HDR Plasmid Cloning Protocol

Updated 01/05/2024

**Part 1 – gRNA insertion**

1. Make 1:1 mix of diluted (20uM) **XXX-gRNA-F** and **XXX-gRNA-R** primers. Anneal the **XXX-gRNA-F** and **XXX-gRNA-R** mix with a gradually decreasing cycle in the PCR machine from 94C to 4C.
2. Insert the primers into the plasmid *pHEE-GT* (cut with *BsaI)* using T4 ligase. Use the following table for creating the mix (volumes in microliters).

|  |  |
| --- | --- |
| H2O | 14.5 |
| 10X T4 Ligase Buffer | 2 |
| Annealed gRNA Primer Mix | 0.5 |
| BsaI cut plasmid | 2 |
| T4 Ligase | 1 |
| **Total** | **20** |

Mix and leave the reaction tube floating on ice which will melt overnight.

1. In the morning, deactivate the reaction for 10 minutes at 65C.
2. Transform the plasmid into E.coli (I typically transform 1ul of ligation mix into our competent cells). Inoculate two colonies and perform mini-prep. Do not do colony PCR. Sequence one of the plasmids using **U6-26-IDF** to confirm single gRNA insertion. You now have a “gRNA plasmid”.
3. Cut the gRNA plasmid with HindIII. (Amount of plasmid may vary based on your concentration):

|  |  |
| --- | --- |
| H2O | 38 |
| 10X NEB Buffer 2.1 | 5 |
| gRNA Plasmid | 5 |
| HindIII | 2 |
| **Total** | **50** |

Leave overnight at 37C.

1. The next morning, deactivate HindIII digest for 20 minutes at 80C. Continue to Step 9 when ready.

**Part 2: Donor creation and insertion**

1. Amplify the fragments of your donor element. I recommend using 2X Q5 Master Mix. Shown is an example for a GFP donor element for gene XXX:
   1. L: **XXX-L-F** + **XXX-L-R** + Col DNA
   2. C: **GFP-F** + **GFP-R** + A plasmid containing GFP
   3. R: **XXX-R-F** + **XXX-R-R** + Col DNA

|  |  |
| --- | --- |
| H2O | 10.1 |
| Primer 1 | 0.7 |
| Primer 2 | 0.7 |
| Template DNA | 1 |
| 2X Q5 MM | 12.5 |
| **Total** | **25** |

98C | 98C 65C 72C | 72C

2:00 | :10 :20 :30\* | 2:00

35 Cycles

\*(Extension time varies based on fragment length)

1. Run gels on the PCRs and cut bands. Do subsequent overlapping PCRs to get one large fragment. If you cannot get one large fragment, you may be able to do the Gibson Assembly with just two fragments, or even three.
2. Insert the donor piece into the HindIII cut gRNA plasmid. Use the 2X Gibson Assembly Master Mix. Only 1ul of Master Mix is needed if you are inserting a single fragment. More is required as the number of fragments increases.
3. Transform the plasmid into E.coli. Select a sufficient number of colonies and perform colony PCR. Check the left side: **pHEE-HindIII-seq1** + **GFP-R** and the right side: **pHEE-d-HindIII-seq2** + **GFP-F**. Inoculate positive colonies and perform mini-prep. Sequence one of the plasmids using **pHEE-HindIII-seq1** and **pHEE-d-HindIII-seq2** to confirm the donor element sequence. You now have the complete gene targeting plasmid.