime is shown in Fig. 1 and is structured around the four types of cloning operations that are possible with plasmid DNA. For each operation, the algorithm requests the minimal amount of information needed as user input and the algorithm subsequently generates the primer sequences. The primers are output in a format of choice, preferably one that can be used for direct ordering.

At the onset of the procedure, the user uploads the original plasmid map. For mutations and insertions, the user selects the region to be modified, enters the DNA or AA sequence to be inserted, and optionally, specifies the desired organism for codon optimization, with *E. coli* being the default. Deletions are performed by selecting a DNA region and choosing the option "Delete selection". For subcloning, the source vector is uploaded in a second tab and the region to be subcloned is marked by selecting it and clicking on "Mark selection for subcloning". The user then selects the destination position in the target vector in the first tab and the algorithm generates the primers. If additional insertions are desired adjacent to the subcloned region, the user can specify their DNA or AA sequence and have them optionally codon optimized. All operations generate a set of primers that can be directly exported to multiple, ready-to-order formats. Additionally, the plasmid is automatically updated with the newly cloned information and can be directly downloaded.

IVA operations

As a central mechanism, IVA cloning employs a recA-independent recombination pathway which assembles linear DNA fragments to a circular plasmid. The molecular mechanism underlying the recombination pathway is poorly understood, but it has been shown to occur in all standard laboratory *E. coli* strains tested with high reliability and efficiency. Hence, IVA cloning does not require specialized bacteria [18]. The DNA fragments need to contain HRs at their ends. The fragments are created from a template plasmid via PCR and are subsequently transformed into competent *E. coli*. To this end, IVA cloning requires primers that are designed to introduce the desired modifications and create the necessary HRs. Four types of cloning operations can be distinguished (Fig. 2). In each case, the primer pairs contain TBRs (gray), that define how the template plasmid will be amplified. An additional HR (shades of green, blue, or orange) is required on at least one primer for the recombination.

(i) For **mutations** (Fig. 2A), the TBRs flank the region to be replaced and the HR includes the new sequence (magenta). **Insertions** are similar, but do not feature offset TBRs. If the inserted sequence (magenta) is longer than easily accommodated with standard primer synthesis, it

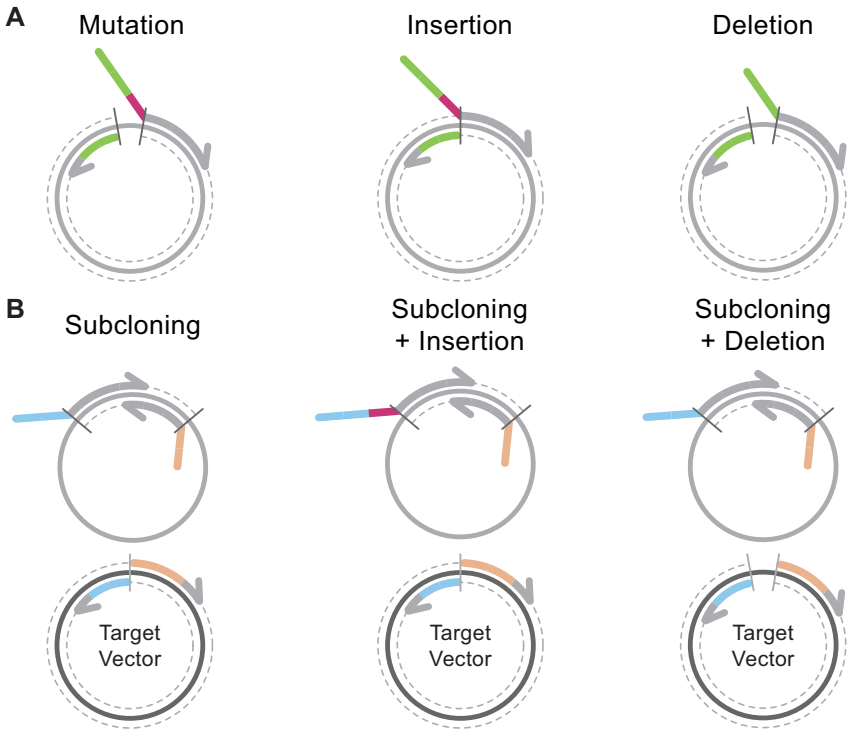

Figure 2. Overview of asymmetric primer design for the operations available in IVA Prime. (**A**) Operations on a single plasmid with a single recombination event. TBRs ($T_m = 60°C$) are shown in gray, newly introduced sequences are shown in magenta, and HRs ($T_m = 50°C$) are shown in shades of green. Segments in the HRs will recombine with other segments of the same shade. (**B**) Operations on two plasmids. The plasmid shown in the top row depicts the source plasmid, from which a gene is to be transferred. The plasmid in the bottom row depicts the target vector, into which the gene is to be inserted. TBRs ($T_m = 60°C$) are shown in gray, newly introduced sequences are shown in magenta, and HRs ($T_m = 55°C$) are shown in shades of blue and orange. Segments in the HRs will recombine with other segments of the same shade. Dashed gray lines indicate the extended sequence of the DNA polymerase.

is distributed between both primers, with overlapping regions serving as HRs. For **deletions**, the HR is comprised of a segment complementary to the 5′ end of the other primer.

(ii) **Subcloning** operations (Fig. 2B) require a set of four primers. One primer set (insert primers) amplifies a specific DNA region from the source plasmid (Fig. 2B, top row), while including HRs (shades of blue and orange) complementary to the target vector. The second primer set (vector primers) amplifies the target vector (Fig. 2B, bottom row). **Subcloning with insertions** is achieved by combining the standard subcloning operation with the short and long insertions by adding the newly introduced sequence to the forward insert primer and the reverse vector primer (insertions at the 5′ end) or to the reverse insert primer and the forward vector primer (insertions at the 3′ end). **Subcloning with deletions** can be achieved by offsetting the starting points of the TBRs of the vector primers.

The IVA Prime interface

IVA Prime accepts all common plasmid file types as primary input: GenBank record (.gb) and SnapGene maps (.dna). It also allows for the creation of a new file from a pasted sequence. By default, specific features of the pasted sequence are automatically detected and annotated. IVA Prime allows for the simultaneous editing of multiple files at the same time (Fig. 3A). After import, the plasmid sequence as well as any annotated features or translated sequences are displayed in the main window (Fig. 3B). A list of annotated features is displayed in the sidebar and can be used to edit the features as well as to navigate quickly to specific features (Fig. 3C). The plasmid sequence can be edited through cloning operations, i.e. new sequences can be inserted, existing sequences can be changed or entirely deleted. For each operation, IVA Prime will generate pairs of primers and display them at the top of the sidebar (Fig. 3D).

The use of IVA Prime is facilitated by a context menu that appears upon right-clicking inside the plasmid sequence. The context menu displays all possible IVA operations, taking into account whether the user has clicked on an insertion point or selected a region (Fig. 4A). The context menu also allows for quick copying or translation of selected sequences. For insertions and mutations, the user will be prompted by a popup window to specify the desired DNA or AA sequence, which can be optimized for the target organism of choice (Fig. 4B). Likewise, when subcloning, additional sequences can be specified to be inserted at the 5′ or 3′ ends (Fig. 4C). Once all modifications are made, the generated primers can be exported to a variety of formats (Fig. 4D), including order forms of oligo suppliers. The plasmid file can also be exported and will include all modifications.

Primer length determination

For each primer, IVA Prime starts from the user-specified sites and extends these in the 5′→3′ direction with bases from the template sequence until the resulting primer is at least 18 bases long, at which point IVA Prime calculates the melting tempera-

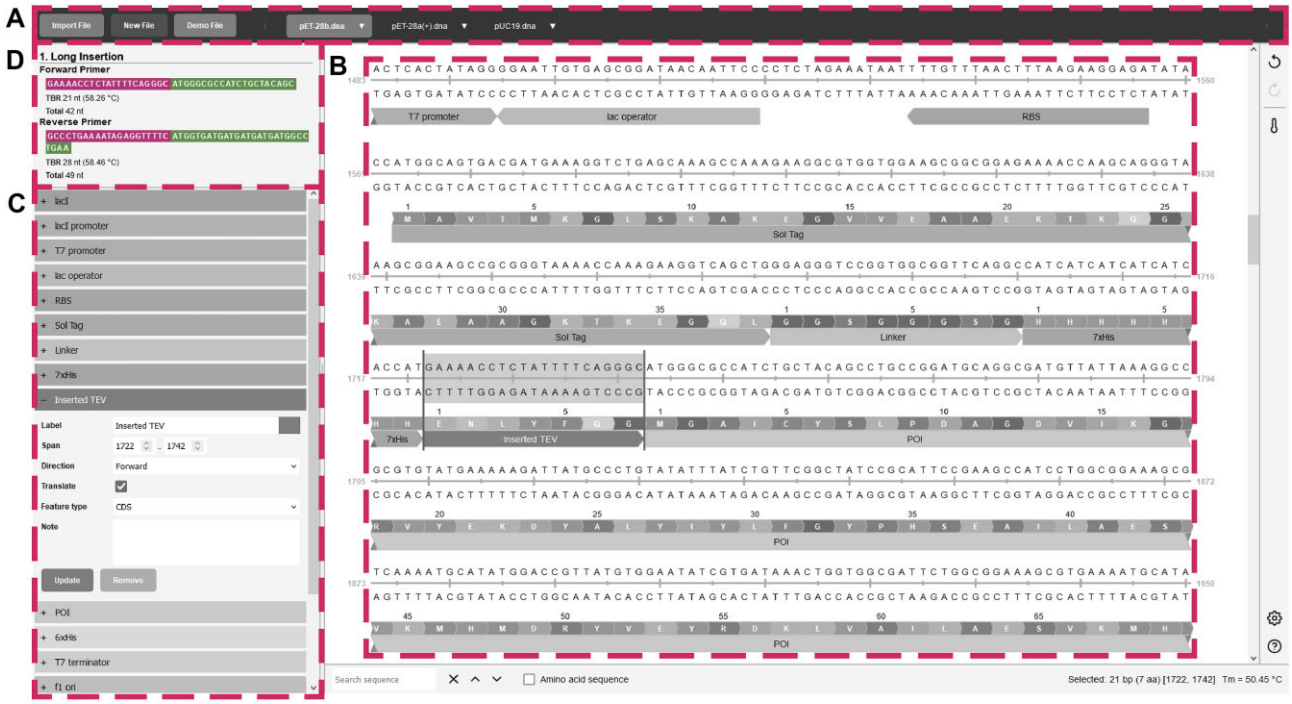

Figure 3. User interface of IVA Prime. (**A**) The navigation bar is used to import plasmid files and switch between opened plasmids. (**B**) The sequence grid displays the plasmid sequence as well as annotated features and serves as the main point of interaction when editing the plasmid and generating primers. (**C**) Annotated features from the plasmid file are displayed as a table, which can be used to quickly navigate to regions of interest. (**D**) Generated primers will be displayed at the top of the sidebar following an operation.

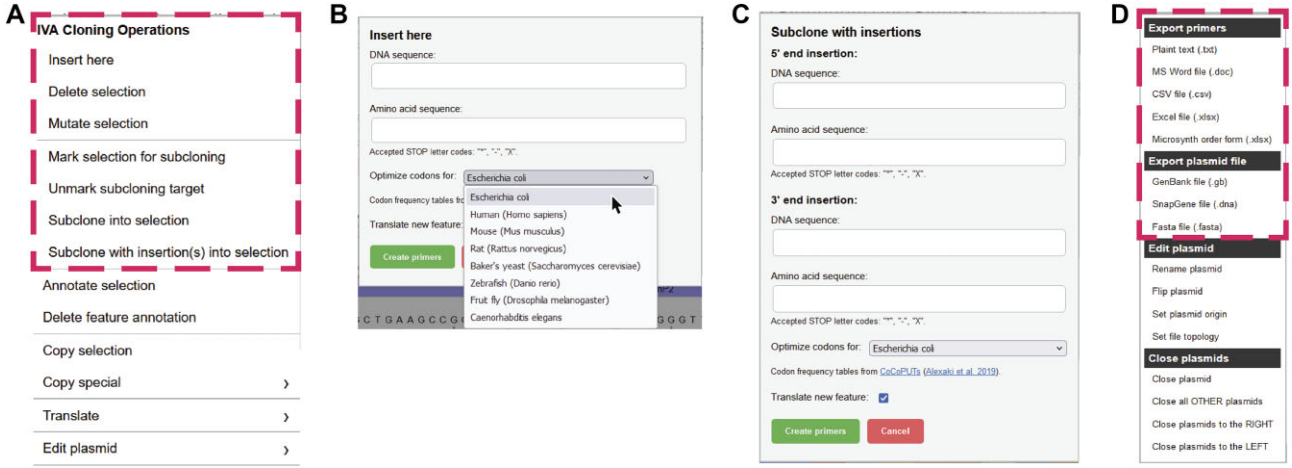

Figure 4. Additional functionality menus. (**A**) Context menu accessed by right-clicking on the main screen, featuring all the necessary IVA cloning operations and further options such as copying and sequence translation. (**B**) Popup window for insertions and mutations/replacements. The DNA or AA sequence to be inserted can be specified. The AA sequence will be codon optimized for the selected organism. (**C**) Popup window for subcloning with simultaneous insertion. (**D**) Generated primers can be exported to a variety of formats from the plasmid tab dropdown menu. The modified plasmid file can also be exported.

ture. It then continues to extend the primer until it has reached the target annealing temperature. IVA Prime will then create a homologous sequence complementary to the reverse primer's 5′ end, denoting the site of recombination. Depending on the IVA operation, IVA Prime will also insert a new sequence between the HR and the TBR, sometimes using the new sequence as part of the HR. It should be noted that the upper bound for the lengths of inserted sequences can be as high as 100 bp; however, alternative approaches that do not use such long primer synthesis might result in more cost-effective options.

By default, the HR will be allocated entirely to the forward primer (asymmetric primers). The procedure can be changed to create symmetric primers, which will distribute the HR among both forward and reverse primers, thus averaging primer lengths and creating a symmetric distribution among both primers (Supplementary Fig. S1). Asymmetric primers are useful when performing multiple similar cloning operations from the same starting position, as it allows for the same reverse primer to be used in all these operations. On the other hand, a distributed HR creates a shorter for-

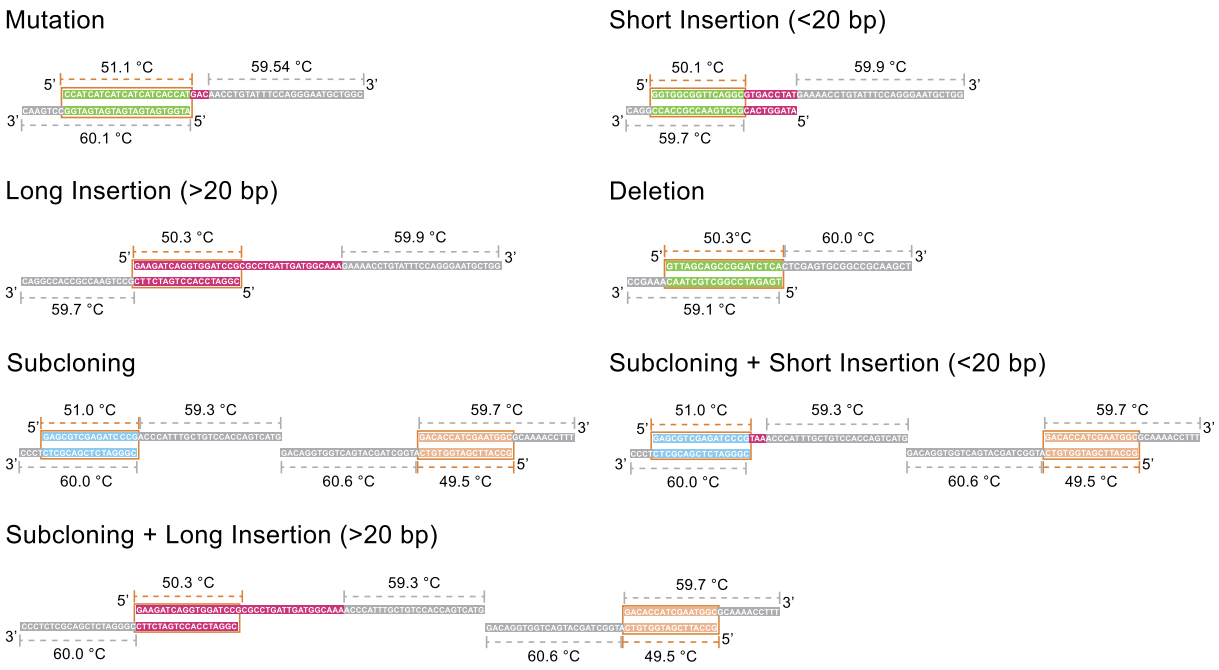

Figure 5. Example asymmetric primers for each type of operation and recombination scheme. TBRs (gray) were generated to be approximately 60°C and HRs (demarcated by orange boxes) to be ∼50°C. Color code as in Fig. 2.

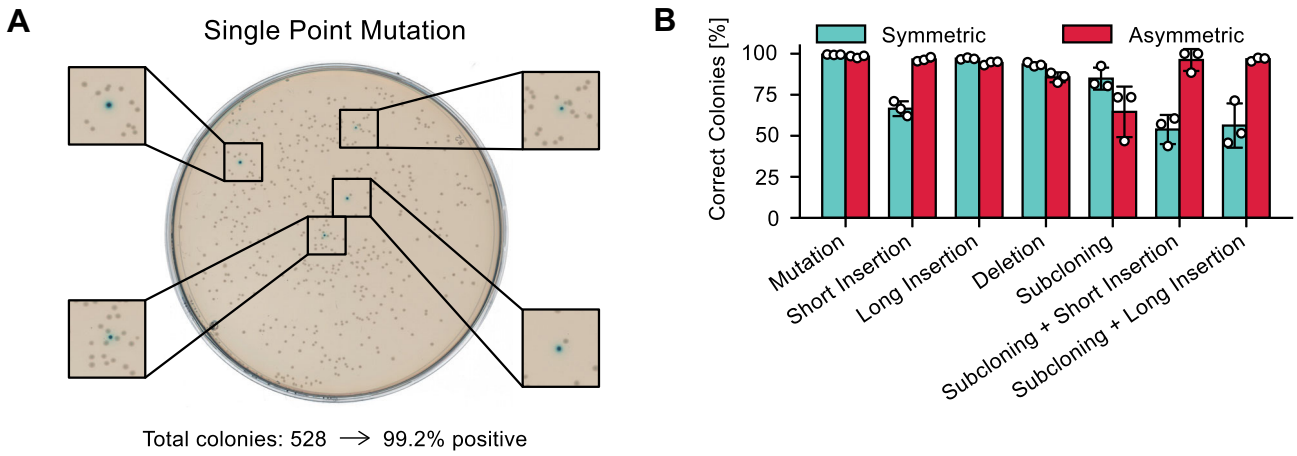

Figure 6. Efficiency of IVA cloning with IVA Prime primer design. (**A**) Blue–white screening used to phenotypically distinguish correct colonies (white) from incorrect ones (blue). A single point mutation to a stop codon was introduced to break the lacZα gene. (**B**) Efficiency of primers designed with IVA Prime. For each cloning operation, symmetric and asymmetric primer pairs were designed and the efficiency determined by blue–white screening ($n = 3$).

ward primer, thus potentially reducing costs. Furthermore, recombination success is sometimes dependent on the nature of the homologous sequence. Although the reasons are unclear, strong secondary structures in this region might lower assembly efficiency. Switching between asymmetric and symmetric distributions has thus shown to improve efficiency in certain cases.

For each IVA cloning operation, we provide a specific example of the designed primer pairs from the output of IVA Prime and annotate the melting temperatures of the individual regions (Fig. 5). The primer sequences given are examples of asymmetric designs. Their symmetric counterparts (Supplementary Fig. S2) produce cloning results that are overall indistinguishable.

Advice on primer design

IVA Prime designs primers for *in vivo* molecular cloning in a fully automated fashion, optimizing (i) the lengths of the HRs and (ii) the overall length of the DNA fragments. First, the length of the HRs determines the efficiency of recombination and therefore the number of colonies. The automatic design targets an HR T_m of 50°C for single-recombination events (insertions, deletions, and mutagenesis) and 55°C for subcloning. The value of 50°C for single-recombination events has been shown to be an optimum to keep transformation efficiency high and primer synthesis costs low [17]. The increased T_m of 55°C for subcloning operations accounts for the tendency of the recombination efficiency to decrease with increasing number of recombination events [17]. These default values can be

changed by the user in the settings panel. An increased T_m resulting in HR lengths of 35–40 bp is recommended for complex procedures such as multiple mutations, deletions, or insertions in a single reaction.

Secondly, the efficiency of *in vivo* DNA assembly is reduced when the linear DNA fragments are shorter than around 400 bp, potentially due to bacterial exonuclease activity. Hence, IVA Prime issues a warning if subcloning fragments become shorter than 400 bp. Similarly, to perform multiple mutations or combinations of modifications in a single reaction, fragments between modification sites should not become shorter than 400 bp.

Practical examples

We assessed the reliability of IVA Prime by performing each IVA cloning operation once with symmetric and once with asymmetric primer types, and quantifying the percentage of correct colonies using blue–white screening. This method relies on the lacZα gene, which leads to a blue colony if correctly expressed in the presence of X-Gal, and a white colony if defective. The ratio of correct and incorrect cloning events can thus be determined phenotypically (Fig. 6A). For each of the IVA cloning operations, we designed a suitable setup with the lacZα gene in a pUC19 vector, where all primers were designed in a way to induce the correct colony phenotype only if the operation occurs flawlessly. The efficiency of single point mutations was measured using a stop codon early in the lacZα coding sequence. For short and long insertions, we used a lacZα gene with a deletion of 8 bp or 32 bp, both needing to be repaired by insertions. Deletion success was evaluated upon its application on the lac promoter driving lacZα expression. Subcloning operations required the lacZα gene to be moved from a vector without promoter back into a pUC19 vector with the lac promoter. Lastly, for subcloning with short and long insertions, the same approach as subcloning was used, but this time using a plasmid with pre-designed deletions for its repair.

As can be seen in Fig. 6B, primers generated using IVA Prime yield a high rate of correct colonies, with all operations displaying recombination efficiencies of >80%. The operations with the lowest recombination efficiencies were the symmetric short insertion, asymmetric subcloning, and symmetric subcloning with insertions. Sequencing showed that the symmetric short insertion primers lead to the insertion of primer tandem repeats, a cloning issue [28] occurring where the primer sequence is repeatedly inserted at the site of interest, which manifested as intermediate light-blue phenotype. The underperforming subcloning primers generally produced less colonies and featured the intermediate phenotype seen on the symmetric short insertion plates. These sequence specific issues are not common and are unpredictable at this time; however, we show that switching primer type from symmetric to asymmetric or vice versa generally rescues the recombination efficiency. Overall, each operation can be run with efficiencies >85%, and most have efficiencies >98%.

Discussion

In this paper, we introduce IVA Prime, an innovative online tool designed to simplify and streamline primer design for IVA cloning. This website is free and open to all users and there is no login requirement. Through systematic primer length adjustments to achieve specific target melting temperatures, IVA Prime transforms a once error-prone and heuristic process into a precise and automated procedure, offering researchers a user-friendly solution for primer design.

Beyond its convenience, automated primer design is a step forward in molecular cloning methodology, as it not only saves time, but also enhances experimental reproducibility and efficiency. By offloading the task of primer design, researchers can avoid spending weeks troubleshooting cloning and instead focus on the core aspects of their investigations, as well as potentially allowing them to attempt studies requiring many different constructs, which would otherwise be too costly or time-consuming.

IVA Prime is feature-complete for the use cases presented, but we nonetheless envision its ongoing development through incremental quality-of-life enhancements and user-driven feature requests. As an open-source project, IVA Prime is open to contributions from the broader community, which may help the tool evolve to support a wider range of use cases and molecular cloning workflows over time.

Since *in vivo* DNA assembly is a homology-based method, IVA Prime will be useful for combination and/or use of alternative homology-based approaches such as Gibson assembly [3] or In-Fusion [29], although we encourage the users to test bacterial IVA since requirements are minimal.

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Supplementary data

Supplementary data are available at NAR online.

Conflict of interest

All authors declare no conflicts of interest.

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Data availability

The data underlying this article are available in the article and in its online supplementary material. IVA Prime can be found at https://ivaprime.com. This website is free

and open to all users and there is no login requirement. The source code for IVA Prime can be accessed at Zenodo (DOI: 10.5281/zenodo.15245669) and GitHub (https://github.com/RaduLeonte/IVA-Prime) and modified under the GNU GPLv3.0 license.

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