# pDG* One-Step Cloning Protocol

Oligos for guide insertion into plasmid need to be of the following form:

```
5′-CACCGNNNNNNNNNNNNNNNNNNN-3′
    3′-CNNNNNNNNNNNNNNNNNNNCAAA-5′
```

And:

```
5′-ACCGNNNNNNNNNNNNNNNNNNNGT-3′
    3′-NNNNNNNNNNNNNNNNNNNCAAAAT-5′
```

If the first N on the top strand for each guide is a G, it should be excluded.

The N's in the top strands comprise the guide sequences, which target the matching gRNA binding sequences followed by PAMs in the genomic DNA.

The overhangs allow the oligos to match the cut sites in the pDG* plasmids caused by *Bbs*I digestion.

The extra G/C in the first pair of oligos completes the U6 promoter, this doubles as the first base in the guide which is why it should be excluded if the guide starts with a G.

The extra GT/CA in the second pair of oligos completes the gRNA scaffold.

1. Mix the following reagents in a PCR tube for each of the two inserts:

| Reagent | | Amount |
|---|---|---|
| MQ $H_2O$ | | 6.5 µL |
| NEB T4 DNA Ligase Reaction Buffer | (10x) | 1 µL |
| bottom oligo | (100 µM) | 1 µL |
| top oligo | (100 µM) | 1 µL |
| NEB T4 Polynucleotide Kinase | (10 U/µL) | 0.5 µL |
| **Total** | | **10 µL** |

T4 Polynucleotide Kinase phosphorylates the 5′ end of each oligo to allow more efficient ligation when cloning into a plasmid.

2. Place each of the two mixtures in a thermocycler with the following parameters:

| | | |
|---|---|---|
| **1** | 37 °C | 30 min |
| **2** | 95 °C | 5 min |
| **3** | Ramp to 25 °C @ 0.1 °C/s | ∞ |

37 °C for optimal T4 PNK activity, 95 °C to melt then ramp down to anneal.

3. Dilute the 2 sets of phospho-annealed oligos 1:125 with MQ $H_2O$ in a 1.5 mL tube.

| Reagent | Amount |
|---|---|
| MQ $H_2O$ | 124 µL |
| phospho-annealed oligo | 1 µL |
| **Total** | **125 µL** |

4. Mix the following reagents in a PCR tube:

| Reagent | | Amount |
|---|---|---|
| MQ $H_2O$ | | 11.5 µL |
| pDG* plasmid | (100 ng/µL) | 1 µL |
| phospho-annealed oligo 1 | (1:125) | 1 µL |
| phospho-annealed oligo 2 | (1:125) | 1 µL |
| NEBuffer 2.1 | (10x) | 2 µL |
| DTT | (10 mM) | 1 µL |
| ATP | (10 mM) | 1 µL |
| NEB *Bbs*I | (5 U/µL) | 1 µL |
| NEB T4 DNA Ligase | (400 U/µL) | 0.5 µL |
| **Total** | | **20 µL** |

- Dithiothreitol (DTT) is a reducing agent used to prevent the formation of disulfide bonds in the Ligase, allowing it to maintain its function.
- ATP is integral to the function of the reaction that the Ligase catalyses.

5. Place in thermocycler with the following parameters:

| | | |
|---|---|---|
| **1** | 37 °C | 5 min |
| **2** | 16 °C | 5 min |
| **3** | Go to step 1 | 5 times |
| **4** | 4 °C | ∞ |

- Cycle between optimal temperature for *Bbs*I which cuts the plasmid open and T4 DNA Ligase (16 °C) which ligates the phospho-annealed oligos into those cut sites and destroys the *Bbs*I sites. Alternatively, the original pDG* plasmid is re-assembled by the Ligase without insertion of the phospho-annealed oligos, in which case the plasmid is cut again in the next 37 °C step. 5 cycles is sufficient to get enough plasmid containing the custom phospho-annealed oligos.

- Transform Invitrogen Subcloning Efficiency DH5α Competent Cells with 5 µL of mixture, following the recommended protocol.
- Verify insertion of both guides are present in the plasmid by one of the following:
  - Digesting with any restriction enzymes that have new binding sites generated by the insertion of the guide.
  - Digesting with *Bbs*I and another restriction enzyme present in the backbone.
  - Sequencing both inserts with **seq gRNA U6 V1**: 5'-GGTTTCGCCACCTCTGACTTG-3' and **bgh PA F**: 5'-TGCATCGCATTGTCTGAGTAGG-3'.