GGAssmbler Library Construction V.3

Version 1 is forked from PCR with Q5® Polymerase (M0491)

Shlomo Yakir Hoch[1], Ravit Netzer[1,2], Lucas Krauss[1,2], Sarel J Fleishman[1,2], Karen Hakeny[1]

[1]Weizmann Institute of Science; [2]Scala Biodesign LTD

Fleishman Lab

Shlomo Yakir Hoch

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ABSTRACT

This protocol describes methods for GGAssembler.

GUIDELINES

Please note that protocols with Q5® High-Fidelity DNA Polymerase may differ from protocols with other polymerases. Conditions recommended below should be used for optimal performance.

Reaction Setup:
We recommend assembling all reaction components on ice and quickly transferring the reactions to a thermocycler preheated to the denaturation temperature (98°C). All components should be mixed prior to use. Q5 High-Fidelity DNA Polymerase may be diluted in 1X Q5 Reaction Buffer just prior to use in order to reduce pipetting errors.

General Guidelines:

1. Template:
Use of high quality, purified DNA templates greatly enhances the success of PCR. Recommended amounts of DNA template for a 50 µl reaction are as follows:

	A	B
	DNA Genomic	1 ng–1 µg
	Plasmid or Viral	1 pg–1 ng

2. Primers:
Oligonucleotide primers are generally 20–40 nucleotides in length and ideally have a

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We use this protocol and it's working

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GC content of 40–60%. Computer programs such as Primer3 can be used to design or analyze primers. The best results are typically seen when using each primer at a final concentration of 0.5 µM in the reaction.

3. Mg^{++} and additives:
Mg^{++} concentration of 2.0 mM is optimal for most PCR products generated with Q5 High-Fidelity DNA Polymerase. When used at a final concentration of 1X, the Q5 Reaction Buffer provides the optimal Mg^{++} concentration.

Amplification of some difficult targets, like GC-rich sequences, may be improved by the addition of 1X Q5 High GC Enhancer. The Q5 High GC Enhancer is not a buffer and should not be used alone. It should be added only to reactions with the Q5 Reaction Buffer when other conditions have failed.

4. Deoxynucleotides:
The final concentration of dNTPs is typically 200 µM of each deoxynucleotide. Q5 High-Fidelity DNA Polymerase cannot incorporate dUTP and is not recommended for use with uracil-containing primers or templates.

5. Q5 High-Fidelity DNA Polymerase concentration:
We generally recommend using Q5 High-Fidelity DNA Polymerase at a final concentration of 20 units/ml (1.0 unit/50 µl reaction). However, the optimal concentration of Q5 High-Fidelity DNA Polymerase may vary from 10–40 units/ml (0.5–2 units/50 µl reaction) depending on amplicon length and difficulty. Do not exceed 2 units/50 µl reaction, especially for amplicons longer than 5 kb.

6. Buffers:
The 5X Q5 Reaction Buffer provided with the enzyme is recommended as the first-choice buffer for robust, high-fidelity amplification. For difficult amplicons, such as GC-rich templates or those with secondary structure, the addition of the Q5 High GC Enhancer can improve reaction performance. The 5X Q5 Reaction Buffer is detergent-free and contains 2.0 mM Mg^{++} at the final (1X) concentration.

7. Denaturation:
An initial denaturation of 30 seconds at 98°C is sufficient for most amplicons from pure DNA templates. Longer denaturation times can be used (up to 3 minutes) for templates that require it.

During thermocycling, the denaturation step should be kept to a minimum. Typically, a 5–10 second denaturation at 98°C is recommended for most templates.

8. Annealing:
Optimal annealing temperatures for Q5 High-Fidelity DNA Polymerase tend to be

higher than for other PCR polymerases. The NEB T_m Calculator should be used to determine the annealing temperature when using this enzyme. Typically, use a 10–30 second annealing step at 3°C above the T_m of the lower T_m primer. A temperature gradient can also be used to optimize the annealing temperature for each primer pair.

For high T_m primer pairs, two-step cycling without a separate annealing step can be used (see note 11).

9. Extension:
The recommended extension temperature is 72°C. Extension times are generally 20–30 seconds per kb for complex, genomic samples, but can be reduced to 10 seconds per kb for simple templates (plasmid, E. coli, etc.) or complex templates < 1 kb. Extension time can be increased to 40 seconds per kb for cDNA or long, complex templates, if necessary.

A final extension of 2 minutes at 72°C is recommended.

10. Cycle number:
Generally, 25–35 cycles yield sufficient product. For genomic amplicons, 30-35 cycles are recommended.

11. 2-step PCR:
When primers with annealing temperatures ≥ 72°C are used, a 2-step thermocycling protocol (combining annealing and extension into one step) is possible.

12. Amplification of long products:
When amplifying products > 6 kb, it is often helpful to increase the extension time to 40–50 seconds/kb.

13. PCR product:
The PCR products generated using Q5 High-Fidelity DNA Polymerase have blunt ends. If cloning is the next step, then blunt-end cloning is recommended. If T/A-cloning is preferred, the DNA should be purified prior to A-addition, as Q5 High-Fidelity DNA Polymerase will degrade any overhangs generated.

Addition of an untemplated -dA can be done with *Taq* DNA Polymerase (NEB #M0267) or Klenow exo$^-$ (NEB #M0212).

MATERIALS

Materials

Q5 High-Fidelity DNA Polymerase - 100 units **New England Biolabs Catalog #M0491S**

DpnI - 5,000 units **New England Biolabs Catalog #R0176L**

T4 DNA Ligase Reaction Buffer - 6.0 ml **New England Biolabs Catalog #B0202S**

T4 DNA Ligase - 20,000 units **New England Biolabs Catalog #M0202S**

BsaI-HFv2 **New England Biolabs Catalog # R3733S**

Nuclease-free Water - 25 ml **New England Biolabs Catalog #B1500S**

Monarch® PCR & DNA Cleanup Kit (5 µg) **New England Biolabs Catalog #T1030**

Equipment

Equipment

NAME	BRAND	SKU	SPECIFICATIONS
Qubit 2.0 Fluorometer instrument		Q33226	with Qubit RNA HS Assays

Equipment

4200 TapeStation System NAME

Electrophoresis tool for DNA and RNA sample quality control. TYPE

TapeStation Instruments BRAND

G2991AA SKU

https://www.agilent.com/en/product/automated-electrophoresis/tapestation-systems/tapestation-instruments/4200-tapestation-system-228263 LINK

SAFETY WARNINGS

Please refer to the Safety Data Sheets (SDS) for health and environmental hazards.

BEFORE START INSTRUCTIONS

Please note that protocols with Q5® High-Fidelity DNA Polymerase may differ from protocols with other polymerases. Conditions recommended below should be used for optimal performance.

Amplify constant fragments

1 Set up the following reaction On ice:

Note

All components should be mixed prior to use. Q5 High-Fidelity DNA Polymerase may be diluted in 1X Q5 Reaction Buffer just prior to use in order to reduce pipetting errors.

	A	B	C
	COMPONENT	25 µl REACTION	FINAL CONCENTRATION
	5X Q5 Reaction Buffer	5 µl	1X

	A	B	C
	10 mM dNTPs	0.5 µl	200 µM
	10 µM Forward Primer	1.25 µl	0.5 µM
	10 µM Reverse Primer	1.25 µl	0.5 µM
	Template DNA	variable	~ 5 ng
	Q5 High-Fidelity DNA Polymerase	0.25 µl	0.02 U/µl
	5X Q5 High GC Enhancer (optional)	(5 µl)	(1X)
	Nuclease-Free Water	to 25 µl	

2 Gently mix the reaction.

3 Collect all liquid to the bottom of the tube by a quick spin if necessary.

4 Quickly transfer PCR tubes to a PCR machine preheated to the denaturation temperature (98 °C) and begin thermocycling.

Thermocycling Conditions for a Routine PCR:

	A	B	C
	STEP	TEMP	TIME
	Initial Denaturation	98°C	30 seconds
	25–35 Cycles	98°C	5–10 seconds
		*50–72°C	10–30 seconds
		72°C	20–30 seconds/kb
	Final Extension	72°C	2 minutes
	Hold	4–10°C	

*Use of the NEB Tm Calculator is highly recommended.

5 Digest the lead gene templates with DpnI at 37°C for 2 hours. Heat-inactivate at 80°C for 20 minutes.

6 Perform rapid PCR cleanup (see: https://dx.doi.org/10.17504/protocols.io.bg9xjz7n)

7 Perform PCR DNA cleanup (see: https://dx.doi.org/10.17504/protocols.io.bg9rjz56)

8 Quantify DNA concentration by Qubit Fluorometer (Qubit 2.0 Fluorometer, ThermoFisher Scientific).

9 Measure DNA length using TapeStation (Agilent 2200 TapeStation).

Variable fragments fill in

10 Follow the steps for Amplify constant fragments (steps 1-9), skipping step 5.

11 Combine variable fragments of the same segment in equal concentration.

Golden Gate Assembly

12 Set up 25 µl assembly reactions as follows:

	A	B
	REAGENTS	ASSEMBLY REACTION
	DNA inserts 100 ng/ul each	1 µl (~ 100 ng) each, (up to 39 µl)
	T4 DNA Ligase Buffer (NEB #B0202) (10X)	5 µl
	T4 DNA Ligase (NEB #M0202), 2000 U/µl	1 µl (2000 units)
	BsaI-HFv2 (NEB #R3733), 20 U/µl	3 µl (60 units)
	Nuclease-free H2O (NEB #B1500)	to 50 µl

13 Mix gently by pipetting up and down 4 times.

14 Briefly microcentrifuge (1 second) to bring material to the bottom of tube.

15 Transfer to thermocycler and program as follows: (5 min 37ºC → 10 min 16ºC) x 60 cycles followed by 5 minutes 60ºC. If reactions are done overnight, add a 4ºC terminal hold to the protocol, but repeat the final 5 minutes 60ºC step the next day before the transformations.

Golden Gate Assembly Cleanup

16 Perform PCR DNA Cleanup (https://dx.doi.org/10.17504/protocols.io.bg9sjz6e) or follow a size selection protocol.

Amplify Golden Gate Assembly Prodcuts

17 For 1x reaction set up the following reaction On ice:

Note

Using GG assembly products in concentration of 3-5ng/µl is highly recommended.

	A	B	C
	Component	25 µl Reaction	Final Concentration
	Q5 High-Fidelity 2X Master Mix	12.5 µl	1X
	10 µM Forward Primer	1.25 µl	0.5 µM
	10 µM Reverse Primer	1.25 µl	0.5 µM
	GG assembly products	5 µl	
	Nuclease-Free Water	to 25 µl	

18 Gently mix the reaction.

19 Collect all liquid to the bottom of the tube by a quick spin if necessary.

20 Quickly transfer PCR tubes to a PCR machine preheated to the denaturation temperature (98 °C) and begin thermocycling.

Thermocycling Conditions for a Routine PCR:

	A	B	C

	A	B	C
	STEP	TEMP	TIME
	Initial Denaturation	98°C	30 seconds
	25–35 Cycles	98°C	5–10 seconds
		*50–72°C	10–30 seconds
		72°C	10 seconds/kb
	Final Extension	72°C	2 minutes
	Hold	4–10°C	

*Use of the NEB Tm Calculator is highly recommended.

Golden Gate Assembly Amplification Cleanup

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Note

This step is optional but HIGHLY recommended.

Perform Oligonucleotide Cleanup (https://dx.doi.org/10.17504/protocols.io.bg9sjz6e) or follow a size selection protocol.

Golden Gate Assembly Amplification Quantification

22 Quantify DNA concentration by Qubit.

23 Measure DNA length using TapeStation.