clone smarter technologies

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Not intended for human diagnostic use

Seamless Assembly Cloning Kit

-for multi fragments assembly

Rev. A. May. 1 2013

Cat. No. C5891-25 Size: 25 reactions
C5891-50 50 reactions

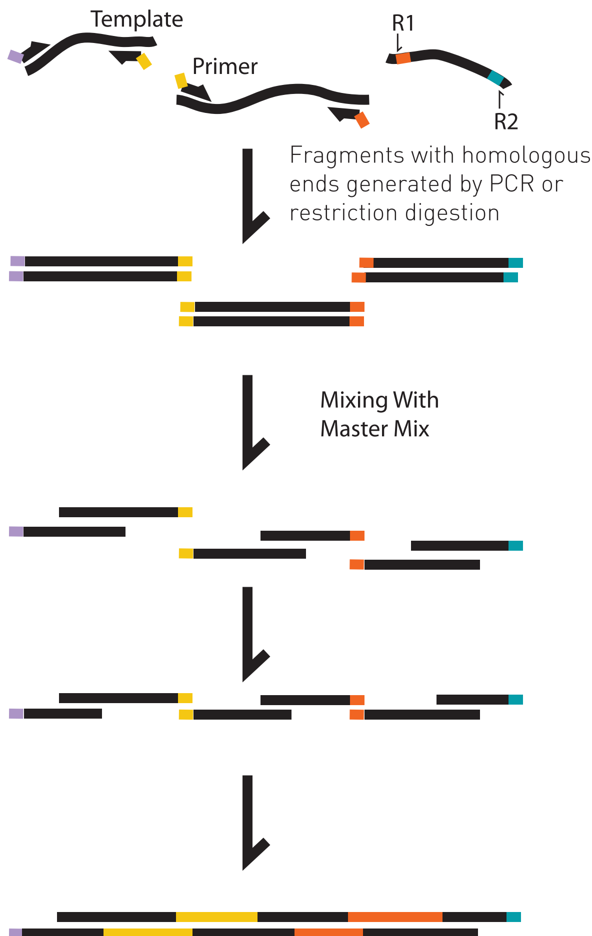
Store at -20°C

Note: Read and follow the instruction carefully to ensure optimal performance.

Description:

Originally developed for the synthetic biology community, Seamless Assembly Cloning Kit is the method of choice for joining multiple fragments up to hundreds of kilo bases without relying on specific restriction sites. Isothermal joining reaction is achieved by homologous recombination through concerted actions of multiple enzymes in a single tube. Double strand DNA fragments with overlapping homologous ends of various lengths (15 bp — 80 bp) can be effectively joined together. The product can be amplified in vivo by transforming E. coli or in vitro by PCR or RCA. This kit can also be used efficiently for site directed mutagenesis of multiple sites.

Fig.1 Seamless Assembly Cloning Mechanism



Components:

	Kit Size	
Components	25 Rxns	50 Rxns
2X Assembly Master Mix	125 µl	250 µl
Control Fragment I (20ng/µl)	10 µl	10 µl
Control Fragment II (30ng/µl)	10 µl	10 µl
Linearized Control Cloning Vector (30ng/ul)	10 µl	10 µl

Protocol:

Brief Summary (for first time user please refer to the detailed protocol below)

Mix: Fragments + Master Mix + H2O = 10 ul

Standard Protocol (Total time needed: 55 – 115 minutes)

1. Incubate 15 min at 50 °C
2. Transfer 5 µl to competent cell
3. 30 min on ice
4. Heat shock (42°C, 30 seconds)
5. 2 min on ice
6. For Kan^R only: +SOC/LB, incubate @ 37°C, 60 min. For Amp^R go to step 7 directly
7. Plate
8. Incubate @ 37°C O.N.

Fast Protocol (Total time needed: 20 – 80 minutes)

1. Incubate 15 min at 50 °C
2. Transfer 5 µl to competent cell
3. 2 min on ice
4. Heat shock (42°C, 30 seconds)
5. 2 min on ice
6. For Kan^R only: +SOC/LB, incubate @ 37°C, 60 min. For Amp^R go to step 7 directly
7. Plate
8. Incubate @ 37°C O.N.

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Detailed Protocol

⚠ First time users should carefully read through this protocol before starting the experiment.

1. Generate DNA fragments with homologous ends.
Fragments with homologous ends are generated by restriction digestions if such sites are available, or by PCR amplification with compatible primers.

General considerations for primer design.
Primers should be at least 15-20 bp for overlapping and 15-20 bp for target annealing (refer to Fig. 1). The length of overlap correlates with assembly efficiency, with longer overlapping being more effective (Table 1).

Table 1. Overlap length vs. cloning efficiency:

Overlapping Length (bp)	Total transformants (single 3kb fragment into 2.8 kb vector)
15	76
20	192
30	441

Although regions of overlapping can be arbitrary, cautions should be taken when potential hairpin structures happens to be at the end of the fragments: single stranded DNA produced during reaction may fold back and form stable double helix with itself and prohibit homologous joining with adjacent fragment. However GC content of the overlapping region is generally not a consideration.

The length of overlapping should be adjusted depending on the number of fragments need to be joined. With more fragments need to be joined longer overlapping is required. An overlap of 75 bp was used to successfully join 8 fragments (seven 1kb insert into a 3kb cloning vector) into a 10 kb plasmid.

2. Purification of DNA fragments
It is strongly recommended that fragments to be joined first go through gel purification. Gel purification can eliminate a larger number of backgrounds. Direct PCR purification without agarose gel separation will also give reasonable result as long as the desired fragment is the major product. ⚠ Non purified fragments are not recommended for assembly.

3. Measure or estimate the concentration of the DNA fragments.
DNA fragments are preferably measured by a micro volume spectrophotometer such as NanoDrop™ or any equivalent. Fragments can also be roughly estimated on gel using known standards. High quality fragment is key for success.

4. Set up the Seamless Assembly Cloning reaction by mixing the reagents in the order shown. In general, keep 1:1 ratio between inserts and 1:3 ratio between vector and inserts. Use 20-60 ng of linearized vector for optimal result.

Reagent	**Volume**
DNA Fragments + Linearized Vector	0.5–5 μL
2X Seamless Master Mix	5 μL
diH2O	X μL
Final Volume	**10 μL**

DNA fragments Does NOT need be 5′ phosphorylated. Blunt ended DNA fragments, fragments with sticky ends from restriction digestion as well as fragments with 3′- adenosine overhang generated by Taq polymerase can all be effectively assembled and cloned. This single kit can be used for cloning of PCR fragments generated by KOD, Pfu, Phusion as well as Taq DNA polymerase.

A control reaction using 1 μL of each of the fragments (600 and 900 bp insert together with 2.8 kb linerized vector, with 30 bp overlap between each other) included in the kit can be set up. The control reaction should produce better than 99% white colonies when transformed into DH5 or DH10B and plated on X-gal and IPTG containing LB + ampicillin plates.

5. Mix the reaction gently and incubate for 15 minutes at 50°C.
Extended incubation does little benefit if there is any.

Table 2. Effects of incubation time vs. cloning efficiency:

Incubation Time	Total Transformants		
	15bp overlap	20bp overlap	30bp overlap
15 minutes	41	141	281
30 minutes	28	107	200

6. Add 5 μL of the cloning reaction into 50 μL chemically competent E. coli and mix gently. Do not mix by pipetting up and down.

If not used for transformation immediately, reaction mixture can be stored at -20°C until use. Prolonged storage of the reaction mixture is NOT recommended.

7. Incubate on ice for 2–30 minutes.

Recent R&D data indicates extended incubation at this step produce no detectable benefits as evident in the following data. In any case, do not incubate for more than 30 minutes. The length of incubation is at the user's discretion.

Table 3. Effects of incubation duration on ice on the total number of resulting transformants

Overlap Length	Total Number of Transformants	
	30 min. Incubation	2 min. Incubation
15 bp	41	76
20 bp	141	192
30 bp	281	440

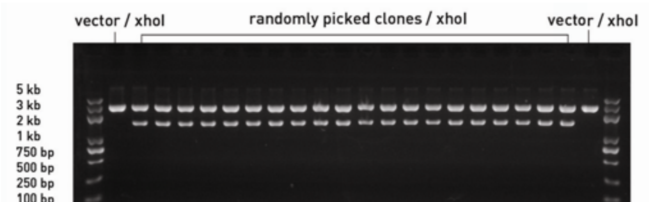
8. Heat-shock the cells for 30 seconds at 42°C without shaking.

9. Immediately transfer the tubes to ice and incubate for 2 minutes.

10. Add 200 ul SOC medium and shake at 37ºC for 60 minutes to allow the cell to recover before plating on plates with appropriate antibiotics. If ampicillin is used for selection immediately spread without recovery in SOC medium is OK if minimum colonies are needed.

11. An efficient assembly cloning reaction will produce hundred colonies. Pick ~10 colonies for analysis.

12. A control reaction set up according to step 4 with fragments supplied will generate a plasmid which, when digested with xhol, will release a 1.5 kb insert together with a 2.8 kb backbone. The result of a typical control reaction is shown below. Clones were picked randomly.



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