Plasmid sequencing library prep: pLCv2-opti-HepmCherry

Introduction
Preparation of Illumina sequencing libraries from pLentiCRISPRv2-HepmCherry-based pooled CRISPR libraries.

NOTE: Modifications to a vector may result in removal of primer binding sites. Verify primer binding and correct predicted amplicon prior to starting the protocol.

Materials

› Plasmid DNA
  › 10 ng/μL dilution

› Forward and reverse PCR primers
  › gPCR primer: 5’-AATGATACGGGACCCACCGAGATCTACACCAGACTCGTGGTCTCTTTTTTACGATCT-3’
  › General Index primer: 5’-CAAGCAGAAGACGGCATACGACGCTATAGCGG-12m-ATTCTTGAGGATGCTTGTGAGCT-3’ (n 6-mer denotes barcode for demultiplexing)

› Takara ExTaq DNA Polymerase
  › 10X ExTaq buffer
  › 2.5 mM dNTPs
  › Polymerase

› Sterile diH₂O stored at 4 °C

› PCR strips and caps

› Microcentrifuge tubes

› MagBio HighPrep PCR beads or Zymo DNA Clean and Concentrator 5 Kit

› Freshly-prepared 85% ethanol

› Qubit DNA High Sensitivity Assay Kit

› Qubit assay tubes

› Qubit fluorometer

› Sequencing primers
  › Opti sequencing primer: 5’-GTTGATAACGGACTAGCCTATTTAAAACATTGCTATGCTTTCCAGCATAGCTTAAAC-3’
  › Index sequencing primer: 5’-TTTCAAGTTACGGTAGTATAGCCATTTAAAACATTGCTATGCTTTCCAGCATAGCTTAAAC-3’

Procedure
Prepare pre-PCR workspace and reagents

**CRITICAL** Pre-PCR reagents can easily become contaminated by CRISPR plasmids or PCR products. These templates will amplify much more readily than gDNA or other templates which will result in amplification of the wrong template. Always take care to handle pre-PCR reagents separately from plasmids and PCR products.

1. Turn on UV light and air circulator in the PCR hood or non-CRISPR-contaminated space.
   - Make sure magnet engages.
   - UV is on a 30min timer.

2. Add ice to the pre-PCR ice bucket. Chill pre-PCR cold block in ice bucket and place in the PCR room. Thaw 10X ExTaq buffer, 2.5 mM dNTPs, and primers at RT in the PCR room.
   - Use new gloves when handling ice bucket and reagents to avoid contamination with plasmid or PCR products.
   - Do not expose reagents to UV.
   - Place reagents on ice as soon as they are thawed.

3. Label and place PCR strips and microcentrifuge tubes in pre-PCR cold block on ice. Place PCR caps in PCR hood.

Prepare post-PCR workspace and reagents

4. Chill post-PCR tube rack and thaw plasmid (10 ng/μL) on ice.
   - **NOTE:** Thaw and pipette plasmid in a separate area from pre-PCR reagents.

5. Pour a 1% TAE gel.

6. Start CRISPR PCR 16 program on thermocycler.
   - Take care to use a block that has space for the appropriate number of tubes.

### Table 1

<table>
<thead>
<tr>
<th></th>
<th>A</th>
<th>B</th>
<th>C</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1 cycle</td>
<td>95 °C</td>
<td>5 min</td>
</tr>
<tr>
<td>2</td>
<td></td>
<td>95 °C</td>
<td>10 sec</td>
</tr>
<tr>
<td>3</td>
<td>16 cycles</td>
<td>60 °C</td>
<td>15 sec</td>
</tr>
<tr>
<td>4</td>
<td></td>
<td>72 °C</td>
<td>45 sec</td>
</tr>
<tr>
<td>5</td>
<td>1 cycle</td>
<td>72 °C</td>
<td>5 min</td>
</tr>
<tr>
<td>6</td>
<td>hold</td>
<td>4 °C</td>
<td>∞</td>
</tr>
</tbody>
</table>

Make master mix in pre-PCR workspace
Set up a no-template control (NTC) reaction for each sample / each set of reagents used. This control will allow you to assess whether your reagents have been contaminated by plasmid or PCR product.

**PCR master mix**

<table>
<thead>
<tr>
<th></th>
<th>A</th>
<th>B</th>
<th>C</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Number of 50 μL reactions:</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>diH₂O</td>
<td>35.75</td>
<td>117.975</td>
</tr>
<tr>
<td>4</td>
<td>10X ExTaq Buffer</td>
<td>5</td>
<td>16.5</td>
</tr>
<tr>
<td>5</td>
<td>2.5 mM dNTPs</td>
<td>4</td>
<td>13.2</td>
</tr>
<tr>
<td>6</td>
<td>gPCR primer</td>
<td>2</td>
<td>6.6</td>
</tr>
<tr>
<td>7</td>
<td>General Index Primer</td>
<td>2</td>
<td>6.6</td>
</tr>
<tr>
<td>8</td>
<td>ExTaq Polymerase</td>
<td>0.25</td>
<td>0.825</td>
</tr>
<tr>
<td>9</td>
<td>diH₂O or plasmid DNA</td>
<td>1</td>
<td>3.3</td>
</tr>
<tr>
<td>10</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>Total volume</td>
<td>50</td>
<td>165</td>
</tr>
</tbody>
</table>

7. Using new gloves, place Extaq directly on ice from the -20 °C freezer.

8. Briefly vortex and centrifuge all reagents in pre-PCR workspace.

9. Remove sterile diH₂O from 4 °C and place in PCR hood.

   **CRITICAL**  H₂O must be stored at 4 °C because this ExTaq polymerase is NOT hot-start. Incompletely chilled master mix can result in mispriming and reaction failure.

10. Add H₂O, 10X ExTaq buffer, and both primers to chilled microcentrifuge tube. Briefly vortex and centrifuge mix.

11. Add ExTaq polymerase to mix. Briefly vortex and spin.

12. Aliquot 49 μL to 3 PCR tubes. Add 1 μL chilled diH₂O to one PCR tube on the end. This is your no-template control. Carefully snap the lid on the NTC tube ONLY.

   **CRITICAL**  A no-template control (NTC) reaction is required for each sample / each set of reagents used. This control allows assessment of reagents to determine whether they have been contaminated by plasmid or PCR product.

Put away reagents, add plasmid, start PCR reaction
13. Put away all pre-PCR reagents. Carefully transfer PCR strips to post-PCR ice bucket. Put away pre-PCR ice bucket and cold block.

14. Briefly vortex and spin down plasmid. Using filter tips, carefully add 1 μL plasmid (10 ng/μL) to the remaining two PCR tubes. Close remaining two PCR tubes.

15. Briefly vortex or flick tubes to thoroughly mix reactions and quickly centrifuge. Ensure there are no air bubbles, but work quickly to maintain a 4 °C temperature.

16. Open the thermocycler lid and place tubes in small tube holders in the thermocycler block. Close the thermocycler lid. Press enter to clear the error message. Press enter again to proceed from the 95 °C hold to the first step of the program.

Assess PCR reaction

17. Chill a microfuge tube on ice. When PCR reaction is complete and block has cooled to 4 °C, press enter to end program and place PCR strips on ice.

18. Taking care not to uncap the NTC tube, combine the two template reactions together and pipette into the chilled microfuge tube. Briefly vortex and centrifuge.

19. Prepare two aliquots of 10X DNA loading dye. Add 5 μL NTC reaction to one aliquot and 5 μL template reaction to the other aliquot.

20. Load 5 μL DNA ladder and all 10 μL of NTC and template aliquots on a 1% TAE gel. Run gel 30 min at 100V or 1h at 50V.

21. Image gel and save each image as an .sgd file and as a .jpg file. Print a physical copy of each .sgd image to insert into notebook. Insert each .jpg image into the experimental spreadsheet on Google Drive.

CRITICAL Assess gel for 1.) appropriate molecular weight band in the template reaction and its intensity; 2.) absence of appropriate molecular weight band in the NTC reaction; 3.) presence and intensity of primer and primer dimer. Longer exposures may be necessary to visualize potential products in the NTC lane.

Clean/size-select PCR reactions

NOTE: Protocol below uses HighPrep PCR beads to clean and size-select the sequencing library. Using a Zymo DNA Clean and Concentrator 5 or other column-based PCR purification kit has also yielded high-quality sequencing libraries.

22. Warm HighPrep PCR beads to RT. Prepare 500 μL of fresh 85% ethanol in H₂O per sequencing library.

23. Mix beads thoroughly. To 95 μL combined PCR reactions, add 1X volume (95 μL) beads. Vortex 30 sec - 1 min. Briefly centrifuge.

24. Incubate 10 min at RT.
25. Place tube on magnetic rack for 5 min.

26. Remove and discard supernatant. Add 200 μL fresh 85% ethanol to the tube, taking care not to dislodge the beads. If ethanol does not cover the beads, gently rock the magnetic rack back and forth several times to wash the beads.


28. Remove and discard ethanol wash. Using a P20, carefully remove excess ethanol residue from the tube. Air dry 5 min at RT.

    CRITICAL Do not allow beads to dry more than 5 min.

29. Remove tube from magnetic rack. Add 20 μL diH₂O to beads, pipetting up and down to dislodge the beads from the side of the tube and mix. Vortex 30 sec - 1 min. Briefly centrifuge.

30. Incubate 10 min at RT.

31. Place tube on magnetic rack for 5 min. Transfer the eluate to a new tube, taking care not to transfer any beads.

    Recovery is typically 15 μL - 18 μL.

Quantify PCR reactions

32. Quantify 1 μL of library by nanodrop.

    Qubit DNA High Sensitivity Assay can quantify DNA at ~0.2 - 100 ng/μL. If necessary, dilute a portion of the sequencing library so it falls within this range.

33. Equilibrate all Qubit reagents to RT. Vortex and briefly spin reagents.

    NOTE: protect Qubit reagent from light.

34. Set up 3 Qubit assay tubes (one for sample and two for standards).

35. To prepare Qubit working solution, aliquot 700 μL Qubit assay buffer to a plastic tube (200 μL for sample plus two standards, plus 100 μL extra). Add 3.5 μL Qubit reagent and vortex to mix.

    NOTE: Do not prepare in a glass container.

36. Aliquot 190 μL Qubit working solution to each standard Qubit assay tube. Aliquot 199 μL Qubit working solution to sequencing library tube.

37. Add 10 μL Standard 1 to first standard tube. Vortex to mix.

38. Add 10 μL Standard 2 to second standard tube. Vortex to mix.

39. Add 1 μL sequencing library to sample tube. Vortex to mix.

40. Incubate 2 min at RT. Read on Qubit fluorometer, selecting DNA High Sensitivity Assay and 1 μL sample.
Submit libraries with custom sequencing primers

41. Use custom sequencing primers Opti sequencing primer and Index sequencing primer to sequence the sgRNA and the demultiplexing barcode.

**NOTE:** This sequencing library is designed such that the orientation of the sequence output will be the reverse complement of the sgRNA sequence and the reverse complement of the barcode sequence.