

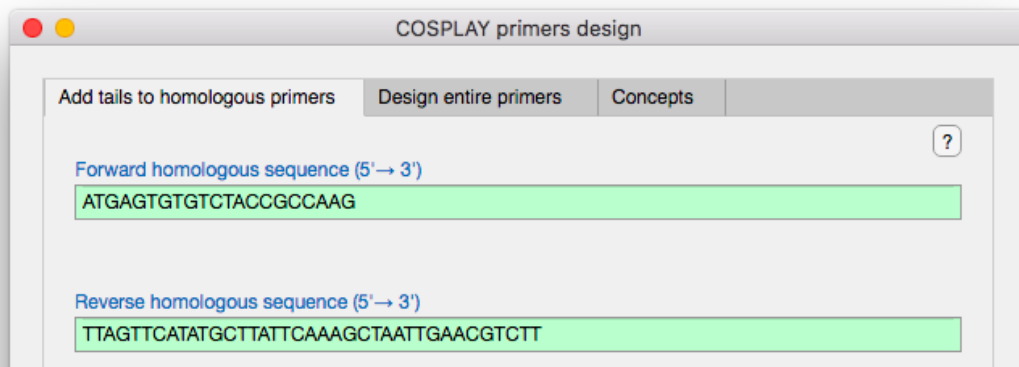
When generating a new module the first step is to design primers that will amplify the module. These primers contain specific tails which drive the integration of the PCR product into a carrying plasmid and determine the future position of the module in a multi-modules assembly. The COSPLAY software propose 2 options to build primers: designing only the tails of the primers while the user freely choose the module homologous regions or designing the full tailed primers including optimized homologous regions.

In order to generate tailed primers when the homologous primer sequences are already available, the tab “Add tails to homologous primers” should be used:

The screenshot shows the 'COSPLAY primers design' window. The 'Add tails to homologous primers' tab is selected, indicated by a red arrow. The interface contains the following elements:

- Forward homologous sequence (5' → 3')**: An empty text input field.
- Reverse homologous sequence (5' → 3')**: An empty text input field.
- Module position**: A dropdown menu currently showing '1'.
- Generate tailed primers**: A button to process the input sequences.
- Diagram**: A schematic showing a 'module' (homology) flanked by 'homology' regions. The 5' primer is labeled 'PUC57-Bsal-homology' and the 3' primer is labeled 'homology-Bsal-PUC57'.
- Tailed primers (5' → 3')**: An empty text area for the output.
- Buttons**: 'Clear', 'Copy forward primer', 'Copy reverse primer', and 'Save'.

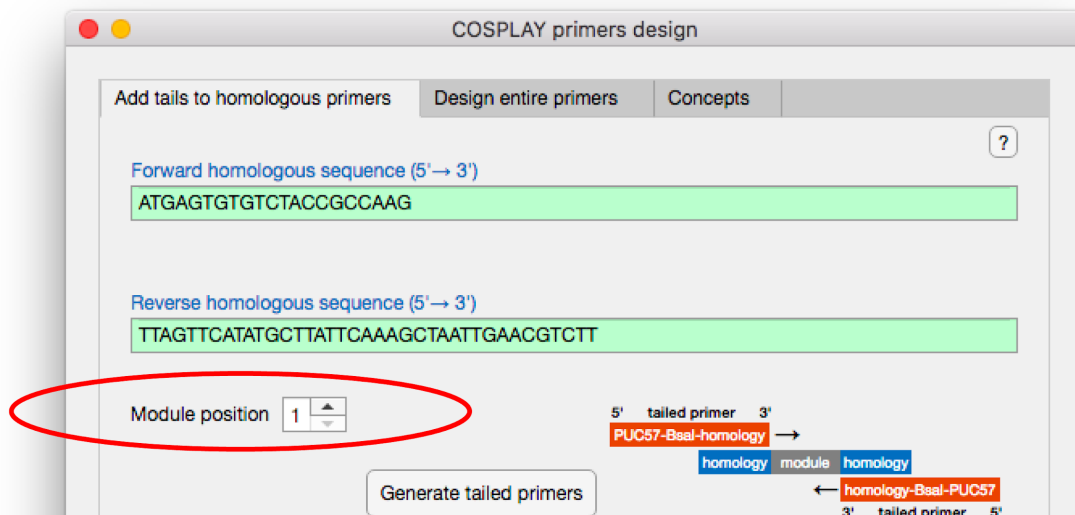
In this tab, the user is invited to enter 2 DNA sequences that are homologous to the 5' and 3' module regions. Only A/a T/t G/g C/c characters and blank spaces are allowed. A minimum length of 4 nt is required (minimum of 11 is recommended):



The next step is to choose what position the module should have in an assembled vector. In our COSPLAY toolbox the structure of an assembled vector is based on 6 different modules:

position 1 - plasmide type position 3 - gene position 4 - degen
position 2 - promoter position 5 - terminator position 6 - selection

The selection of the module position is achieved by using the corresponding spinner:



The button “Generate tailed primers” builds the tailed primers by integrating the provided information- homologous sequences and module position. The resulting sequences are displayed in the text area on the bottom. The primer Tm for Q5 pol is also shown:

COSPLAY primers design

Forward homologous sequence (5' → 3')

ATGAGTGTGTCTACCGCCAAG

Reverse homologous sequence (5' → 3')

TTAGTTCATATGCTTATTCAAAGCTAATTGAACGTCTT

Module position

5' tailed primer 3'
 PUC57-Bsal-homology →
 homology module homology
 ← homology-Bsal-PUC57
 3' tailed primer 5'

Tailed primers (5' → 3')

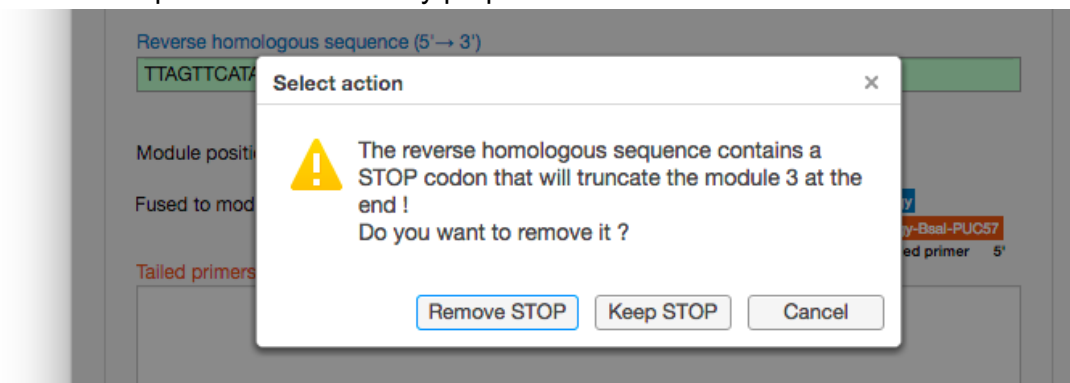
Forward tailed primer (Q5 pol Tm = 66.7°C):
 CGAATGCATCTAGATATCGGATCCCGGTCTCGAGCAATGAGTGTGTCTACCGCCAAG

Reverse tailed primer (Q5 pol Tm = 66.6°C):
 GCAGGCCTCTGCAGTCGACGGGCCCGGTCTCTATGGTTAGTTCATATGCTTATTCAAAGCTAATTGAACGTCTT

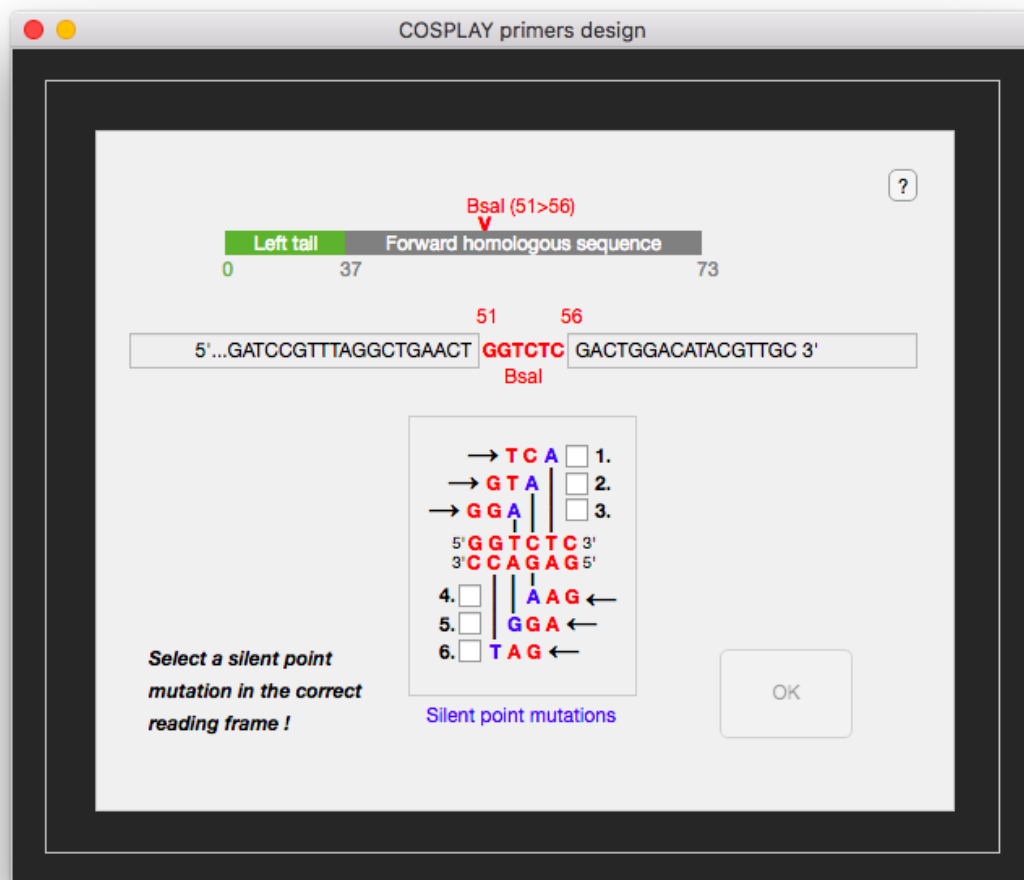
The sequences of the tailed primers generated by the COSPLAY software can be directly copied in the clipboard or saved as text file by using the corresponding buttons under the results text area.

Additional option is available for module position 3. Indeed, the module 3 may contain a gene that has to be fused to a destabilizing sequence contained in module 4. Therefore, the linker site between module 3 and 4 should be adjusted to preserve the reading frame. If no fusion is needed the standard linker sequence is [module 3]-^{5'}CGCA^{3'}-[module 4]. In the case of fusion the linker is extended by a gly-gly bond: [module 3]-^{5'}GGAGGCGCA^{3'}-[module 4]. To indicate that fusion is required the checkbox "Fused to module 4" should be checked:

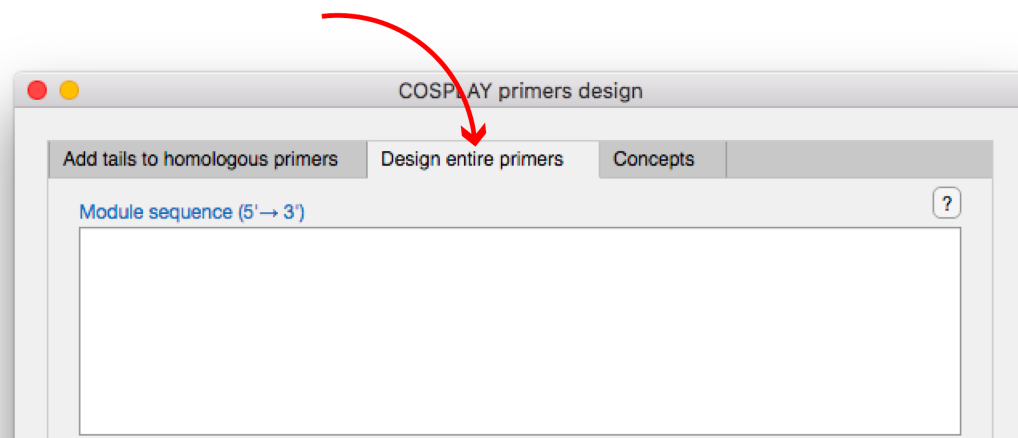
If the fusion option is validated the software tests whether a STOP codon is present in the reverse primer and eventually propose to eliminate it:



One limitation of the COSPLAY toolbox is the presence of additional Bsal sites in the modules sequence. Such sites have to be muted to avoid unintended cutting during assembly reactions (Bsal digestion + T4 ligase activity). Our software automatically detect the problematic Bsal sites present in the tailed primers and propose several silent substitutions depending on the reading frame:



If the user can not provide the homologous sequences the tab “Design entire primers” may be used to find optimized homologous primers that amplify a module:



In this tab, the module sequence has to be communicated in order to generate the primers. For this the upper text area should be used:

The screenshot shows the 'COSPLAY primers design' web application. It features a top navigation bar with three tabs: 'Add tails to homologous primers' (selected), 'Design entire primers', and 'Concepts'. Below the tabs, the 'Module sequence (5' → 3')' is displayed in a green box, containing a 20-nucleotide DNA sequence. To the right of the sequence is a help icon (?). Below the sequence, the 'Module position' is set to 1. A 'Single integration' checkbox is present. A 'Generate tailed primers' button is located below the checkbox. To the right of the button is a diagram showing the primer design: a blue box labeled 'module' with a red box labeled 'PUC57-Bsal-homology' to its left and a red box labeled 'homology-Bsal-PUC57' to its right. Arrows indicate the 5' to 3' direction for the primers. An 'Options' button is located to the right of the diagram. Below the diagram, the 'Tailed primers (5' → 3')' section is empty. At the bottom, there are four buttons: 'Clear', 'Copy forward primer', 'Copy reverse primer', and 'Save'.

The forward and reverse homologous primer sequences always start respectively at the first (5' end) and last (3' end) nucleotide of the module. This guarantees that the entire module sequence will be amplified. The length of the homologous sequences is optimized to obtain the most efficient primers pair. Many criteria are considered during the optimisation process. The balance between different criteria is ensured via a system of weights that determine the importance of each criterion. The weights are numbers that multiply the optimisation effect of the criteria. Default values for all weights is 1 but this can be modified $[0; +\infty]$ by using the button "Option" situated under the module sequence text area. This button opens a new window that allow to change the weights as well as the optimal T_m :

Options

Optimal Tm (°C)

Penalty Weights:

Tm

Size

Primers self and cross complementarity

Primers self and cross 3' complementarity

Template ectopic complementarity

Template ectopic 3' complementarity

Poly-X

G/C %

G/C clamps

3' stability

Primers Tm difference

Single integration:

Restriction site linker

OK Default Cancel

After the module sequence is communicated the user has to select the module position which determine the sequences of the tails that will be added to the homologous sequences in order to generate tailed primers. The button “Generate tailed primers” integrate all the input information to build the entire primers. The sequences of these primers and corresponding Tms are displayed in the bottom text area. In addition a full report on the primers quality is showed. In this report the different optimisation criteria are evaluated and results are based on 3 categories- “Ok”, “Mediocre” and “Bad” :

COSPLAY primers design

Add tails to homologous primers
Design entire primers
Concepts

Module sequence (5' → 3')

ATGAGTGTGTCTACCGCCAAGAGGTCGCTGGATGTCGTTTCTCCGGGTTTCATTAGCGGAG
TTTGAGGGTTCAAATCTCGTCACGATGAAATAGAAAATGAACATAGACGTAAGTGGTACA
CGTGATGGCGAGGATAGCGAGCAACCGAAGAAGAAGGGTAGCAAACTAGCAAAAAGCAA
GATTTGGATCCTGAACTAAGCAGAAGAGGACTGCCCAAAATCGGGCCGCTCAAAGAGCT
TTAGGGAACGTAAGGAGAGGAAGATGAAGGAATTGGAGAAGAAGGTACAAAGTTTAGAG
AGTATTCAGCAGCAAAATGAAGTGGAAGCTACTTTTTGAGGGACCAGTTAATCACTCTG
GTGAATGAGTTAAAAAATATAGACCAGAGACAAGAAATGACTCAAAAGTGCTGGAATAT
TTAGCAAGGCGAGATCCTAATTGCATTTTCAAAAAATACGTTAACCACAGCAATAGC
GAGCCAATTGACACACCCAATGATGACATACAAGAAAATGTTAAACAAAAGATGAATTC

Module position
1

Single integration
☐

Generate tailed primers

Options

5' tailed primer 3'
PUC57-Bsal-homology → module ← homology-Bsal-PUC57
3' tailed primer 5'

Tailed primers (5' → 3')

Forward tailed primer (Q5 pol T_m = 66.7°C):
CGAATGCATCTAGATATCGGATCCCGGTCTCGATCCATGAGTGTGTCTACCGCCAAG

Reverse tailed primer (Q5 pol T_m = 66.6°C):
GCAGGCCTCTGCAGTCGACGGGCCCGGTCTCTCATATTAGTTCATATGCTTATTCAAAGCTAATTGAA
CGTCTT

FULL REPORT:

Clear
Copy forward primer
Copy reverse primer
Save

FULL REPORT:

Primers pair

Primers T _m difference	OK
Primers cross complementarity	MEDIOCRE
Primers 3' cross complementarity	OK

Individual primers	Forward	Reverse
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Primer size	OK	BAD
Self complementarity	MEDIOCRE	OK
3' self complementarity	OK	OK
G/C %	OK	MEDIOCRE
G/C clamps	OK	OK
3' stability	OK	OK
Poly-X	OK	OK
Template ectopic complementarity	OK	OK
Template 3' ectopic complementarity	OK	OK

The forward and reverse tailed primers can be copied or exported as a text file:

Forward tailed primer (Q5 pol Tm = 66.7°C):
CGAATGCATCTAGATATCGGATCCCGGTCTCGATCCATGAGTGTGTCTACCGCCAAG

Reverse tailed primer (Q5 pol Tm = 66.6°C):
GCAGGCCTCTGCAGTCGACGGGCCCGGTCTCTCATATTAGTTCATATGCTTATTCAAAGCTAATTGAACGTCTT

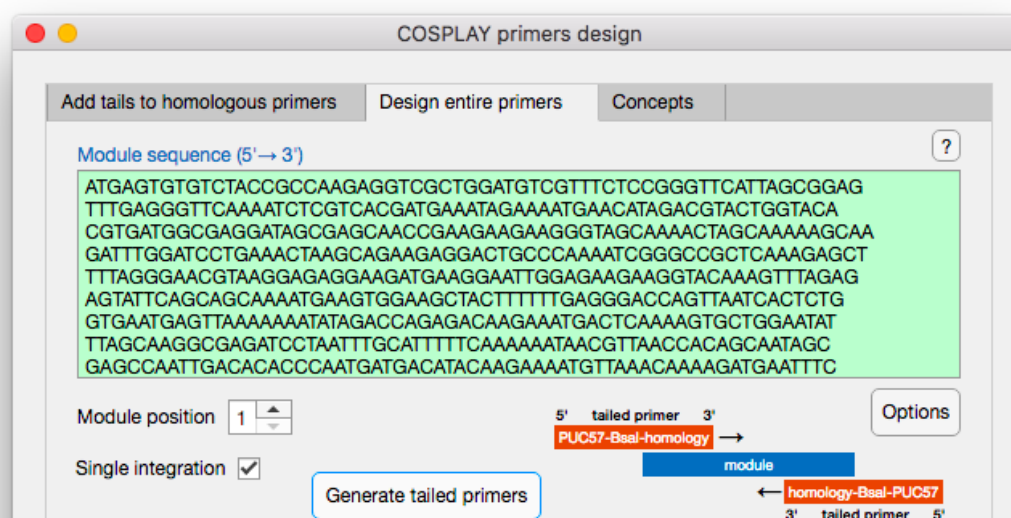
FULL REPORT:

Primers pair

Primers Tm difference	OK
Primers cross complementarity	MEDIOCRE
Primers 3' cross complementarity	OK

Individual primers	Forward	Reverse
Tm	OK	OK
Primer size	OK	BAD
Self complementarity	MEDIOCRE	OK
3' self complementarity	OK	OK
G/C %	OK	MEDIOCRE
G/C clamps	OK	OK
3' stability	OK	OK
Poly-X	OK	OK
Template ectopic complementarity	OK	OK
Template 3' ectopic complementarity	OK	OK

When the selected module position is 1 there is an additional checkbox determining whether the module is intended to perform single integrations.



Indeed, the COSPLAY toolbox provides the possibility to do single genomic integrations of assembled vectors through a specific module 1. This module is based on 2 sequences homologous to the 5' and 3' regions of a target locus. The particularity ensuring the single integration is that these sequences are inverted (the 3' region is upstream the 5' region). If the checkbox "Single integration" is validated the software will generate the primers required for the production of a module 1 containing inverted 5' and 3' target locus regions (40bp each). The sequences are separated by a cassette containing *Ascl* and *FseI* restriction sites used to open the plasmid prior to the genomic integration. These primers share 20bp of homology and should be directly dimerised by PCR (matrix not required!):

COSPLAY primers design

[Add tails to homologous primers](#)
[Design entire primers](#)
[Concepts](#)

Module sequence (5' → 3')

```

ATGAGTGTGTCTACCGCCAAGAGGTGCTGGATGTCGTTTCTCCGGGTTCTTAGCGGAG
TTTGAGGGTTCAAATCTCGTCACGATGAAATAGAAAATGAACATAGACGTAAGTACA
CGTGATGGCGAGGATAGCGAGCAACCGAAGAAGAAGGGTAGCAAACTAGCAAAAAGCAA
GATTTGGATCCTGAAACTAAGCAGAAGAGGACTGCCCAAAATCGGGCCGCTCAAAGAGCT
TTTAGGGAACGTAAGGAGAGGAAGATGAAGGAATTGGAGAAGAAGGTACAAAGTTTAGAG
AGTATTCAGCAGCAAAATGAAGTGAAGCTACTTTTTTGAAGGACCAGTTAATCACTCTG
GTGAATGAGTTAAAAAATATAGACCAGAGACAAGAAATGACTCAAAAGTGCTGGAATAT
TTAGCAAGGCGAGATCCTAATTTGCATTTTCAAAAAATAACGTTAACCACAGCAATAGC
GAGCCAATTGACACACCCAATGATGACATACAAGAAAATGTTAAACAAAAGATGAATTC
  
```

Module position

Single integration ☒

Generate tailored primers

Options

Tailed primers (5' → 3')

Forward tailed primer:
CGAATGCATCTAGATATCGGATCCCGTCTCGATCCAGAAGACGTTCAATTAGCTTTGAATAAGCATAT
GAACTAAGGCGCGCCGGCCGGCCATGA

Reverse tailed primer:
GCAGGCCTCTGCAGTCGACGGGCCCGGTCTCTCATAAACGACATCCAGCGACCTCTTGCGGTA
GACACACTCATGGCCGGCCGGCCGCGCCTTAG

Use Ascl or FseI to open the single integration plasmid !

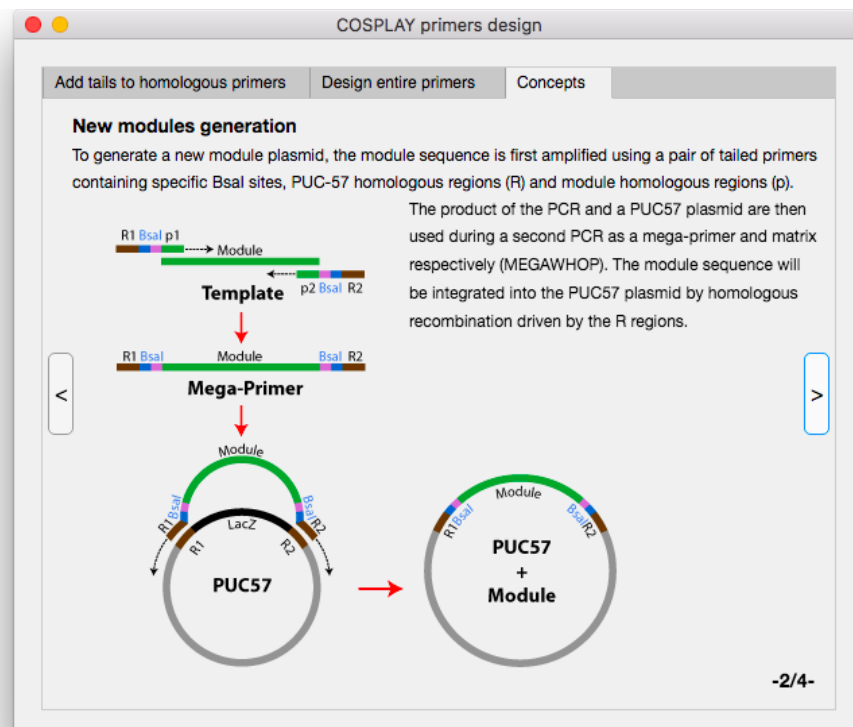
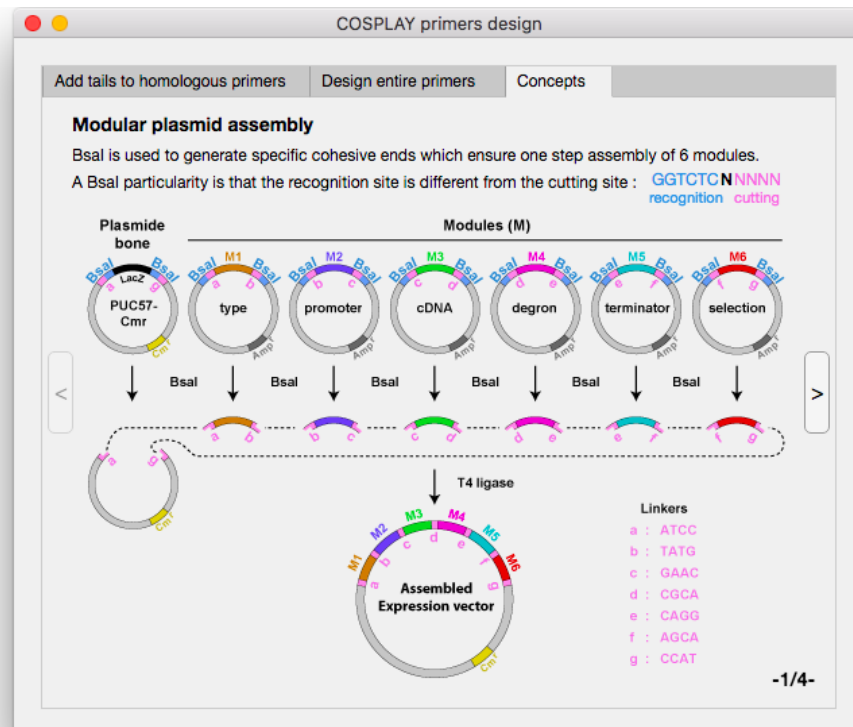
The diagram illustrates the module sequence with two homologous regions. The top region is labeled 'PUC57-Bsal-homology' and the bottom region is labeled 'homology-Bsal-PUC57'. Arrows indicate the direction of the sequence, with 5' and 3' ends marked. The 'module' is shown in the center, flanked by these homologous regions.

In case Ascl and FseI are both present in the module 1 sequence outside the restriction cassette or in other modules that will be assembled with the module 1 it is possible to modify the restriction sites of the cassette through the button “Option”:

Single integration:

Restriction site linker

The last pad of the software contain informations about the main principles of the COSPLAY tool box:



COSPLAY primers design

Add tails to homologous primers Design entire primers Concepts

Modules mega-primers

Module specific tails containing BsaI sites and PUC57 homologous regions.

PUC57-R1 : 5' CGAATGCATCTAGATATCGGATCCC 3'
PUC57-R2 : 5' GCAGGCCTCTGCAGTCGACGGGCC 3'

Forward tail	Reverse tail
5' PUC57-R1 GGTCTCGATCC 3'	3' Module 1 ATACTCTCTGG PUC57-R2 5'
5' PUC57-R1 GGTCTCGTATG 3'	3' Module 2 CTTGTCTCTGG PUC57-R2 5'
5' PUC57-R1 GGTCTCGGAAC 3'	3' Module 3 GCGTTCTCTGG PUC57-R2 5'
5' PUC57-R1 GGTCTCGGCA 3'	3' Module 4 CCTCCGCGTTCTCTGG.. if fusion) PUC57-R2 5'
5' PUC57-R1 GGTCTCGCAGG 3'	3' Module 5 GTCCTCTCTGG PUC57-R2 5'
5' PUC57-R1 GGTCTCGAGCA 3'	3' Module 6 TCGTTCTCTGG PUC57-R2 5'
	3' Module 6 GGTATCTCTGG PUC57-R2 5'

-3/4-

COSPLAY primers design

Add tails to homologous primers Design entire primers Concepts

Single integration

Single integration is achieved by inverting 5' and 3' regions of the genomic target locus.

Classical multi-copies integration

Enzymatic digestion

First recombination

Seconds recombination

Single integration (inverted regions)

Enzymatic digestion

First recombination

-4/4-