Synthesize sgRNA by GeneArt Precision Synthesis Kit

1. Design and synthesize a pair of target forward (F) and reverse (R) primers based on the USER GUIDE.
2. Prepare a 0.3 μM target oligonucleotide mix working solution in nuclease-free water.
3. Set up the PCR assembly reaction:

 Phusion High-Fidelity PCR Master Mix (2X) 12.5 μL

 Tracr Fragment + T7 Primer Mix 1 μL

 0.3 μM Target F/R oligonucleotide mix 1 μL

 Nuclease-free water 10.5 μL

1. Perform assembly PCR using the cycling parameters below.

|  |  |  |  |
| --- | --- | --- | --- |
|  Cycle step  | Temperature | Time  | Cycles |
| Initial denaturation | 98°C | 10 seconds | 1X |
| Denaturation | 98°C | 5 seconds | 32X |
| Annealing | 55°C | 15 seconds |
| Final extension | 72°C | 1 minute | 1X |
| Hold  | 4°C | Hold\* | 1X |

**Perform in vitro transcription**

1. Set up the following in vitro transcription reaction, adding the reaction components in the order given.

 NTP mix (100 mM each of ATP, GTP, CTP, UTP) 8 μL

 gRNA DNA template (from PCR assembly, page 13) 6 μL

 5X TranscriptAid™ Reaction Buffer 4 μL

 TranscriptAid Enzyme Mix 2 μL

1. Incubate at 37°C for 2–3 hours.
2. Add 1 μL of DNase I into the reaction mix after the transcription reaction and incubate at 37°C for 15 minutes.

**Purify in vitro transcribed (IVT) gRNA**

1. Adjust the volume of the IVT reaction to 200 μL with nuclease-free water.
2. Add 100 μL of Binding Buffer. Mix thoroughly by pipetting.
3. Add 300 μL of ethanol (>96%) and mix by pipetting.
4. Transfer the mixture to the GeneJET RNA Purification Micro Column and centrifuge for 30–60 seconds at 14,000 × g. Discard the flow-through.
5. Add 700 μL Wash Buffer 1(diluted with 13 mL of >96% ethanol) and centrifuge for 30–60 seconds at 14,000 × g. Discard the flow-through.
6. Add 700 μL Wash Buffer 2 (diluted with 30 mL of >96% ethanol) centrifuge for 30–60 seconds at 14,000 × g. Discard the flow-through and repeat.
7. Centrifuge the empty purification column for an additional 60 seconds at 14,000 × g to completely remove any residual Wash Buffer and transfer the purification column to a clean 1.5-mL collection tube.
8. Add 10 μL of nuclease-free water to the center of the purification column filter, and centrifuge for 60 seconds at 14,000 × g to elute the gRNA.