Generating the Double-Stranded Oligo (ds oligo)

Introduction

Once you have synthesized the appropriate complementary single-stranded DNA oligos (*i.e.* top and bottom strand oligos), you will anneal equal amounts of each single-stranded oligo to generate a double-stranded oligo (ds oligo) that is suitable for cloning into the pENTR™/U6 or pENTR™/H1/TO vector. Guidelines and instructions are provided in this section to generate the ds oligo. For instructions to clone the ds oligo into pENTR™/U6 or pENTR™/H1/TO, refer to the BLOCK-iT™ U6 RNAi Entry Vector Kit or the BLOCK-iT™ Inducible H1 RNAi Entry Vector Kit manual, respectively.

Amount of DNA Oligo to Anneal

You will anneal equal amounts of the top and bottom strand oligos to generate the ds oligos. We generally perform the annealing reaction at a final single-stranded oligo concentration of 50 µM. Annealing at concentrations lower than 50 µM can significantly reduce the efficiency. Note that the annealing step is not 100% efficient; approximately half of the single-stranded oligos remain unannealed even at a concentration of 50 µM.

Resuspending the Oligos

If your single-stranded oligos are supplied lyophilized, resuspend them in water or TE Buffer to a final concentration of 200 µM before use.

Materials Needed

Have the following materials on hand before beginning. Make sure that all solutions are RNase-free.

- Your "top strand" single-stranded oligo (200 µM in water or TE Buffer)
- Your "bottom strand" single-stranded oligo (200 µM in water or TE Buffer)
- 10X Oligo Annealing Buffer (100 mM Tris-HCl, pH 8.0, 10 mM EDTA, pH 8.0, 1 M NaCl)
- DNase/RNase-Free Water
- 0.5 ml sterile microcentrifuge tubes
- 95°C water bath or heat block

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Annealing Procedure

Follow this procedure to anneal your single-stranded oligos to generate the ds oligo. Note that the final concentration of the oligo mixture is 50 µM.

1. In a 0.5 ml sterile microcentrifuge tube, set up the following annealing reaction at room temperature.

Reagent	Amount
"Top strand" DNA oligo (200 µM)	5 µl
"Bottom strand" DNA oligo (200 µM)	5 µl
10X Oligo Annealing Buffer	2 µl
DNase/RNase-Free Water	8 µl
Total volume	20 µl

2. Incubate the reaction at 95°C for 4 minutes.
3. Remove the tube containing the annealing reaction from the water bath or the heat block and set on your laboratory bench.
4. Allow the reaction mixture to cool to room temperature for 5-10 minutes. The single-stranded oligos will anneal during this time.
5. Place the sample in a microcentrifuge and centrifuge briefly (~5 seconds). Mix gently.
6. Remove 1 µl of the annealing mixture and dilute the ds oligo as directed in **Diluting the ds Oligo**, below.
7. Store the remainder of the 50 µM ds oligo mixture at -20°C.

Diluting the ds Oligo

To clone your ds oligo into pENTR™/U6 or pENTR™/H1/TO, you **must** dilute the 50 µM stock to a final concentration of 5 nM (*i.e.* 10,000-fold dilution). Perform two 100-fold serial dilutions, the first into DNase/RNase-free water and the second into the 1X Oligo Annealing Buffer. Follow the procedure below to dilute the ds oligo.

1. Dilute the 50 µM ds oligo mixture (from **Annealing Procedure**, Step 5, above) 100-fold into DNase/RNase-free water (*i.e.* 1 µl of 50 µM ds oligo into 99 µl of DNase/RNase-free water) to obtain a final concentration of 500 nM. Vortex to mix thoroughly.
2. Dilute the 500 nM ds oligo mixture (from Step 1) 100-fold into 1X Oligo Annealing Buffer as follows to obtain a final concentration of 5 nM. Vortex to mix thoroughly. Store the remaining 500 nM ds oligo stock at -20°C.

500 nM ds oligo	1 µl
10X Oligo Annealing Buffer	10 µl
DNase/RNase-free water	89 µl
Total volume	100 µl

3. Aliquot the 5 nM ds oligo stock and store at -20°C.

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Important

The undiluted ds oligos are 10,000-fold more concentrated than the working concentration. **When performing the dilutions, be careful not to cross-contaminate the different ds oligo stocks.** Remember to wear gloves and change pipette tips after every manipulation.

Storing the ds Oligo

Once you have diluted your ds oligo, you should have three stocks of annealed ds oligo. Use each stock as follows:

- **50 µM ds oligo (undiluted):** Use this stock to prepare new diluted ds oligo stocks if existing stocks become denatured or cross-contaminated.
- **500 nM ds oligo (100-fold dilution):** Use this stock for gel analysis (see **Checking the Integrity of the ds Oligo**, below).
- **5 nM ds oligo (10,000-fold dilution):** Use this stock for cloning into pENTR™/U6 or pENTR™/H1/TO.

Store the three ds oligo stocks at -20°C .

Important

When using the diluted ds oligo stock solutions (*i.e.* 100-fold or 10,000-fold diluted stocks), thaw the solutions on ice. **Do not** heat or allow the ds oligo solutions to reach greater than room temperature as this will cause the ds oligos to melt. The concentration of the oligos in the diluted solutions is not sufficiently high to permit re-annealing and instead favors the formation of intramolecular hairpin structures. These intramolecular hairpin structures **will not clone** into pENTR™/U6 or pENTR™/H1/TO.

If your diluted ds oligo stock solution(s) is heated, discard the ds oligo solution and prepare new diluted stocks using the procedure on the previous page.

Note: If the 50 µM ds oligo solution (undiluted stock) becomes heated, the oligos are sufficiently concentrated and may be re-annealed following the annealing procedure on the previous page.

Checking the Integrity of the ds Oligo

Before proceeding to cloning, we recommend verifying the integrity of your annealed ds oligo using agarose gel electrophoresis. Run an aliquot of the annealed ds oligo (5 µl of the 500 nM stock) and compare it to an aliquot of each starting single-stranded oligo (dilute the 200 µM stock 4000-fold to 500 nM; use 5 µl for gel analysis). Be sure to include an appropriate molecular weight standard. We recommend using the following gel and molecular weight standard:

- **Agarose gel:** 4% E-Gel® (Invitrogen, Catalog no. G5000-04)
- **Molecular weight standard:** 10 bp DNA Ladder (Invitrogen, Catalog no. 10821-015)

You should see the following results when performing agarose gel analysis;

- A detectable higher molecular weight band representing annealed ds oligo.
- A detectable lower molecular weight band representing unannealed single-stranded oligos. Note that this band is detected since a significant amount of the single-stranded oligo remains unannealed.