Amplification of G-Blocks by PCR

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PfuTurbo Hotstart PCR Master Mix (Agilent Technologies 600600) 2.5 ml of 2x Master Mix (250 U)

Storage conditions: stored at -20°C. The 2.5 mL is aliquoted in Johan fridge in 100 µL. The aliquots are to be stored at -4°C after thawing. Once thawed, full activity is guaranteed for 6 months.

Unit activity: one unit of activity is the amount of polymerase required to incorporate 10 nmoles of [3H]TTP into an acid insoluble form in 30 minutes at 72°C.

Note:

- Always work on ice.
- Wear gloves.
- Use nuclease-free water and sterile tubes.
- Completely thaw and thoroughly mix all reagents before use.
- Avoid repeated freeze-thaw cycles of reagents.
- **Important:** The first thing you do should be reading through the protocol and thawing all reactants on ice. This will take some time so the earlier the better.

PCR PROTOCOL

1. Add the PCR reaction components in order while mixing gently.

G-Blocks from IDT are resuspended at 20 ng/µL (unless otherwise specified on the tube). The recipe listed is for one reaction and must be adjusted for multiple samples or different insert concentrations.

Table 1.

Component	Amount per reaction (µL)	Final concentration
Pfu Hotstart Master Mix (2x)	25	1x
Primer Forward (10 µM)	x	0.5 µM
Primer Reverse (10 µM)	x	0.5 µM
DNA Insert (20 ng/µL)	x	10 ng
MilliQ H2O	x	Fill up to 50 µL
Total volume	50	-

*primer forward is the same for all constructs (His_tag_Fw_for_ampl), g-block depends on the insert (AKR1D1_rev_for_ampl, ci2350_rev_for_ampl).

2. Aliquot 50 µL of the reaction mixture into the appropriate number of sterile thin-wall PCR tubes or standard 0.5-mL microcentrifuge tubes.

3. Perform PCR using optimized cycling conditions. Suggested cycling parameters for PfuTurbo hotstart PCR master mix are indicated in Table 2.

Primers	***Annealing temperature***
His_tag_Fw_for_ampl	62°C
AKR1D1_rev_for_ampl	62°C
ci2350_rev_for_ampl	62°C

Table 2.

Segment	Number of cycles	Temperature	Duration
Initial Denaturation	1	95°C	2 minutes
Denaturation	30	95°C	30 seconds
Anealing		Primer Tm – 5°C = 62 - 5 = 57 °C	30 seconds
Elongation		72°C	2-3 minutes*
Final elongation	1	72°C	10 minutes
Holding	-	4°C	Infinite

* - Recommended time is 1 minute per kb of insert

4. Analyze the PCR amplification products on a 1.0% (w/v) agarose gel

Troubleshooting (Agilent Technologies)

Observation	Solution(s)
No product or low yield	Increase extension time to 2 minutes per kb of PCR target.
	Use cosolvents such as DMSO in a 1–10% (v/v) final concentration for GC-rich templates.
	Lower the annealing temperature in 5°C increments.
	Use higher denaturing temperatures (94–98°C) if the template DNA contains a high GC content or secondary structures.
	Denaturation times of 30–60 seconds at 94–95°C are usually sufficient while longer denaturation times may damage the DNA template; use the shortest denaturation time compatible with successful PCR on the thermal cycler.
	Remove extraneous salts from the PCR primers and DNA preparations.
	Excessive template DNA can be inhibitory. Follow the recommendations given for template amount.
	Use the recommended primer concentrations between 0.3 and 0.5 µM (corresponding to 100–200 ng for typical 18- to 25-mer oligonucleotide primers in a 50-µl reaction volume).
	Check the melting temperature, purity, GC content, and length of the primers.
Multiple bands	Increase the annealing temperature in 5°C increments.
Artifactual smears	Reduce the extension time.

REFERENCE

1. Innis, M. A., Gelfand, D. H., Sninsky, J. J. and White, T. J. (1990). PCR Protocols: A Guide to Methods and Applications.
Academic Press, New York.