Silencer siRNA cocktail kit (RNaseIII)

For the first time you use this kit:
Add 12ml of 100% ethanol (ASC) to the 2X wash solution and check the label. From now, it will be called 1X Wash solution and store it at RT.

(1) Bring an ice bucket.
(2) Get the T7 Enzyme Mix out of the freezer and place it quickly on the ice.

(3) Vortex 10X T7 Rxn buffer as well as the four NTP solutions.
(4) Thaw the 4 NTP solutions on the ice while keep the 10X T7 Rxn buffer at RT.
(5) Spin these microfuges prior to open them to: (a) avoid contamination and (b) prevent loss.

In vitro transcription

The *in vitro* Rxn should be done at RT.This is to avoid Spremidine (present in transcription buffer) to precipitation of DNA at low temperature.

The order of adding materials should be:
Water + DNA template, then add 10X Rxn buffer.

For 20µl transcription Rxn:
Water up to20µl
0.2-2µg DNA (with T7 promoter at the ends).
2µl of 10X T7 Rxn buffer
2µl of each NTP
2µl of T7 polymerase Mix

(1) Flick the tube or gently pipet up and down→ spin it down→ incubate for 2hrs at 37C.
(2) Reserve 0.5µl of this transcription Rxn for 1% agarose electrophoresis. Run 1/400 in non-denaturing agarose and dilute the sample in TE
(3) Incubate the sample at 75C for 5minutes and cool at RT. This maximizes the duplex RNA formation. In our case where a small DNA (i.e. <800bp) and both ends have T7 promoter sequence, no need for this step. However, it is still better to do it since there will be no drawback.

Purification of dsRNA (by nucleic acid digestion and filtration):

Nucleic acid digestion of DNase I and RNase A
For 20µl of in vitro transcription tube:
-20µl of dsRNA
-21µl Nuclease free water
-5µl 10X digestion buffer.
-2µl DNaseI
-2µl RNase A

50µl total

Incubate not more than 2hours. In the first 15minute incubation, ssRNA will be digested by RNase A while any DNA can be digested in 1hr.

Purification step:
To remove proteins (the enzymes), free nucleotides and oligonucleotides but retains dsRNA until it is eluted:
(1) Preheat the elution solution to 95C
(2) Assemble the following:
-50µl of dsRNA (obtained from digestion step)
-50µl 10X Binding buffer
-150µl Nuclease free water
250µl 100% ethanol

(3) Gently mix
(4) Pipet this 500µl onto a Transcription Rxn filter cartridge
(5) Centrifuge at max speed for 2min→ Discard the flow through and place the cartridge in a new collection tube.
(6) Add 500µl of wash solution→ Centrifuge→ Discard the flow through.
(7) Repeat step (6) → Centrifuge→ Discard the flow through
(8) Centrifuge again without any wash solution.

To recover dsRNA:
(1) Transfer the rxn filter cartridge to a new collection tube.
(2) Add 50µl of **hot elution buffer** (preheated to 95C). If you use a cold elution buffer, let it stand for 2 minutes.
(3) Centrifuge for 2minute at highest speed.
(4) Repeat steps 2 and 3 on the same collection tube.

To quantitate dsRNA:
Measure dsRNA at 260nm.

Storage:
dsRNA is stable at -20C when it is stored in the elution buffer.

- Run 1/400 of clean dsRNA on 2% Agarose (non-denaturing) to check up the integrity and efficiency of dsRNA formation.
- To have 1/400 of dsRNA from the original 100µl in elution buffer, do the following:

(1) take 1µl of 100µl dsRNA + 9µl (10mM Tris and 1mM EDTA), i.e. 1:10
(2) Take 2.5µl of this 1:10 + 7.5µl of TE→ 1:400 dilution.

dsRNA → siRNA (by RNase III digestion) and the purification of siRNA
One rxn can digest up to 15µg of dsRNA/rxn. This is enough to get siRNA for about 120 transfections in a 24well dish at 50nM final concentration.
-dsRNA up to 15µg (up to 30µl)
-15µl RNase III.
- 5µl of 10X RNase III buffer.

-to 50µl water (nuclease free).
(1) Incubate in water bath at 37C for 1hr
(2) Pre-wet siRNA purification unit with 50µl of nuclease free water, centrifuge for 8 minutes for 14,000 x g.