STITCHER: A web resource for high-throughput design of primers for overlapping PCR applications

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BioTechniques 58:325-328 (June 2015) doi 10.2144/000114301

Keywords: PCR; overlapping PCR; primer design; web-tool; PCR fusion

Supplementary material for this article is available at www.BioTechniques.com/article/114301.

Overlapping PCR is routinely used in a wide number of molecular applications. These include stitching PCR fragments together, generating fluorescent transcriptional and translational fusions, inserting mutations, making deletions, and PCR cloning. Overlapping PCR is also used for genotyping by traditional PCR techniques and in detection experiments using techniques such as loop-mediated isothermal amplification (LAMP). STITCHER is a web tool providing a central resource for researchers conducting all types of overlapping PCR experiments with an intuitive interface for automated primer design that's fast, easy to use, and freely available online (<http://ohalloranlab.net/STITCHER.html>). STITCHER can handle both single sequence and multi-sequence input, and specific features facilitate numerous other PCR applications, including assembly PCR, adapter PCR, and primer walking. Field PCR, and in particular, LAMP, offers promise as an on site tool for pathogen detection in underdeveloped areas, and STITCHER includes off-target detection features for pathogens commonly targeted using LAMP technology.

Overlapping PCR is widely employed by researchers in diverse experimental designs (1–8). Although numerous primer design tools are available that provide automated design features for traditional PCR, there is no single web tool that provides automated design for diverse overlapping PCR applications. Here I present such

a tool, called STITCHER (Figure 1). STITCHER is freely available online at <http://ohalloranlab.net/STITCHER.html>. It can handle both single sequence and multi-sequence input provided in FASTA format. The upper and lower primer lengths, 5' and 3' search areas, percentage GC, and primer T_m are all set by the user. Primer T_m is calculated

using nearest neighbor thermodynamic calculations as described previously (9). Each parameter contains a default setting on STITCHER's homepage so as to provide a guide for researchers new to primer design; however, all settings can (and should) be changed by the user to meet specific requirements. The addition of a GC clamp to primers and the stringency level of primer self-complementarity are also defined by the user.

Unlike other platforms that do not take into account overlapping fragments, STITCHER incorporates the overlap fragment when examining primer self-complementarity. When searching the user defined search areas, STITCHER moves along in increments defined by the user. Although the default increment is 2 bp, the forward and reverse increments can be modified independently to facilitate specific applications such as nested PCR, loop-mediated isothermal amplification (LAMP), or primer walking experiments (Figure 1). By default, STITCHER also excludes repetitive sequences from primer design; however the user may de-select this exclusion. A repetitive sequence is defined as a stretch of more than four identical base pairs in a row (e.g., AAAAA or GGGGG), or more than three repetitive dinucleotide repeats (e.g., ATATATAT or GCGCGCGC).

Overlapping PCR is often used to generate transcriptional and translational fluorophore reporters. To facilitate this application, STITCHER includes a drop-down selection of pre-defined overlapping fragments for commonly used fluorescent proteins (see Supplementary Table S1) and also a widely used *Caenorhabditis elegans* specific overlap called "GFP_C_elegans" (1,10). Within the same drop-down menu is a list of adapters commonly used for Illumina (San Diego, CA) sequencing projects (Supplementary Table S1). Users may also want to design primers with a specific overlap to their DNA sequence of interest. This can be achieved by inputting any sequence into the user-defined overlap text-box for

METHOD SUMMARY

Here I present STITCHER, an intuitive web based resource that automates primer design for overlapping PCR applications.

Data input & application	Representative Overview	STITCHER features
Primer design for multiple DNA input sequences		Multi-sequence parser with default settings
Overlap PCR to target gene for chimeric fusion		User defined reverse overlap
Overlap PCR to target fluorophore for transcriptional or translational reporter		Pre-defined fluorophore overlaps
Multiple primers (nested and/or overlap) for LAMP, Assembly PCR or PCR walking		Step feature with user-defined overlap forward and reverse
Overlap PCR for deletion mutagenesis or substitution mutagenesis		Deletion feature with reverse overlap automatically appends forward overlap

Figure 1. Overview of STITCHER facilitated PCR applications. The left column indicates input type and the desired applications, which are graphically represented in cartoon form. The right column indicates the STITCHER specific features that enable each application.

the reverse overlap (e.g., to generate a translational fusion to a specific gene) or for both the reverse and forward overlap (e.g., for assembly PCR or LAMP applications). Overlapping PCR can also be used to delete segments of DNA (2). For this application, the user will have a specific area in mind for deletion, so using 5' and 3' search areas will not make sense. Instead, if the user selects the deletion mutation feature, the values entered in the 5' and 3' search area text-boxes will be used to generate a single primer pair that starts from the base pair entered (Figure 2). Furthermore, by selecting the deletion mutation feature, the forward primer will automatically

contain a reverse complement overlap at its 5' end matching the reverse primer's overlapping segment. Thus the user only needs to enter an overlap sequence for the reverse primer overlap and not the forward primer overlap. Once a primer pair of interest is selected using the features outlined above, they can then be screened for cross complementarity and off-target hits by clicking on the link to these tools in STITCHER's navigation menu at the top of each page (Figure 2). The cross complementarity tool allows the user to input forward and reverse primers and calculate the free energy, ΔG° (kJ/mol), of DNA duplexes using the formula:

$$\Delta G^\circ(\text{total}) = \Delta H^\circ \text{total} - T \Delta S^\circ \text{total} \quad [\text{Eq. 1}]$$

where enthalpic (ΔH°) and entropic (ΔS°) parameters are as described in (11). The ΔG value is then normalized by the ratio of total duplex matches to primer length (N).

The cross complementarity tool will return a warning if significant complementarity exists between each primer, with significance tuned based upon Primer3Plus (12) complementarity values from 100 randomly generated primer pairs. The off-target detection tool allows the user to input each primer

and search for possible complement matches on each strand from species commonly used in overlapping PCR detection. This feature also allows the user to define the number of mismatches allowed in the Search. (If no mismatches are desired, enter 0.) As of now, this tool permits screening against the following ten bacterial genomes: *Tannerella forsythia*, *Staphylococcus aureus*, *Candidatus Liberibacter*, *Bacillus anthracis*, *Bordetella pertussis*, *Plasmodium falciparum*, *Streptococcus pneumonia*, *Mycobacterium tuberculosis*, *Escherichia coli*, and *Klebsiella pneumonia*. These specific species were selected based upon their use in diagnostic PCR assays using overlapping PCR approaches (4,6,13–20).

In designing STITCHER, one of the goals was to help researchers automate the process of primer design for pathogen detection using overlapping PCR applications. To support this goal, extra species can be added to STITCHER's off-target detection tool based on user feedback. Users can enter their species of interest and their e-mail address into the text boxes on the off-target detection page, and the genome for that species will be added to STITCHER's off-target detection feature, making STITCHER an interactive and dynamic resource for researchers performing diverse PCR applications. (Users that request a genome to be added to STITCHER's off-target detection feature will receive an e-mail from STITCHER to which they will need to reply in order to validate all requests.)

Instead of searching locally, researchers can also search for off-target matches to their primer sequence of interest by remotely accessing the National Center for Biotechnology Information (NCBI) nucleotide database using the blast-off tool. Output from blast-off is printed to the browser. STITCHER also includes a tool called fetch that will return sequences from NCBI using Entrez Programming Utilities [www.ncbi.nlm.nih.gov/books/NBK25501/]. To use fetch, the user inputs one or more accession numbers into the text-box, and after pushing "submit," the program returns the nucleotide sequences in

sticher | fetch | off-target | blast-off | cross-comp | articles | help

STITCHER: a web-based resource to generate primers for overlapping PCR applications from datasets of any size

Enter any number of sequences in FASTA format:

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>sample fastA format
ATGACCAAGTTGAAAATCTACTTGTTCCTTGTGTCTCGTTGACTACACTTGGGCA
ATACGCGAGCTGAGCCGCAAAATGGAGAAATATTCACGTATCTTCCCAACGTATA
CCCGGGCCCTGAGCCGGCTTGTGCTCCGGCTAAGCCATGTTCCCGGAGTTATC
GTACCAAGTTTGGCAGCCATCGGAAACCTGTCAGAAATGCAAAATATGGTCCGTG
CAATTGTCTATTTAATCGCATTGGCCTATTATTCTTTGGTGTCTCAATTGTGGCGG
ATCGATTATGCGCGTCTATTGAAGTGATCACTTCTCAGCAGAAATCTGTGAAATG
AAGAAGATAACCGGTGAACATTTACAAATATGTTACGTGTCTGGAATGAAACAGT
CAGTAACCTGACGCTAATGGCTCTCGGATCCTCAGCCCCGAGATTTTGTCTCG
GTCATTGAAATTTGCGGAAATAATTTGAAGCTGGAGAGCTGGGACCATCGACAA
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Primer size:
Max: 28 Min: 18

5' and 3' search areas (bp):
5': 100 3': 100

Primer GC %:
Upper GC: 60 Lower GC: 40

Primer Tm:
Upper Tm: 68 Lower Tm: 55

Salt concentration (mM):
☐ 10 ☒ 50

Increment forward:
Steps (bps): 2

Increment reverse:
Steps (bps): 2

GC clamp:
☐ Yes ☒ No

Self complementarity stringency:
☒ Regular ☐ High ☐ None

Exclude repetitive sequence:
☒ Yes ☐ No

Overlapping fragment:
none

User-defined overlapping reverse sequence:

User-defined overlapping forward sequence:

Deletion Mutation:
☐ Yes ☒ No

Figure 2. Screenshot from the STITCHER home page. Default settings are inserted for each feature, and sample sequence input is provided in FASTA format. The navigation menu at the top of the page can be used to obtain sequences from NCBI (fetch), search for off-target matches (off-target and blast-off), examine cross complementary between primers (cross-comp), read about overlapping PCR technology (articles), and learn more about STITCHER's features and output (help).

FASTA format by printing to the browser. If multiple sequences are required, the list should be comma delimited without white space. Users can also stay up to date with recent articles related to overlapping PCR technology through STITCHER's articles page. Clicking "get papers" will return matches for articles related to overlapping PCR. Users can also click on the help avatar on STITCHER's help page to learn more about each feature as well as STITCHER's output.

STITCHER was written using Perl, CSS, JavaScript, and HTML, and uses the Perl CGI module to generate output HTML. STITCHER has been tested successfully on PC and Mac, as well as iPhone and Android mobile devices, using the following browsers: Safari, Chrome, Internet Explorer, Firefox, and Atomic. STITCHER generated primers have been extensively validated in PCR experiments (see Supplementary Figure S1 and Supplementary Table S2 for primer sequences, and Supplementary Figure S2 for an overview of the STITCHER process).

STITCHER's main advantage is that it offers an intuitive platform simply requiring a web browser and internet connection, instead of being command line driven and relying on dependencies. To my knowledge, STITCHER represents the first web-based resource for diverse overlapping PCR applications. The utility of this resource is that it automates the primer design process for single- or multi-sequence input, thus facilitating high throughput approaches for various overlapping PCR experiments to address biological questions in diverse fields.

Acknowledgments

I thank members of the O'Halloran lab for critical reading of the manuscript, and would like to thank the George Washington University Columbian College of Arts and Sciences, the GW Office of the Vice-President for Research, and the Department of Biological Sciences for Funding.

Competing interests

The author declares no competing interests.

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Received 30 January 2015; accepted 09 March 2015.

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