Jun 13, 2025

Primer Design

DOI
dx.doi.org/10.17504/protocols.io.n92ldz3znv5b/v1

Dakota Betz[1], Dakota Betz[1]

[1]ucsd

Rouse Lab

Marina McCowin
UCSD- Scripps Institution of Oceanography

DOI: dx.doi.org/10.17504/protocols.io.n92ldz3znv5b/v1

Protocol Citation: Dakota Betz, Dakota Betz 2025. Primer Design. **protocols.io https://dx.doi.org/10.17504/protocols.io.n92ldz3znv5b/v1**

Protocol status: Working
We use this protocol and it's working

Created: December 15, 2021

Last Modified: June 13, 2025

Protocol Integer ID: 55983

Abstract

Protocol for designing primers in Geneious.

Programs and Dependencies

1 **Geneious**
Version 11.1.5

> Note
>
> *NOTE: This protocol assumes you have sequence files already in Geneious. If they are coming from another source, be sure to import them first.*

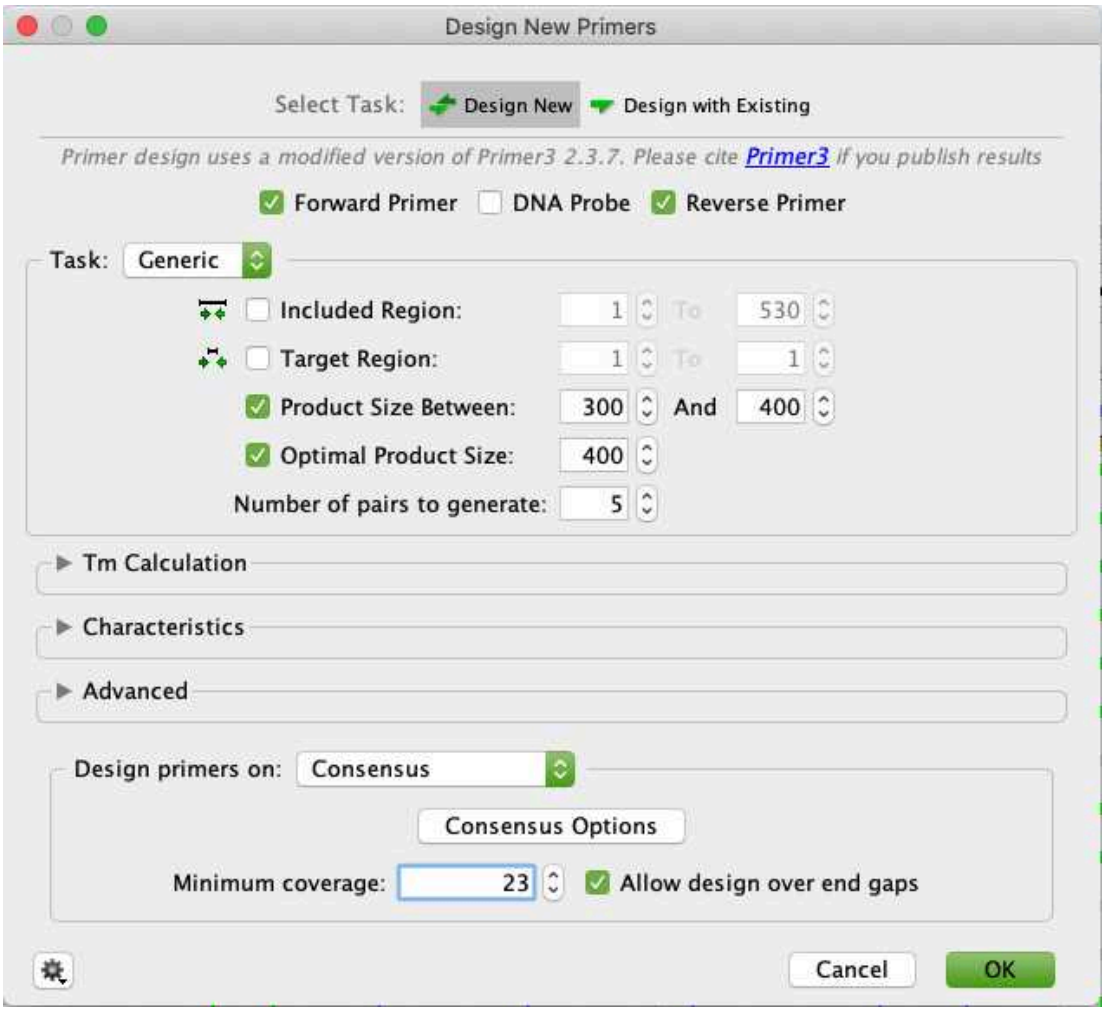
File preparation

2 You will an alignment of sequences in Geneious to design primers. Select the sequences you want to design primers for/align, then **Align/Assemble > Multiple Align** and then select the alignment software you prefer (usually Mafft, Geneious works too).

> Note
>
> *NOTE: There are a variety of options for which sequences you choose depending on your needs. You can use sequences from a single species or multiple species, but it is recommended not to attempt to design primers beyond the family level for most applications.*

Primer Design

3 Select your new alignment file, then **Primers > Design New Primers**.
Select **Design New** and check the boxes for **Forward Primer** and **Reverse Primer**.
The "task" should remain at the default **Generic**.
If you have a target region of interest, you can input that. Input your product size by checking the **Product Size Between** box and entering the basepair range you're aiming for (300-400 in the example below). You can also select **Optimal Product Size** if you would like to aim for a particular number of basepairs.
Leave the default number of pairs to generate at **5** and Design Primers On should be set to **Consensus**.
The **Minimum Coverage** value below **Consensus Options** is arbitrary--choose something that's around 1/3 to 1/2 of the number of sequences in your alignment. Click **OK** when done.



Example of primer design settings.

4 To evaluate the success of your primer set, check the primers that Geneious has generated. If you see that they're all in roughly the same region, that region is likely conserved and will work for your target. If you see sets in different regions, take a closer look at coverage and consider ordering multiple sets.

Troubleshooting

5 If your primer design does not work, consider tweaking the following parameters:

- **Product Size Between** and/or **Optimal Product Size**: you may need to choose a smaller number of basepairs
- **Minimum Coverage**: you may need to reduce this number if you don't have many sequences in your alignment or if they are highly variable
- Consider making a primer set for a more specific group (e.g. if you are trying to design primers at the family level, consider making multiple sets at the genus level

instead).

Ordering Primers

6

> Note
>
> *NOTE: Ordering primers from Marketplace requires access to Marketplace. If you do not have access, contact Avery or Marina.*

Once on the Marketplace website select **All Punchouts > IDT**.

7 Select **Custom DNA Oligos** and input the number of items (note that forward and reverse primers are ordered separately, so one primer set will be 2 items). Give your primers a useful name (includes what they're for, for example).

8 Copy the primer sequences (forward and reverse) from Geneious and paste them in the **sequence** box.

9 Select **Standard Desalting**. Check the box to **select all** and then select **Checkout**.

10 Check the box for a **Paper Spec Sheet** (this is very important--will have the info you need for programming the thermal cycler). Select your PI (Greg) as **P**, then edit the product description to explain your order (designed primers, your project, etc etc). Check that the room number and address match those for our lab at UCSD (**Hubbs Hall 2350**).

11 You will have to fill out the billing information *for each line item* (select each one from the list at the top, then scroll down to fill out the info). Be sure to select each item and edit the **Expense Type** to **522401 (lab supplies)**.

PCR Program Design

12 If you're not sure how your PCR program should look, look at similar primer sets for similar genes/organisms. The following is a generic PCR program example and a good starting point. Edit the parts in bold to match your primer information (the rest can remain the same and/or be edited to your preferences):

Initial Denaturation (1 cycle)

- 94 °C 4 minutes

Denaturation, Annealing, Extension (30 cycles)

- Denaturation: 94 °C 30-40 seconds
- Annealing**: Tm** 40-50 seconds
- Extension: 72 °C 40-60 seconds

Final Extension (1 cycle)

- 72 °C 5-10 minutes

Hold (temperature to maintain after program is done)

- 4 °C

Note

Tm should be 5 °C *lower* than the lowest Tm (melting temperature) in your primer set.

13 If you're interested in creating a **touchdown PCR** modify the Denaturation, Annealing, and Extension portion of the above program as follows:

- Record the final annealing temperature for your primer set (this should be 5 °C lower than the lowest Tm of your primer set).
- Modify the first 10-15 cycles of DAE so that the annealing temperature begins 10-15 °C above your **Tm** (annealing temperature you calculated) and decreases 1 °C per cycle until you reach your final **Tm**
- The remaining 15-20 cycles of DAE will proceed as in the above PCR program example with the final **Tm**. Be careful not to exceed ~35 cycles total to avoid primer dimers.

Note

NOTE: The Initial Denaturation, Final Extension, and Hold Phases of the program will remain the same as the above example.