Preparation of ssDNA via RNA as intermediate

Introduction

dsDNA (Syntentic gene + hology arms) → PCR (1h) → SPRI beads → T7 transcription (5h, no primer)→ (**RNA + dsDNA**) + DNAse → SPRI beads (**RNA**) → Reverse transcription → (**DNA:RNA hybrid**) RNA hydrolysis (NaOH) → **ssDNA** purification by SPRI beads
The total ssDNA yield 50 ug > 15 µg

Materials

- Primers
  - Primers to Generate
  - Forward (T7 universal site) - for PCR
  - Reverse (unique) - for PCR, Reverse transcription
- Phusion HF DNA Polymerase (2 units/µl) + buffer
- SuperScript™ IV Reverse Transcriptase (Thermo)
- SUPRERase In Rnase inhibitor (Thermo)
- Deoxynucleotide Solution Set
- T7 Quick High Yield RNA Synthesis Kit (included T7 pol, NTP, buffer, DNAse I)
- Magnetic Separation Rack
- SPRI Beads (AmpureXP beads, Beckman Coulter)
- RNAse free plast, tips
- TE Buffer or Nuclease-Free Water, Nuclease-Free IDTE Buffer, Nuclease-Free Duplex Buffer
- 1 M NaOH..autoclave
- 0.5 M EDTA pH 8.0 in H20 (The disodium salt of **EDTA** will not go into solution until the pH of the solution is adjusted to ~8.0 by the addition of NaOH).. autoclave

Procedure

Commercial dsDNA fragment

1. Prepare dsDNA template containing the template sequence preceded by a T7 promoter (For CRISPR knock in gene + ideally 300 pb flanking homology sequences) using gBlock (KRD/IDT) or GeneArt (Thermo)

dsDNA donor amplification

2. Clone donor sequence to plasmid (highly recommended) or amplify it using PCR. We recommend to use cloning kit: pJET1.2 vector (CloneJET PCR Cloning Kit. #K1231; Thermofisher). It allows easy blunt end cloning. be sure that you have the option to linearize plasmid by vector specific restriction enzyme later (cut should be as close as possible to the 3′ end of donor sequence)

We recommend plasmid DNA as it can be prepared as a maxiprep, properly sequenced and used multiple times. Furthermore, we found hard to amplify neccessary amount of dsDNA prior transcription of highly GC rich sequences (usually present in homologous arms).

3. Amplify plasmid by Maxiprep. You need DNA around 1ug/ul concentration, at least 50 ug total (you can concentrate DNA by precipitation if necessary). Sequence donor sequence from both sides (final ssDNA can be sequenced only from one side)

4. CUT 50 ug DNA from both sides using right restriction enzymes (BglII for pJET vector and so on)... 50 ul total overnight (use at least 1 ul of enzyme)

   You can linearize plasmid by cuting only one place (just behind 3′ end of donor sequence) as long as you know the orientation of cloned fragment to pJet vector.

5. Check digested DNA on gel - ALL should be cleaved... if not, leave longer or add additional enzyme

6. Amplified dsDNA purify using SPRI beads (AMPure XP resin) at 1:1 DNA:resin volue ratio, wash 2x times with 70 % ethanol, elute in X ul RNase-free H20 (you wan to have 2.5 pmol per 10 ul for in vitro transcription).... See calculation (around 50-60 ul)

   Fill orange fields, use dsDNA: Mass to/from Moles Convertor to calculate how many ug = 2.5 pmol https://nebiocalculator.neb.com/#!/dsdnaamt

Table1

	A	B	C	D	E	F	G
1	Example				**2.5pmol = Xug Use https://nebiocalculator.neb.com/#!/dsdnaamt**		10 ul of templates to 25 ul of IVT (15 % loses from purif. Included)
2	plasmid size	T7-donor cut size	x ug/ 50 ug plasmid DNA	conc. ug/ ul	X ug = 2.5 pmol for IVT (25 ul reaction)	concentr. Needed (at least 10 ul)	ELUTION from beads (Volume)
3	5097.0	2400.0	23.54	0.47	3.70	0.37	54.09

You do not need to remove unwanted parts of the dsDNA from plasmid. They will be removed by DNAse

T7 transcription

"We strongly recommend wearing gloves and using nuclease-free tubes and reagents to avoid RNase contamination. Reactions are typically 20 µl but can be scaled up as needed. Reactions should be assembled in nuclease-free microfuge tubes or PCR strip tubes"

7. T7 transcription using T7 HiScribe reagents (New England Biolabs), transcription mix (25 ul):

Table2

	A	B	C	D
1		Volume (µl)	Final Conc.	
2	**Nuclease free water**	0		
3	**NTP Buffer mix**	12.5	**1x, 10 mM**	Alter. 2.5ul 10x buffer + 4x 2.5 ul each NTP
4	**DNA template**	10	**2.5 pmol**	
5	**T7 RNA polymerase mix**	2.5		
6	Total Volume (µl)	**25**		
7	The kit contains sufficient materials for 50 reactions with 20 ul volume			
8				
9				

8. Incubate transcription mix for 4 hours at 37˚ in a thermal cycler

   Reaction time depends on template amount, quality and RNA transcript length. For reactions with transcripts longer than 0.3 kb, 2 hour incubation should give you the maximum yield. For reaction times of 60 minutes or less, a water bath or heating block may be used; for reaction times longer than 60 minutes, we recommend using a dry air incubator or a thermocycler to prevent evaporation of the sample. For reactions with short RNA transcripts (< 0.3 kb), we recommend an incubation time of 4 hours or longer. It is safe to incubate the reaction for 16 hours (overnight). For example, we have achieved good yield with only 0.2 µg plasmid template encoding a 50-mer RNA by incubating the reaction overnight at 37°C

   04:00:00

   This step can be extremely efficient (yield can exceed 1 mg RNA). For high-yield reactions, magnetic purification can be challenging because RNA amount exceeds the binding capacity of the beads. In such cases, the reaction can be scaled down or more beads added.

9. For 25 ul reaction add 2 units (1 ul) of TURBO DNAseI, incubate 15 min 37C, then added 20 ul of RNASE-free water and incubate additionaly 5 min RT...

   DNase treatment to remove DNA template. Standard reactions are capable of generating large amounts of RNA, at concentrations up to 10 mg/ml. As a result, the reaction mixture is quite viscous. It is easier to perform DNase treatment after the reaction mixture is diluted. If you plan to use standard DNAseI (RNase-free), add 30 µl nuclease-free water to each 20 µl reaction, followed by 2 µl of DNase I (RNase-free), mix and incubate for 15 minutes at 37°C.

10. purify RNA using SPRI beads (AMPure XP resin) at 1:1 RNA:resin volue ratio, elute in 30 ul RNase-free H20.... In some cases it is neccessary to increase amout of resin (capacity of beads is 1ul per 1ug nucleic acid). After 30 ul elution, add additional 15 ul (second elution - run this second elution on gel)

11. Run agarose gel (use RNA loading dye and denature RNA by 5 min at 70 °C). Run only RNA from second elution, keep first elution for RT

12. Quantification of RNA product by UV Light Absorbance

RNA concentration can be determined by measuring the ultraviolet light absorbance at 260 nm, however, any unincorporated nucleotides and template DNA in the mixture will affect the reading. Free nucleotides from the transcription reaction must be removed before the RNA concentration can be quantified = step 9. A 1:200 dilution of a sample of the purified RNA should give an absorbance reading in the linear range of a spectrophotometer. RNA dilution may not be necessary if using a NanoDrop Spectrophotometer. A NanoDrop Spectrophotometer can directly read RNA concentrations from 10 ng/µl to 3000 ng/µl. For single-stranded RNA, 1 A260 is equivalent to an RNA concentration of 40 µg/ml. The RNA concentration can be calculated as follows:

A260 x dilution factor x 40 = __ µg/ml RNA
Nucleic Acids: OD260 Convertor

Reverse transcription using SuperScript™ IV Reverse Transcriptase

13. For RT, you desire 500 pmol of RNA (if you have less, do not add water). Calculate 500 pmol using ssRNA: Mass to Moles Convertor https://nebiocalculator.neb.com/#!/ssrnaamt to know how many ug you need. Example: 500 pmol = 240 ug size 1500 pb.

14. Mix:

Table5

	A	B	C
1		Volume (µl)	Final Concentration (mM)
2	Nuclease free water	0	
3	RNA template	19	500 pmol (240 ug size 1500 pb)
4	Reverse Unique primer_100 uM	10	1 nmol (6.5 ug size 21 nt)
5	dNTP 10 mM each	10	2.4 mM each or more (5 mM)
6			
7	Total Volume (µl)	39	

15. Incubate 5 min at 65 °C , transfer on ice for 5 min to allow primer annealing

16. Add as in table, Cap the tube, mix, and then briefly centrifuge the contents.:

Table7

	A	B	C
1		Volume (µl)	Final Concentration (mM)
2	Mixture from table 3	40	
3	5x Buffer	12	1x
4	100 mM DTT	3	
5	SuperScript IV Reverse transcriptase (200 U/µL)	3	
6	RNAse inhibitor SUperase	2	
7	Total Volume (µl)	60	

17. Incubate 45 (1.5h) min at 50 °C

00:45:00

18. Hydrolyze RNA by the addition 12 ul of 0.5 M EDTA (pH 8.0) + 12 ul NaOH (1 M NaOH) followed by incubation a 95 °C for 10 min.. Neutralize it by 24 ul 0.5M HCl (pH 7-8) or 12 ul of 1M HCl

    CRITICAL EDTA (Mg chelator) needs to be added first before NaOH or Mg ions can precipitate with DNA. With high pH (greater than pH 11) only RNA hydrolyzes,DNA will denature but the phosphodieser backbone remains intact ....

19. Final ssDNA product purify using SPRI beads at 1:1.2 resin volume ratio and elute in 15-17 uL H20...2nd elution in 10 ul (run this on gel)

20. Measure on nanodrop, sequence it and directly use or store it at –20°C.