Opentrons
for Education

Prep Sheet

Lab Module 7: Primer Design for PCR and Cloning

Dr. Cătălin Voiniciuc, Dr. Moni Qiande, and Abigail Lin

University of Florida

Getting Started

This lesson plan uses values from an Excel template to customize the Primer Dilution protocol. Directions are included in this lesson plan to create and use the Excel template. Values from the template are copied and pasted into labeled sections of the Python protocol in a code editing program. Questions about using the Excel template for protocol customization can be directed to cvoiniciuc@ufl.edu.

This course does not require previous coding experience. For guidance working with Python code in this Opentrons protocol, you can refer to the following resources:

- ☐ Python Protocol API Tutorial
- ☐ Python Protocol API- Labware
- ☐ Python Protocol API- Temperature Module
- ☐ Python Protocol API- Loading Labware on Adapters

Additional Support and Resources

OT-2 Manual
Running a protocol on the OT-2

For technical support, please check our Opentrons Help Center for relevant articles. If you need further support, please contact support@opentrons.com. Inform them that you are a part of the Opentrons for Education program and provide the date of your next laboratory class.

If you have questions related to the lesson plan, please reach out to Dr. Cătălin Voiniciuc at cvoiniciuc@ufl.edu.

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Educator Guide

Lab Module 7: Primer Design for PCR and Cloning

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Contents

Included in this document are the following sections:

Purpose

Students will design primers for PCR and cloning using an attached worksheet, guiding them through common pitfalls in primer efficiency and compatibility. Primer activities in this lab class prepare for the following classes, in which students will amplify their DNA via PCR and complete one-pot digestion and ligation according to Golden Gate (and MoClo) assembly methods. Primer solution preparation and dilution is then automated using the OT-2.

In this lab class, students learn about and gain experience with:

- Primer design in modular (Golden Gate) cloning, including the use of online design tools
- Experimental planning ahead of complex experiments
- Laboratory automation of dilution tasks

Core Competencies

Laboratory Skills:

- Primer design for PCR and cloning experiments
- Basics of Golden Gate cloning methods

Automation Skills:

- Automation of dilution tasks
- Protocol customization

Background Knowledge

Students should begin this lab with an understanding of the cloning workflow used in this course from previous weeks, as well as a basic understanding of primers and their use in PCR. An included pre-lab reading introduces basics of PCR and standard primer design rules. *No coding experience is required for this lab,* but students and/or instructors will need to edit a Python protocol file.

Supplies

Opentrons Equipment

- [ ] OT-2 automated liquid handling robot
- [ ] OT-2 P300 Single-Channel GEN2 pipette (in right mount)

Non-Opentrons Equipment

- [ ] Primer Dilution protocol

Non-Opentrons Equipment

- [ ] Benchtop thermocycler

Labware

- [ ] Opentrons OT-2 Tips, 300µL in deck slot 9
- [ ] Opentrons 10 Tube Rack with Falcon 4x 50 mL and 6x15 mL Conical tubes in deck slot 2
- [ ] Opentrons 24 Tube Rack with NEST 1.5 mL Snapcap tubes in deck slot 5
- [ ] Opentrons 24 Tube Rack with Generic 2 mL Screwcap tubes in deck slot 4

Reagents and Other Materials

- [ ] Primers (dry oligos, ordered from Eurofins Scientific or similar) in 2 mL screwcap tubes
- [ ] Molecular grade water, such as HyClone Water

Experimental Duration

Required Class Sessions

1

Lab Run Time

This lesson plan was prepared for a traditional laboratory class time of 80-90 minutes. Students can complete the attached worksheet to guide them in primer design, including the use of online design tools and avoiding common pitfalls. Primers should be ordered ahead of time (as dry oligos) for dilution in class.

Basic Troubleshooting and Tips

- We recommend completing a trial run of the protocol used in this lesson plan prior to class. On the OT-2 robot, this trial run can be completed with or without tips.

- To save time in class, an Excel template for entering data for protocol customization can be created ahead of time (see Step 1 of **Primer Dilution** in the **Procedure Guide**).

- Customizing your protocol can result in changes to labware and liquids. Import your customized protocol into the Opentrons App and check the setup instructions, including labware and liquid locations and initial volumes.

- If planning to amplify and genotype new genes and primers are not already commercially available, Express Oligos can be ordered from Eurofins by 5 PM ET and are shipped overnight in the United States.

- Resources such as the User's Guide to Golden Gate Cloning Methods and Standards can be helpful in student understanding of primer and experimental design.

- When designing PCR thermocycler reaction steps, the initial 5x 3-step cycles are used when primers contain adapters or overhangs that do not appear in the template. In the example shown in the attached worksheet (Section **A**), annealing temperature for step 3 should match the melting temperature (TM) of the **gene-specific** portion of primers. If primers bind fully to the template (or after initial 3-step cycles), we recommend performing 2-step cycles with simultaneous annealing of primers and extension of product. Extension time (in each extension step) should be calculated as 30 kilobase pairs/second.

Procedure Guide

Before Class

1. Order appropriate primers based on chosen plasmid backbones and inserts. Follow primer design guidelines for Golden Gate assembly (one-pot digestion and ligation), such as including appropriate restriction sites for the chosen enzyme in the primer sequence. Primers should be ordered as dry oligos, from Eurofins Scientific or similar.

Primer Design

1. Students can follow the attached Primer and PCR Design worksheet:
    a. Section **A** demonstrates primer (and PCR experiment) design for classical cloning using an online tool (such as the Multiple Primer Analyzer from ThermoFisher). Students should understand why Phusion DNA polymerase is a more appropriate choice for cloning reactions (high fidelity, used in instances where a precise sequence is absolutely required). An example table from Section **A** with answers provided is shown below.

# Reactions	1	Thermocycler Conditions	°C	min:sec	
Water	13.35	1. Initial Denaturation	98	0:30	
Green HF Buffer (5x)	4	2. Denaturation	98	0:10	5x
dNTPs (10 mM)	0.4	3. Annealing	60	0:20	
F Primer (10 µM)	1	4. Extension	72	0:40	
R Primer (10 µM)	1	5. Denaturation	98	0:10	30x
DNA		6. Anneal and Extend	72	0:40	
Phusion DNA Polymerase	0.25	7. Final Elongation	72	5:00	
Total	20	8. Storage	12	∞	

b. Section **B** directs students to design primers that could be used to genotype a finished construct (in this case, the pPICZ B plasmid backbone and added gene construct students worked with in the Benchling virtual digest; Lab Module 5). Students should understand *why* Taq DNA polymerase is a more appropriate choice for this application, and *how* they would run a reaction with gene- and vector-specific primer pairs in a single thermocycler.

Primer Dilution

Students will use the OT-2 to prepare 100 µM and 10 µM primer stocks from dry oligos: first, preparing a 100 µM stock, followed by dilution to a 10 µM working solution.

1. Create an Excel template to insert volumes to be used in the protocol. This can be done before or during class. The Python protocol, using your entered values, can dilute up to 24 primers in a single run. *Note:* if diluting less than 24 samples, all subsequent empty rows must be deleted for Steps 2, 3, and 4.
    a. First, create a table with columns B-G to specify initial liquid volumes for transfers.

Step 1: Put columns "B-G" into "csv_volume_data_raw" This stocks the robot with Hyclone water in falcon tube(s) for all primer diutions. Calculate how much water is needed before running. Can Leave column "D" value as is.	Labware	Initial_Wells	Initial_Volume	Liquid_Name	Description	Color
	Falcon_Water	A4	25000	Hyclone Water	Hyclone Water	#00FF00

For this protocol, the table should include a Falcon tube (Falcon_Water) in well A4 (of the tube rack this Falcon tube is located in) with an initial volume of 25 mL of Hyclone water. *Note:* here, API names are not needed. Instead, a common name (Falcon_Water) is defined in a labware dictionary (labware_dict) function in the protocol. If you change values in the Labware column, be sure to make the equivalent changes in the labware dictionary section of the protocol.

b. Next, create a table with columns I-N to specify transfer volumes of water to prepare for primer dilution.

Step 2: Put columns "I-N" into "Prepare_Final_Dilution_raw". Volume is in uL. This is the table for preparing solvent in 1.5 mL snapcap tubes for dilution (180uL water + 20uL 100uM primer = 10uM working primer dilution).	Source_Labware	Source_Well	Destination_Labware	Destination_Well	Transfer_Volume	Pick_Up_Tip
	Falcon_Water_R	A4	Solvent_SnapCaps	A1	180.0	TRUE
	Falcon_Water_R	A4	Solvent_SnapCaps	A2	180.0	FALSE
	Falcon_Water_R	A4	Solvent_SnapCaps	A3	180.0	FALSE
	Falcon_Water_R	A4	Solvent_SnapCaps	A4	180.0	FALSE
	Falcon_Water_R	A4	Solvent_SnapCaps	A5	180.0	FALSE
	Falcon_Water_R	A4	Solvent_SnapCaps	A6	180.0	FALSE
	Falcon_Water_R	A4	Solvent_SnapCaps	B1	180.0	FALSE
	Falcon_Water_R	A4	Solvent_SnapCaps	B2	180.0	FALSE
	Falcon_Water_R	A4	Solvent_SnapCaps	B3	180.0	FALSE
	Falcon_Water_R	A4	Solvent_SnapCaps	B4	180.0	FALSE
	Falcon_Water_R	A4	Solvent_SnapCaps	B5	180.0	FALSE
	Falcon_Water_R	A4	Solvent_SnapCaps	B6	180.0	FALSE
	Falcon_Water_R	A4	Solvent_SnapCaps	C1	180.0	FALSE
	Falcon_Water_R	A4	Solvent_SnapCaps	C2	180.0	FALSE
	Falcon_Water_R	A4	Solvent_SnapCaps	C3	180.0	FALSE
	Falcon_Water_R	A4	Solvent_SnapCaps	C4	180.0	FALSE
	Falcon_Water_R	A4	Solvent_SnapCaps	C5	180.0	FALSE
	Falcon_Water_R	A4	Solvent_SnapCaps	C6	180.0	FALSE
	Falcon_Water_R	A4	Solvent_SnapCaps	D1	180.0	FALSE
	Falcon_Water_R	A4	Solvent_SnapCaps	D2	180.0	FALSE
	Falcon_Water_R	A4	Solvent_SnapCaps	D3	180.0	FALSE
	Falcon_Water_R	A4	Solvent_SnapCaps	D4	180.0	FALSE
	Falcon_Water_R	A4	Solvent_SnapCaps	D5	180.0	FALSE
	Falcon_Water_R	A4	Solvent_SnapCaps	D6	180.0	FALSE

For this protocol, the table should include transfer volumes from the source labware (Falcon tubes with Hyclone water) to the destination labware (Solvent_SnapCaps), or snapcap tubes that dry primer oligos will eventually be diluted to a 10 µM working dilution in. Be sure to specify source and destination wells. Here, the Pick_Up_Tip function details when the OT-2 will pick up a new tip.

c. Next, create a table with columns P-U to specify volume to dilute screwcap tubes (from primer order from Eurofins or similar) to 100 µM.

	Source_Labware	Source_Well	Destination_Labware	Destination_Well	Transfer_Volume	Pick_Up_Tip
Step 3: Put columns "P-U" into "Stock_Dilution_raw". Volume is in uL. This dilutes screwcap tubes from Eurofins to 100uM stock solution. Input volumes for dilution from primer order here.	Falcon_Water_Ra	A4	Dilute_100uM_ScrewCaps	A1	158	TRUE
	Falcon_Water_Ra	A4	Dilute_100uM_ScrewCaps	A2	164	FALSE
	Falcon_Water_Ra	A4	Dilute_100uM_ScrewCaps	A3	159	FALSE
	Falcon_Water_Ra	A4	Dilute_100uM_ScrewCaps	A4	153	FALSE
	Falcon_Water_Ra	A4	Dilute_100uM_ScrewCaps	A5	172	FALSE
	Falcon_Water_Ra	A4	Dilute_100uM_ScrewCaps	A6	208	FALSE
	Falcon_Water_Ra	A4	Dilute_100uM_ScrewCaps	B1	175	FALSE
	Falcon_Water_Ra	A4	Dilute_100uM_ScrewCaps	B2	186	FALSE
	Falcon_Water_Ra	A4	Dilute_100uM_ScrewCaps	B3	175	FALSE
	Falcon_Water_Ra	A4	Dilute_100uM_ScrewCaps	B4	176	FALSE
	Falcon_Water_Ra	A4	Dilute_100uM_ScrewCaps	B5	176	FALSE
	Falcon_Water_Ra	A4	Dilute_100uM_ScrewCaps	B6	171	FALSE
	Falcon_Water_Ra	A4	Dilute_100uM_ScrewCaps	C1	156	FALSE
	Falcon_Water_Ra	A4	Dilute_100uM_ScrewCaps	C2	187	FALSE
	Falcon_Water_Ra	A4	Dilute_100uM_ScrewCaps	C3	175	FALSE
	Falcon_Water_Ra	A4	Dilute_100uM_ScrewCaps	C4	166	FALSE
	Falcon_Water_Ra	A4	Dilute_100uM_ScrewCaps	C5	173	FALSE
	Falcon_Water_Ra	A4	Dilute_100uM_ScrewCaps	C6	180	FALSE
	Falcon_Water_Ra	A4	Dilute_100uM_ScrewCaps	D1	179	FALSE
	Falcon_Water_Ra	A4	Dilute_100uM_ScrewCaps	D2	181	FALSE
	Falcon_Water_Ra	A4	Dilute_100uM_ScrewCaps	D3	169	FALSE
	Falcon_Water_Ra	A4	Dilute_100uM_ScrewCaps	D4	154	FALSE
	Falcon_Water_Ra	A4	Dilute_100uM_ScrewCaps	D5	182	FALSE
	Falcon_Water_Ra	A4	Dilute_100uM_ScrewCaps	D6	190	FALSE

As shown here, the table should include transfer volumes from the source labware (Falcon tubes with Hyclone water) to the destination labware (Dilute_100uM_ScrewCaps), or screwcap tubes with dry oligos from Eurofins or a similar source. Volumes for dilution from the primer order should be placed into the column shown here in pink. Remind students to take notes on which primers correspond to each well of the respective tube racks.

d. Finally, create a table with columns W-AB. This table specifies the transfer volume from the screw cap tubes (dry primer oligos, now diluted) to the snap cap tubes (final dilution to 100 µM).

	Source_Labware	Source_Well	Destination_Labware	Destination_Well	Transfer_Volume	Pick_Up_Tip
Step 4: Put columns "W-AB" into "Working_Dilution_raw". Volume is in uL. This is the table for diluting solute in 1.5 mL snapcap tubes (180 uL water) for dilution (180uL water + 20uL 100uM primer = 10uM working primer dilution).	Dilute_100uM_ScrewCaps	A1	Solvent_SnapCaps	A1	20.0	TRUE
	Dilute_100uM_ScrewCaps	A2	Solvent_SnapCaps	A2	20.0	TRUE
	Dilute_100uM_ScrewCaps	A3	Solvent_SnapCaps	A3	20.0	TRUE
	Dilute_100uM_ScrewCaps	A4	Solvent_SnapCaps	A4	20.0	TRUE
	Dilute_100uM_ScrewCaps	A5	Solvent_SnapCaps	A5	20.0	TRUE
	Dilute_100uM_ScrewCaps	A6	Solvent_SnapCaps	A6	20.0	TRUE
	Dilute_100uM_ScrewCaps	B1	Solvent_SnapCaps	B1	20.0	TRUE
	Dilute_100uM_ScrewCaps	B2	Solvent_SnapCaps	B2	20.0	TRUE
	Dilute_100uM_ScrewCaps	B3	Solvent_SnapCaps	B3	20.0	TRUE
	Dilute_100uM_ScrewCaps	B4	Solvent_SnapCaps	B4	20.0	TRUE
	Dilute_100uM_ScrewCaps	B5	Solvent_SnapCaps	B5	20.0	TRUE
	Dilute_100uM_ScrewCaps	B6	Solvent_SnapCaps	B6	20.0	TRUE
	Dilute_100uM_ScrewCaps	C1	Solvent_SnapCaps	C1	20.0	TRUE
	Dilute_100uM_ScrewCaps	C2	Solvent_SnapCaps	C2	20.0	TRUE
	Dilute_100uM_ScrewCaps	C3	Solvent_SnapCaps	C3	20.0	TRUE
	Dilute_100uM_ScrewCaps	C4	Solvent_SnapCaps	C4	20.0	TRUE
	Dilute_100uM_ScrewCaps	C5	Solvent_SnapCaps	C5	20.0	TRUE
	Dilute_100uM_ScrewCaps	C6	Solvent_SnapCaps	C6	20.0	TRUE
	Dilute_100uM_ScrewCaps	D1	Solvent_SnapCaps	D1	20.0	TRUE
	Dilute_100uM_ScrewCaps	D2	Solvent_SnapCaps	D2	20.0	TRUE
	Dilute_100uM_ScrewCaps	D3	Solvent_SnapCaps	D3	20.0	TRUE
	Dilute_100uM_ScrewCaps	D4	Solvent_SnapCaps	D4	20.0	TRUE
	Dilute_100uM_ScrewCaps	D5	Solvent_SnapCaps	D5	20.0	TRUE
	Dilute_100uM_ScrewCaps	D6	Solvent_SnapCaps	D6	20.0	TRUE

After completing your data entry, calculate the total volume of Hyclone water required for all dilutions. Update the value in the Initial_Volume column as needed.

2. Save your Excel template. Download and open the Primer Dilution protocol in a code editing program.
3. Beginning at line 19, begin copying and pasting from the Excel template into the Python script. *Note:* when copying and pasting, you will be replacing previous data in the protocol.
    a. **Step 1**: Paste values from columns B-G into csv_volume_data_raw to specify the initial volume of Hyclone water for all dilutions.
    b. **Step 2**: Paste values from columns I-N into Prepare_Final_Dilution_raw (volume is in µL) to

specify volumes in snap cap tubes for final dilution (for example, 180 µL water + 20 µL 100 µM primer = 10 µM working primer dilution).

c. **Step 3:** Paste values in columns P-U into Stock_Dilution_raw (volume is in µL) to specify volumes to initially dilute dry primer oligos in screw cap tubes to 100 µM stock solution.
d. **Step 4:** Paste values from columns W-AB into Working_Dilution_raw (volume is in µL) to specify volumes for final dilution of dry primer oligos to 10 µM working primer dilution (for example, 180 µL water + 20 µL 100 µM primer = 10 µM working primer dilution).

4. Save your changes to the protocol and import the protocol in the Opentrons App.
5. Load your labware and liquids. Wells, or liquid locations, depend on values entered into your protocol.
    a. Opentrons OT-2 96 Tip Rack 300 µL in deck slot 9
    b. Opentrons 10 Tube Rack with Falcon tube containing HyClone water in deck slot 2
    c. Opentrons 24 Tube Rack with 1.5 mL Snapcap tubes in deck slot 5
    d. Opentrons 24 Tube Rack with Generic 2 mL Screwcap tubes in deck slot 4 (containing dry primer oligos)

6. Before running your protocol, specify the starting tip row and column for tip boxes to be used in the protocol in the available runtime parameter options in the app.
7. First, the OT-2 will transfer Hyclone water from the specified Falcon 4x50 mL conical tube to the specified 1.5 mL snapcap tubes in the tube rack.
8. Next, the OT-2 will transfer Hyclone water from the specified Falcon 4x50 mL conical tube to the specified 2 mL screwcap tubes in the tube rack (containing dry primer oligos).
9. Finally, the OT-2 will transfer the specified volumes from 2 mL screwcap tubes into the 1.5 mL snapcap tubes for final dilution, after a mix step in each screwcap tube.

Discussion Questions

Direct students to discuss the lab activities with one another. Example prompts might include:

- Name three rules of primer design that were important for designing your primers today. Consider melting temperature, GC content, and sequence.
- Which online tool was the most helpful *for you* during primer design? Why?

Opentrons
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Student Guide

Lab Module 7: Primer Design for PCR and Cloning

Dr. Cătălin Voiniciuc, Dr. Moni Qiande, and Abigail Lin

University of Florida

Pre-Lab Reading

Today in lab, you will prepare to amplify your DNA in a polymerase chain reaction (PCR). You'll prepare in two ways:

1) Designing primers to accurately amplify your DNA
2) Using the OT-2 to dilute primers to working stocks for PCR

The polymerase chain reaction (PCR) was a major scientific breakthrough in the mid-1980s. This reaction *amplifies* DNA, or a specific segment of DNA. Using this reaction, you can produce millions (or even billions) of copies of your specific gene or region of interest! Your PCR reaction will consist of the following:

-**your starting DNA template** (here, your plasmid backbone and gene of interest)
-**DNA polymerase (**an enzyme that assembles new pieces of DNA)
-**dNTPs** (*deoxynucleotide triphosphates*; single bases [dATP, dCTP, dGTP, or dDTP] to assemble new DNA with)
-**forward and reverse primers** (templates for the polymerase to construct new copies of each strand of DNA)

These components are generally mixed with a buffer and water. It's also common to use a *master mix* containing some of the above components to simplify the process. The reaction

mix is placed into a *thermocycler* and proceeds through several steps:

1. **Denaturation:** heat is used to separate the double-stranded DNA into individual strands
2. **Annealing:** primers bind to complementary sequences in the DNA template
3. **Extension:** DNA polymerase *extends* primer sequences to build new copies of the DNA

This is repeated over multiple cycles in the thermocycler: (*thermo* (heat) and *cycler* (multiple cycles)) to obtain those millions or billions of copies of your DNA. PCR is a crucial reaction in many biology processes, because DNA is a critical component that scientists often don't have enough of. In this class, you'll use PCR in two ways: first, amplifying your DNA for cloning, and second, to *genotype* (begin to confirm) your final construct (plasmid + inserted gene).

The first step in preparing for PCR is often primer design. The PCR reaction can amplify any gene, as long as a template is supplied via the forward and reverse primers. These primers are usually designed using online tools (some of which you'll get to try in class) and ordered to be custom-made by entering their specific sequence. For today's lab, you can follow the attached Primer and PCR Design Worksheet. The worksheet

will help to guide you through some key considerations in primer design, such as:

- **Melting temperature:** the temperature at which 50% of the double-stranded DNA is dissociated into single strands in the **denaturation** step. The difference in melting temperatures for primers used in your reaction should be less than 5°C.
- **CG** (cytosine/guanine) **content:** percentage of cytosine and guanine bases in your DNA (and primer) sequences. A higher GC content results in stronger binding between the primers and the template DNA. Percentages should be between 35-80%, and ideally, the primer percentage closely matches the template DNA percentage.
- **Specificity:** primers should be specific to a single DNA sequence, and primer pairs should not have complementary regions (to prevent *primer-dimer formation*, or primers binding to one another, and not the template DNA).

In this course, we are designing primers not only for DNA amplification, but for cloning purposes. This means that sequences for restriction sites are also added to the primer sequence, ensuring that new copies of DNA are able to be cut by the restriction enzyme. In today's class, you can follow the Primer and PCR Design Worksheet to build your primer sequences with all of the above considerations. Then, you'll

use the OT-2 to dilute your primers to working stock concentrations ahead of the PCR reaction.

Purpose

In this lab, you will design primers for PCR and cloning using an attached worksheet. Primer activities in this lab will prepare you for the following classes, in which we'll amplify your DNA via PCR and complete digestion and ligation of your plasmid and gene of interest at the same time. Preparation of your primers in solution and dilution to stocks we can use in PCR is automated using the OT-2.

Learning Outcomes

- Understand how to design appropriate primers for your experiment
- Be able to plan for cloning experiments using PCR
- Calculate dilution volumes
- Automate dilution tasks in the laboratory

Supplies

Opentrons Equipment

- ☐ OT-2 automated liquid handling robot
- ☐ OT-2 P300 Single-Channel GEN2 pipette (in right mount)

Non-Opentrons Equipment

- [ ] Primer Dilution protocol

Non-Opentrons Equipment

- [ ] Benchtop thermocycler

Labware

- [ ] Opentrons OT-2 Tips, 300µL in deck slot 9
- [ ] Opentrons 10 Tube Rack with Falcon 4x 50 mL and 6x15 mL Conical tubes in deck slot 2
- [ ] Opentrons 24 Tube Rack with NEST 1.5 mL Snapcap tubes in deck slot 5
- [ ] Opentrons 24 Tube Rack with Generic 2 mL Screwcap tubes in deck slot 4

Reagents and Other Materials

- [ ] Primers (dry oligos, ordered from Eurofins Scientific or similar) in 2 mL screwcap tubes
- [ ] Molecular grade water, such as HyClone Water

Procedure Guide

Before Class

1. Complete the pre-lab reading.

Primer Design

1. Follow the attached Primer and PCR Design worksheet:
2. Starting with section **A,** you will design primers for classical cloning based on your gene of interest.
    a. You will need the DNA sequence (nucleotide sequence, including start and end regions) of your gene of interest to be amplified.
    b. You can use an online tool (such as the Multiple Primer Analyzer from ThermoFisher) to help design your primers.
        i. Enter your forward and reverse primer sequences (and a name for your primer) into the Multiple Primer Analyzer.
        ii. Read your results (the Analyzer provides melting temperature, CG content, and other considerations, including *primer-dimer estimation*) and fill out the provided table for your forward and reverse primers.
    c. Fill in the provided table with the amounts of each component (buffer, dNTPs, primer, etc.) that would be needed for your PCR reaction.

d. Follow section **B** to design primers that could be used to *genotype* (confirm and identify) your finished construct (in this case, we'll use the pPICZ B plasmid backbone and added gene construct you worked with in the Benchling virtual digest in Lab Module 5). You can use Primer3 or Primer3Plus to pick primers from your DNA sequence.
e. Fill in the provided table with your primer details.
f. Next, you'll consider your four primers, and how you could run your genotyping PCR in a single thermocycler. Fill in the provided table with details for one gene-specific primer pair and one vector-specific primer pair.

Primer Dilution

Now, you'll use the OT-2 to prepare 100 µM and 10 µM primer stocks from dry oligos: first, preparing a 100 µM stock, followed by dilution to a 10 µM working solution.

1. With your class, you will create an Excel template to insert volumes to be used in the protocol. This can be done before or during class. The Python protocol, using your entered values, can dilute up to 24 primers in a single run. *Note:* if diluting less than 24 samples, all subsequent empty rows must be deleted for Steps 2, 3, and 4.
    a. First, create a table with columns B-G to specify initial liquid volumes for transfers.

Step 1: Put columns "B-G" into "csv_volume_data_raw" This stocks the robot with Hyclone water in falcon tube(s) for all primer diutions. Calculate how much water is needed before running. Can Leave column "D" value as is.	Labware	Initial_Wells	Initial_Volume	Liquid_Name	Description	Color
	Falcon_Water	A4	25000	Hyclone Water	Hyclone Water	#00FF00

For this protocol, the table should include a Falcon tube (Falcon_Water) in well A4 (of the tube rack this Falcon tube is located in) with an initial volume of 25 mL of Hyclone water. *Note:* here, API names are not needed. Instead, a common name (Falcon_Water) is defined in a labware dictionary (labware_dict) function in the protocol. If you change values in the Labware column, be sure to make the equivalent changes in the labware dictionary protocol section.

b. Next, create a table with columns I-N to specify transfer volumes of water to prepare for primer dilution.

Step 2: Put columns "I-N" into "Prepare_Final_Dilution_raw". Volume is in uL. This is the table for preparing solvent in 1.5 mL snapcap tubes for dilution (180uL water + 20uL 100uM primer = 10uM working primer dilution).	Source_Labware	Source_Well	Destination_Labware	Destination_Well	Transfer_Volume	Pick_Up_Tip
	Falcon_Water_R	A4	Solvent_SnapCaps	A1	180.0	TRUE
	Falcon_Water_R	A4	Solvent_SnapCaps	A2	180.0	FALSE
	Falcon_Water_R	A4	Solvent_SnapCaps	A3	180.0	FALSE
	Falcon_Water_R	A4	Solvent_SnapCaps	A4	180.0	FALSE
	Falcon_Water_R	A4	Solvent_SnapCaps	A5	180.0	FALSE
	Falcon_Water_R	A4	Solvent_SnapCaps	A6	180.0	FALSE
	Falcon_Water_R	A4	Solvent_SnapCaps	B1	180.0	FALSE
	Falcon_Water_R	A4	Solvent_SnapCaps	B2	180.0	FALSE
	Falcon_Water_R	A4	Solvent_SnapCaps	B3	180.0	FALSE
	Falcon_Water_R	A4	Solvent_SnapCaps	B4	180.0	FALSE
	Falcon_Water_R	A4	Solvent_SnapCaps	B5	180.0	FALSE
	Falcon_Water_R	A4	Solvent_SnapCaps	B6	180.0	FALSE
	Falcon_Water_R	A4	Solvent_SnapCaps	C1	180.0	FALSE
	Falcon_Water_R	A4	Solvent_SnapCaps	C2	180.0	FALSE
	Falcon_Water_R	A4	Solvent_SnapCaps	C3	180.0	FALSE
	Falcon_Water_R	A4	Solvent_SnapCaps	C4	180.0	FALSE
	Falcon_Water_R	A4	Solvent_SnapCaps	C5	180.0	FALSE
	Falcon_Water_R	A4	Solvent_SnapCaps	C6	180.0	FALSE
	Falcon_Water_R	A4	Solvent_SnapCaps	D1	180.0	FALSE
	Falcon_Water_R	A4	Solvent_SnapCaps	D2	180.0	FALSE
	Falcon_Water_R	A4	Solvent_SnapCaps	D3	180.0	FALSE
	Falcon_Water_R	A4	Solvent_SnapCaps	D4	180.0	FALSE
	Falcon_Water_R	A4	Solvent_SnapCaps	D5	180.0	FALSE
	Falcon_Water_R	A4	Solvent_SnapCaps	D6	180.0	FALSE

For this protocol, the table should include transfer volumes from the source labware (Falcon tubes with Hyclone water) to the destination labware (Solvent_SnapCaps), or snapcap tubes that dry primer oligos will eventually be diluted to a 10 µM working dilution in. Be sure to specify source and destination wells. Here, the Pick_Up_Tip function details when the OT-2 will pick up a new tip.

c. Next, create a table with columns P-U to specify volume to dilute screwcap tubes (from primer order from Eurofins or similar) to 100 µM.

	Source_Labware	Source_Well	Destination_Labware	Destination_Well	Transfer_Volume	Pick_Up_Tip
Step 3: Put columns "P-U" into "Stock_Dilution_raw". Volume is in uL. This dilutes screwcap tubes from Eurofins to 100uM stock solution. Input volumes for dilution from primer order here.	Falcon_Water_Ra	A4	Dilute_100uM_ScrewCaps	A1	158	TRUE
	Falcon_Water_Ra	A4	Dilute_100uM_ScrewCaps	A2	164	FALSE
	Falcon_Water_Ra	A4	Dilute_100uM_ScrewCaps	A3	159	FALSE
	Falcon_Water_Ra	A4	Dilute_100uM_ScrewCaps	A4	153	FALSE
	Falcon_Water_Ra	A4	Dilute_100uM_ScrewCaps	A5	172	FALSE
	Falcon_Water_Ra	A4	Dilute_100uM_ScrewCaps	A6	208	FALSE
	Falcon_Water_Ra	A4	Dilute_100uM_ScrewCaps	B1	175	FALSE
	Falcon_Water_Ra	A4	Dilute_100uM_ScrewCaps	B2	186	FALSE
	Falcon_Water_Ra	A4	Dilute_100uM_ScrewCaps	B3	175	FALSE
	Falcon_Water_Ra	A4	Dilute_100uM_ScrewCaps	B4	176	FALSE
	Falcon_Water_Ra	A4	Dilute_100uM_ScrewCaps	B5	176	FALSE
	Falcon_Water_Ra	A4	Dilute_100uM_ScrewCaps	B6	171	FALSE
	Falcon_Water_Ra	A4	Dilute_100uM_ScrewCaps	C1	156	FALSE
	Falcon_Water_Ra	A4	Dilute_100uM_ScrewCaps	C2	187	FALSE
	Falcon_Water_Ra	A4	Dilute_100uM_ScrewCaps	C3	175	FALSE
	Falcon_Water_Ra	A4	Dilute_100uM_ScrewCaps	C4	166	FALSE
	Falcon_Water_Ra	A4	Dilute_100uM_ScrewCaps	C5	173	FALSE
	Falcon_Water_Ra	A4	Dilute_100uM_ScrewCaps	C6	180	FALSE
	Falcon_Water_Ra	A4	Dilute_100uM_ScrewCaps	D1	179	FALSE
	Falcon_Water_Ra	A4	Dilute_100uM_ScrewCaps	D2	181	FALSE
	Falcon_Water_Ra	A4	Dilute_100uM_ScrewCaps	D3	169	FALSE
	Falcon_Water_Ra	A4	Dilute_100uM_ScrewCaps	D4	154	FALSE
	Falcon_Water_Ra	A4	Dilute_100uM_ScrewCaps	D5	182	FALSE
	Falcon_Water_Ra	A4	Dilute_100uM_ScrewCaps	D6	190	FALSE

As shown here, the table should include transfer volumes from the source labware (Falcon tubes with Hyclone water) to the destination labware (Dilute_100uM_ScrewCaps), or screwcap tubes with dry oligos from Eurofins or a similar source. Volumes for dilution from the primer order should be placed into the column shown here in pink. Remind students to take notes on which primers correspond to each well of the respective tube racks.

d. Finally, create a table with columns W-AB. This table specifies the transfer volume from the screw cap tubes (dry primer oligos, now diluted) to the snap cap tubes (final dilution to 100 µM).

	Source_Labware	Source_Well	Destination_Labware	Destination_Well	Transfer_Volume	Pick_Up_Tip
Step 4: Put columns "W-AB" into "Working_Dilution_raw". Volume is in uL. This is the table for diluting solute in 1.5 mL snapcap tubes (180 uL water) for dilution (180uL water + 20uL 100uM primer = 10uM working primer dilution).	Dilute_100uM_ScrewCaps	A1	Solvent_SnapCaps	A1	20.0	TRUE
	Dilute_100uM_ScrewCaps	A2	Solvent_SnapCaps	A2	20.0	TRUE
	Dilute_100uM_ScrewCaps	A3	Solvent_SnapCaps	A3	20.0	TRUE
	Dilute_100uM_ScrewCaps	A4	Solvent_SnapCaps	A4	20.0	TRUE
	Dilute_100uM_ScrewCaps	A5	Solvent_SnapCaps	A5	20.0	TRUE
	Dilute_100uM_ScrewCaps	A6	Solvent_SnapCaps	A6	20.0	TRUE
	Dilute_100uM_ScrewCaps	B1	Solvent_SnapCaps	B1	20.0	TRUE
	Dilute_100uM_ScrewCaps	B2	Solvent_SnapCaps	B2	20.0	TRUE
	Dilute_100uM_ScrewCaps	B3	Solvent_SnapCaps	B3	20.0	TRUE
	Dilute_100uM_ScrewCaps	B4	Solvent_SnapCaps	B4	20.0	TRUE
	Dilute_100uM_ScrewCaps	B5	Solvent_SnapCaps	B5	20.0	TRUE
	Dilute_100uM_ScrewCaps	B6	Solvent_SnapCaps	B6	20.0	TRUE
	Dilute_100uM_ScrewCaps	C1	Solvent_SnapCaps	C1	20.0	TRUE
	Dilute_100uM_ScrewCaps	C2	Solvent_SnapCaps	C2	20.0	TRUE
	Dilute_100uM_ScrewCaps	C3	Solvent_SnapCaps	C3	20.0	TRUE
	Dilute_100uM_ScrewCaps	C4	Solvent_SnapCaps	C4	20.0	TRUE
	Dilute_100uM_ScrewCaps	C5	Solvent_SnapCaps	C5	20.0	TRUE
	Dilute_100uM_ScrewCaps	C6	Solvent_SnapCaps	C6	20.0	TRUE
	Dilute_100uM_ScrewCaps	D1	Solvent_SnapCaps	D1	20.0	TRUE
	Dilute_100uM_ScrewCaps	D2	Solvent_SnapCaps	D2	20.0	TRUE
	Dilute_100uM_ScrewCaps	D3	Solvent_SnapCaps	D3	20.0	TRUE
	Dilute_100uM_ScrewCaps	D4	Solvent_SnapCaps	D4	20.0	TRUE
	Dilute_100uM_ScrewCaps	D5	Solvent_SnapCaps	D5	20.0	TRUE
	Dilute_100uM_ScrewCaps	D6	Solvent_SnapCaps	D6	20.0	TRUE

After completing your data entry, calculate the total volume of Hyclone water required for all dilutions. Update the value in the Initial_Volume column as needed.

2. Save your Excel template. Download and open the Primer Dilution protocol in a code editing program.
3. Beginning at line 19, begin copying and pasting from the Excel template into the Python script. *Note:* when copying and pasting, you will be replacing previous data in the protocol.
    a. **Step 1**: Paste values from columns B-G into csv_volume_data_raw to specify the initial volume of Hyclone water for all dilutions.
    b. **Step 2**: Paste values from columns I-N into Prepare_Final_Dilution_raw (volume is in µL) to

specify volumes in snap cap tubes for final dilution (for example, 180 µL water + 20 µL 100 µM primer = 10 µM working primer dilution).

c. **Step 3:** Paste values in columns P-U into Stock_Dilution_raw (volume is in µL) to specify volumes to initially dilute dry primer oligos in screw cap tubes to 100 µM stock solution.
d. **Step 4:** Paste values from columns W-AB into Working_Dilution_raw (volume is in µL) to specify volumes for final dilution of dry primer oligos to 10 µM working primer dilution (for example, 180 µL water + 20 µL 100 µM primer = 10 µM working primer dilution).

4. Save your changes to the protocol and import the protocol in the Opentrons app.
5. Load your labware and liquids. Wells, or liquid locations, depend on values entered into your protocol.
    a. Opentrons OT-2 96 Tip Rack 300 µL in deck slot 9
    b. Opentrons 10 Tube Rack with Falcon tube containing HyClone water in deck slot 2
    c. Opentrons 24 Tube Rack with 1.5 mL Snapcap tubes in deck slot 5
    d. Opentrons 24 Tube Rack with Generic 2 mL Screwcap tubes in deck slot 4 (containing dry primer oligos)

6. Before running your protocol, specify the starting tip row and column for tip boxes to be used in the protocol in the available runtime parameter options.
7. First, the OT-2 will transfer Hyclone water from the specified Falcon 4x50 mL conical tube to the specified 1.5 mL snapcap tubes in the tube rack.
8. Next, the OT-2 will transfer Hyclone water from the specified Falcon 4x50 mL conical tube to the specified 2 mL screwcap tubes in the tube rack (containing dry primer oligos).
9. Finally, the OT-2 will transfer the specified volumes from 2 mL screwcap tubes into the 1.5 mL snapcap tubes for final dilution, after a mix step in each screwcap tube.

Discussion Questions

Discuss today's lab activities with a neighbor.

- Name three rules of primer design that were important for designing your primers today. Consider melting temperature, GC content, and sequence.
- Which online tool was the most helpful *for you* during primer design? Why?

Primer and PCR Design Worksheet

Section A: Classical (restriction enzyme) cloning

Design primers for classical cloning manually using Multiple Primer Analyzer .

✔ Example: **AtCSLA2** (ATG to TAG + 8 bp of 3' UTR); Total size: 1613 bp

Primer Name	Final Sequence (RE underlined)	Tm°C	CG %	nt	Problems *
CV22F	gta**TTCGAA**ATGGACGGTGTATC ACCAAAG	72.2	43.3	30	none
CV22R	aat**CACGTG**CACAACTACTAACTC GGGACATAAG	73.6	44.1	34	none

***** 'Problems' refers to the results shown in 'Results for primer dimer detection' using Multiple Primer Analyzer.

For your gene, fill in the provided table:

Gene #_____ (selected DNA _____); **Total size**: _____ bp

Primer Name	Final Sequence (RE underlined)	Tm° C	CG %	nt	Problems
1F					
1R					

Why should you use Phusion DNA polymerase to clone your gene of interest?

Fill in all the relevant blanks for your designed primers. For step 3, consider only the Tm of the gene-specific portion of your primer sequence.

# Reactions and µL needed	**1**	**4**		**Thermocycler Conditions**	**°C**	**min: sec**	
HyClone Water				1. Initial Denaturation			
Green HF Buffer (5x)				2. Denaturation			
dNTPs (10 mM)				3. Annealing			5x
F Primer (10 µM)				4. Extension			
R Primer (10 µM)				5. Denaturation			30x
DNA Template	1.35			6. Anneal and Extend			
Phusion DNA Polymerase				7. Final Elongation			
Total	20.00			8. Storage	12	∞	

Section B: Genotyping of desired constructs using Taq DNA polymerase

Design primers to genotype your pPICZ B + Gene construct using **Primer3** or **Primer3Plus**.

Primer Name (e.g. CV001**F**)	**5' to 3' Sequence**	**Information** (e.g. Tm = 59°C, specificity)
2F		°C, Vector Specific
3R		°C, Gene Specific
4R		°C, Vector Specific

Why use Taq DNA polymerase instead of Phusion for genotyping purposes?

Draw the relative binding positions of your 4 primers using arrows and labels (Arrow direction: 5’ 3’)

Fill in the blanks for the primers and the PCR conditions required to run these reactions *in parallel in a **single** thermocycler*.

There are two options for primers, both of which may need to be used.

1. 2 vector-specific primers (to determine the size of your DNA insert), or:
2. 1 vector-specific and 1 gene-specific primer (to confirm that the correct gene was cloned in the desired direction).

You can use the Benchling “Attach Primers” function to help find the best primer combinations.

F Primer	R Primer	Amplicon (bp) for pPICZ B	Amplicon (bp) for pPICZ B + **Gene**

Now, add in your thermocycler conditions. For the Extension step, calculate the time based on a rate of 1 min/kilobase pair of the desired *amplicon* (in other words, how large is the sequence you are amplifying? How fast could it be amplified at a rate of 1 minute/ kilobase pair?).

# Reactions and volumes (µL) needed	**1**	**18**		**Thermocycler Conditions**	**°C**	**min:sec**	
HyClone Water				1. Initial Denaturation			
Red Taq 2x MM (1.5 mM $MgCl_2$)				2. Denaturation			25x
F Primer (10 µM)				3. Annealing			
R Primer (10 µM)				4. Extension			
DNA Template	1.0			5. Final Extension			
Total Volume	10.0			6. Storage	12	∞	