**Design and Synthesis of GFP gRNAs: Optimizing the CRISPR-Cas9 System**

**4)** Transformation & Plating

* Transform plasmid into chemically competent cells, following Life Technologies One Shot Stbl3 Chemically Competent E. coli protocol.
* Plate two different volumes of transformed cells (50-100 μL and remaining volume) onto LB Carb plates
* Incubate plated bacteria overnight

**2)** Assemble oligos of this form, ensuring they have the same overhang

5’-CACCGGTCAGATCCGCTAGCGCTAC-------3’

3’-------CCAGTCTAGGCGATCGCGATGCAAA-5’

* Prepare the following PCR Reaction

|  |  |
| --- | --- |
| 1.5 μL | Oligo F |
| 1.5 μL | Oligo R |
| 25 μL | Kapa HiFi (2x)\* |
| 22 μL | ddH2O |
| TOTAL: 50 μL |  |

\*Keep Kapa HiFi on ice

* Anneal using the following PCR Program

|  |  |  |
| --- | --- | --- |
| 95 °C | 3 min | Denature |
| 98 °C | 20 sec | Denature |
| 65 °C | 15 sec | Anneal |
| 72 °C | 15 sec | Extension |
|  | 5 Cycles |  |
| 72 °C | 1 min | Extension |

* Purify & Store at -20°C

**3)** Combine vector and insert with Gibson Assembly

* Prepare the following PCR Reaction

|  |  |
| --- | --- |
| 100 ng | Vector |
| 100 ng | Insert |
| 10 μL | Gibson Assembly Master Mix (2x)\* |
| X μL | ddH2O |
| TOTAL: 20 μL |  |

\*Keep GAMM on ice

* Assemble by keeping sample at 50°C for 1 hour (PCR)
* For best results, do PCR purification directly after Gibson Assembly
* Store final plasmid at -20°C

**5)** Final Plasmid

* Use non-filtered pipette tip to pick up isolated colony from plate. Place in 5 mL LB Carb medium and place in shaking incubator overnight
  + For best results, do not allow bacteria to overgrow
* Use Qiagen Miniprep kit to extract and purify DNA plasmid
* Store at -20°C
* Verify purified DNA through sequencing

1. Use Restriction Enzyme Digestion to create stock solution of linearized vector (pLK0-1 gSP-puro)

* Prepare the following PCR Reaction

|  |  |
| --- | --- |
| 10 μg | gSP-puro |
| 5 μL | Cutsmart Buffer (10x) |
| 3 μL | Age-I |
| X μL | ddH20 |
| TOTAL: 50 μL |  |

* Linearize by keeping sample at 37°C for 6 hours (PCR)
* Purify using Qiagen PCR Purification Kit, following manufacturer’s instructions
* Store stock at -20°C

The CRISPR-Cas9 system serves as a powerful tool in genetic engineering. Specific guide-RNAs (gRNAs) are key players in the CRISPR-Cas9 system; efficient design of gRNAs optimize its performance. This protocol describes a method for synthesizing specific gRNAs which target the GFP cassette in order to disrupt the cassette and thus GFP expression. This protocol utilizes the plasmid pLKo-1 gSP puro.