Synthesis of sgRNA for crispor.org

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All handlings are carried out wearing gloves, with filter tips and RNase Free tubes.
If needed you can decontaminate surfaces (pipets, plastics for electrophoresis and bench) with RNAse zap (Ambion)) or 100mM NaOH/0,1% SDS mix (incubate 6min at Room Temp) and wash well with milliQ water and EtOH.

1. **Prepare DNA template**

- Using Plasmid Construct DR274 (http://www.addgene.org/42250/):
  - Digest with DraI (20-40U) 15µg of the 2,1kb plasmid DR274 cloned with your sgRNA in 50 to 100µL 3h-ON.
  - Purify your digestion using a kit like PCR clean up kit (Omega Biotek EZNA Cycle pure kit, Qiagen or Macherey Nagel kits).
  - Note: Wash well (2x minimum) to remove all salts from binding buffer. Let dry the silica membrane before elution (5 min at high speed).
  - Elute in 30µL max of « clean » water (incubate >10min at room temp before centrifugation).
  - Note: You should obtain at least 0.2 to 0.3µg/µL of DraI digested plasmid. If necessary you can precipitate your digestion. Make sure the DraI fragment (around 200pb) is well precipitated.

- Using oligonucleotide based template (adapted from Bassett et al, Cell Reports, 2013)

  You can also synthetize your sgRNA template by PCR using the following overlapping oligonucleotides

  - Specific Forward primer **T7crTARGET**
    5'-GAAATTAATACGACTCACTATA**GN$_{19-20}$**GTTTTAGAGCTAGAAATAGC

  and

  - common reverse primer **sgRNAT7common**
    5'-AAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCT TATTTTAACTTGCTATTTCTAGCTCTAAAAC

  Run a the PCR with the following conditions:

  PCR mix:

  - 77µL H2O
  - 20µL HF 5X Buffer
  - 1µL dNTPmix @ 25mM
  - 1µL Phusion (New England Biolabs)
  - 0,5µL Primer **T7crTARGET** @100µM
  - 0,5µL Primer **sgRNAT7common** @100µM

Program:

- 98°C 30s
- 98°C 10s
- 60°C 30s
- 72°C 15s

} 35X

- 72°C 10min
- RT ∞

Precipitate PCR product:

- From 85µL of PCR add 17µL of AcONa 3M pH 5.5 and 80µL Isoproanol
- Store at -80°C for 30 min-1h
- Centrifuge at max speed at 4°C for 30min
- Wash with 200µL of 95% EtOH
- Centrifuge at max speed for 5min
- Remove the maximum of EtOH and let pellet dry at RT.
- Resuspend in 30µL of MilliQ water RNAse Free (final concentration should be around 300ng/µL)

2. **Carry out the transcription reaction** using Hiscribe T7 High Yield kit (New England Biolabs ref : E2040S).

- Assemble at Room temperature the transcription reaction keeping NTP on ice in the following order :

◦ RNase free H2O qsp 20µL
◦ ATP 1,5µL
◦ UTP 1,5µL
◦ GTP 1,5µL
◦ CTP 1,5µL
◦ 10X reaction Buffer 1,5µL
◦ Enzyme Mix 1,5µL
◦ Purifed DNA (usually around 6-8µL of DraI digest @ 0.3µg/µL or 2µL of T7 PCR product @ 200-300ng/µL)

Note: Make sure 10X buffer does not have any precipitate (vortex until everything is dissolved).

- Mix well by pipetting, centrifuge and incubate in an OVEN at 37°C overnight (not in water bath to avoid condensation on the cap)

- Next morning add 70µL Rnase free H2O, 10µL of 10X DNase I buffer, 1µL of Murine RNase Inhibitor (NEB) and 2 µL of DNase I (NEB) digest 30 min at 37°C.

3. **Proceed to purification** using Microelute RNA clean UP kit (Omega Biotek, ref : 6247-01) (Macherey Nagel, ref: 740948.50, work fine as well)

- Add 350µL of QLV buffer (or Lysis buffer RA1 for MN kit) mix well and add 300µL EtOH 100%. Mix well by pipetting.
- Wait for 5 min at RT or on ice. If the transcription works well a white precipitate can be seen. If centrifugation is needed to remove droplets, make sure to make it fast

and slow not to pellet the precipitate.
- Transfer to the column (with the pellet if present!). Follow the supplier protocol with the following advices:
- Wash well (2X minimum) to remove the large quantities of NTP present in the reaction and DRY WELL (!) THE SILICA MEMBRANE before elution (5 min at high speed up to 20000g).
- Elute with 30µL of RNase free water at room temperature with 30 min to 1h of incubation at room temperature before centrifugation. This is critical for the recovery of the large quantity of RNA precipitated on the silica membrane.
- After elution keep on ice.
- Store at -80°C.

Note: Dilute 5x your RNA before mesuring OD260 with a nanodrop.
You should have a guide concentration at around 0.5 to 4µg/µL (40.OD260=1µg/µL = 30µM sgRNA).

4. **Check your RNA quality and concentration** by running an agarose gel
Native 3% agarose gel in 1X MAE
RNA denaturation: 2 min at 90°C with Formaldehyde/formamide loading dye
and cooling 1min on ice before centrifugation and loading with loading dye 1-2X = 70% deionized formamide RNase Free/ 20% Formaldehyde solution at 35-37%, 10% MAE 10X)

Note : For size standard you can load 2µg of low range ssRNA ladder (NEB - N0364S)
In all cases, Post stain with SyBR green or EtBr.