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qPCR standard for library quantification V.2

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Abstract

Protocol for the preparation of a standard for DNA library quantification by quantitative real-time PCR (Meyer et al. 2008; Gansauge et al. 2020).

References

Meyer, M., Briggs, A. W., Maricic, T., Höber, B., Höffner, B., Krause, J., Weihmann, A., Pääbo, S., & Hofreiter, M. (2008). From micrograms to picograms: Quantitative PCR reduces the material demands of high-throughput sequencing. Nucleic Acids Research, 36(1): e5.

Gansauge, M.-T., Aximu-Petri, A., Nagel, S. *et al.* Manual and automated preparation of single-stranded DNA libraries for the
sequencing of DNA from ancient biological remains and other sources of highly degraded DNA. Nature Protocols,15, 2279-2300 (2020).

Notes

The qPCR standard comprises of a 60 bp fragment of pUC19 sequence flanked by double-indexed Illumina adapters. Indices are qPCR specific and not used for the preparation of sample libraries. The standard is obtained through two successive PCRs. It is then purified, quantified and diluted to concentrations between 10^2 and 10^8 molecules per micro liter.

Materials

	Reagent/consumable	Supplier	Catalogue number
	Reagents		
	Herculase II Fusion DNA Polymerase, including 5x Herculase buffer and dNTP mix (25 mM each dNTP)	Agilent Technologies	600675
	MinElute PCR Purification Kit	Qiagen	28006
	pUC19 plasmid DNA	NEB	N3041S
	Primer CL105_makeSTD103.F *	IDT	-
	Primer CL106_makeSTD103.R †	IDT	-
	Primer P5_iPCR_qPCR_xxQ188 ‡	IDT	-
	Primer P7_iPCR_qPCR_xxQ §	IDT	-
	Agilent DNA 1000 Kit	Agilent Technologies	5067-1504
	TET buffer ||	self-made	
	TE buffer ¶	self-made	
	Water, HPLC-grade		
	Consumables		
	0.2-ml PCR eight-tube strips	Eppendorf®	EP0030124359
	1.5-ml Eppendorf LoBind Tubes	Eppendorf®	0030108051

* Order oligonucleotide CL105_makeSTD103.F at 1µmol synthesis scale (Integrated DNA Technologies, desalted). Dissolve in TE buffer at a concentration of 100 µM. Prepare a 10 µM working dilution in water. Sequence: ACACTCTTTCCCTACACGACGCTCTTCCTCGTCGTTTGGTATGGCTTC

† Order oligonucleotide CL106_makeSTD103.R at 1µmol synthesis scale (Integrated DNA Technologies, desalted). Dissolve in TE buffer at a concentration of 100 µM. Prepare a 10 µM working dilution in water. Sequence: GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTTCATGTAACTCGCCTTGATCGT

‡ Order oligonucleotide P5_iPCR_qPCR_xxQ188 at 1µmol synthesis scale (Integrated DNA Technologies, HPLC purified). Dissolve in TE buffer at a concentration of 100 µM. Prepare a 10 µM working dilution in water. Sequence:

AATGATACGGCGACCACCGAGATCTACACCAATTCAACACTCTTTCCCTACACGACGCTCTT
§ Order oligonucleotide P7_iPCR_qPCR_xxQ at 1µmol synthesis scale (Integrated DNA Technologies, HPLC purified). Prepare a 10 µM working dilution in water. Dissolve in TE buffer at a concentration of 100 µM. Sequence: AGCAGAAGACGGCATACGAGATACCACAGTGACTGGAGTTCAGACGTGT
|| See document in the Appendix for the preparation of TET buffer.
¶ See document in the Appendix for the preparation of TE buffer.

Equipment

- Thermal cycler for PCR strip tubes (e.g., Bio-Rad C1000 Touch Thermal Cycler, cat. no. 1840197)
- NanoDrop spectrophotometer (Model ND-1000 V3.8.1)
- Agilent 2100 Bioanalyser 2100 expert DNA 1000 Series II (cat. no. G2938C)
- Label printer (e.g. Brady M611, cat. no. M611-EU-LABS) and tube labels (e.g. labels for TLS2200/TLS PC Link/Polyester, cat. no. PTL-82-499)

Protocol

1. In a 0.2-ml PCR eight-tube strip, prepare the following reaction mix using 1ng of pUC19 plasmid DNA as template and primers CL105 and CL106. Mix properly.

	Reagents	Volume	Final concentration in reaction
	5x Herculase buffer	20µl	1x
	25 mM each dNTP	1µl	250µM
	10 µM CL105	4µl	400nM
	10 µM CL106	4µl	400nM
	400 U/µl Herculase II Fusion Polymerase	1µl	4U/µl
	Water	69µl	
	1 ng/µl pUC19 plasmid DNA	1µl	0.01 ng/µl
	sum	100µl	

2. Place the strip tube into a thermal cycler and perform cycling with the following temperature profile.

	Step	Temperature	Duration	# of cycles
	Initial denaturation	95°C	2min	1
	Denaturation	95°C	30s	25
	Annealing	60°C	30s	
	Extension	72°C	30s	
	Final extension	72°C	5min	1
	Cooling	10°C	forever	hold

3. Purify the PCR product using the MinElute PCR Purification Kit following the manufacturer's instructions. Elute in 30 µl TE buffer.

4. Determine the concentration of the PCR product using the NanoDrop spectrophotometer. The expected concentration range is 50–100 ng/µl. Dilute PCR product to 1 ng / µl in TE buffer in a 1.5-ml Eppendorf LoBind tube.

5. In a 0.2-ml PCR eight-tube strip, prepare the following reaction mix use 1ng of the purified PCR product as template for a second amplification with a combination of P5 and P7 indexing primers used only for preparing the qPCR standard. Mix properly.

	Reagent	Volume	Final concentration in reaction
	5x Herculase buffer	20µl	1x
	25 mM each dNTP	1µl	250µM
	10 µM P7_iPCR-qPCR_xxQ	4µl	400nM
	10 µM P5_iPCR-qPCR_xxQ188	4µl	400nM
	400 U/µl Herculase II Fusion Polymerase	1µl	
	Water	69µl	
	1 ng/µl PCR product dilution from step 4	1µl	0.01 ng / µl
	sum	100µl	

6. Place the strip tube into a thermal cycler and perform cycling with the following temperature profile.

	Step	Temperature	Duration	# of cycles
	Initial denaturation	95°C	2min	1
	Denaturation	95°C	30s	25
	Annealing	60°C	30s	
	Extension	72°C	30s	
	Final extension	72°C	5min	1
	Cooling	10°C	forever	hold

7. Purify the PCR product using the MinElute PCR Purification Kit. Elute in 30 µl TE buffer.

8. Measure the DNA concentration on a DNA-1000 chip using the Bioanalyzer 2100. Calculate the concentration of the PCR products in molecules per µl, e.g. using the Promega Biomath Calculator (**https://www.promega.de/en/resources/tools/biomath/**) and the Avogadro constant.

9. Dilute the purified PCR product to 10^9copies/µl in TET buffer in a 1.5 ml Eppendorf LoBind tube.

10. Prepare a ten-fold dilution series in TET buffer ranging from $10^8$to 10^2 copies/µl as follows:

- Prepare a ten-fold dilution (10^8copies/µl) by combining 100µl of the 10^9 dilution with 900µl TET buffer in a 1.5 ml Eppendorf LoBind tube.
- Use the same scheme for further dilutions until reaching 10^2 copies/µl.

11. Aliquot 100 µl from each dilution (10^2 to 10^8 copies/µl) into the first seven wells of a 0.2 ml PCR eight-tube strip, which serves as working dilution. Leave the last well of the eight-tube strip empty. The empty well is used as a no-template-control in qPCR.

Note

[Documentation]

Label the 0.2-ml PCR eight-tube strip containing the working dilutions with "2" to "8" and NTC. Include date of creation and your initials.
Label the 1.5-ml Eppendorf LoBind tubes containing the stock dilutions with the date of creation and your initials.

12. Store stock and working aliquots at −20 °C until used.

Appendix

Document

NAME
TET buffer

CREATED BY
Sarah Nagel

PREVIEW

Document

NAME
TE buffer

CREATED BY
Anna Schmidt

PREVIEW