gBlocks® Gene Fragments Protocol: Megaprimer RF Cloning

Megaprimer restriction-free (RF) cloning uses PCR methods to assemble a double-stranded insert such as a PCR product or gBlocks Gene Fragments into any position, in any plasmid vector, without the need for restriction and ligation (Figure 1). A high-fidelity DNA polymerase such as the Phusion® DNA Polymerase (www.NEB.com/Phusion) is used in this protocol to limit the introduction of sequence errors [1].

gBlocks Gene Fragments

gBlocks Gene Fragments are chemically synthesized, double-stranded DNA, delivered normalized to 250, 500, or 1000 ng, depending on length, and dried down. Order at www.idtdna.com/gblocks.

Resuspending your gBlocks Gene Fragments

The dried down gBlocks Gene Fragment pellet can become displaced from the bottom of the tube during shipping.

- Centrifuge the tube for 3–5 sec at a minimum of 3000 x g to pellet the material to the bottom of the tube.
- Add TE to the tube for your desired final concentration
- Briefly vortex and centrifuge

Final concentration	Resuspension volume of TE buffer (µL) for gBlocks Fragments synthesis scales		
	250 ng	500 ng	1000 ng
10 ng/µL	25	50	100
20 ng/µL	Not recommended	25	50
50 ng/µL	Not recommended	10	20

Storing your gBlocks Gene Fragments

gBlocks Gene Fragments can be stored in TE at –20°C for up to 24 months. If gBlocks Gene Fragments will be stored for less than 1 month, they can be resuspended in nuclease-free water instead of TE.

Required materials

- gBlocks Gene Fragments with 30–80 bp 3' and 5', insertion site overlaps (Figure 1)
- Amplification primers
- Phusion® DNA Polymerase (www.NEB.com/Phusion)
- DpnI enzyme and buffer
- Competent cells and transformation reagents

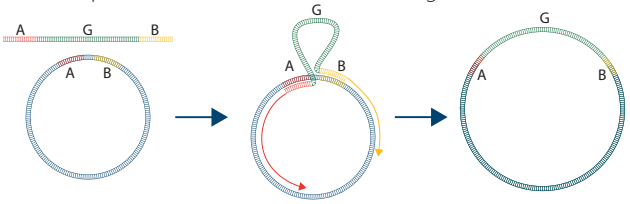

Figure 1. Overview of Megaprimer RF Cloning. A double-stranded, DNA gBlocks® Gene fragment or PCR product is designed with 30–80 bp overlap at the 3' and 5' ends and the vector insertion site (A and B). Using the RF protocol, the fragment is incorporated using PCR methods and a high-fidelity polymerase, as described. The finished plasmid contains the cloned gene (G) with a nick on each strand that is sealed by endogenous *E. coli* mechanisms.

Megaprimer RF cloning procedure

1. Set up the amplification reaction on ice—reaction components for a 50 µL reaction are shown.

	50 µL RF Reaction
Nuclease-free H_2O	Adjust to final 50 µL
5X Phusion HF or GC buffer	10 µL
10 mM dNTPs	1 µL
Cloning plasmid	20 ng
gBlocks® Gene Fragments	100 ng
Phusion® DNA Polymerase	0.8 µL

2. Gently mix the reaction and spin down in a microcentrifuge.
3. Carry out the RF cloning reaction in a thermal cycler with a heated lid.

Cycling conditions

The table shows general guidelines for cloning a gBlocks Gene Fragment into a cloning vector, using the megaprimer RF cloninng procedure.

	Cycling Parameters		
Step	Cycles	Temperature	Time
Initial denaturation	1	95°C	30 sec
Denaturation Annealing Extension	25	95°C 60°C 72°C	30 sec 1 min 5 min
Final extension	1	72°C	7 min
Hold	1	4°C	∞

DpnI Digestion of methylated, empty vector

Remove empty, target-vector template with DpnI digest.

	50 µL DpnI Rxn
Completed RF cloning reaction	10 µL Aliquot
DpnI (20U/ µL)	1 µL
DpnI buffer (10X)	5µL
BSA	0.5 µL
ddH_2O	33.5 µL

1. Incubate the reaction for 1–2 hr at 37°C.
2. Transform directly into *E. coli*.

Reference

1. Unger T, Jacobovitch Y, *et. al*. (2010) Applications of the Restriction Free (RF) cloning procedure for molecular manipulations and protein expression. J Struct Biol. 172(1). 34–44.