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PCR amplification of the barcode region

In 1 collection

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We use this protocol and it's working

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Abstract

This protocol details the gDNA amplification and plasmid DNA amplification.

Guidelines

This protocol will be written as if running a single sample through. Calculations will need to be made for any optimizations

- Annealing temperature, extension time, annealing time, and DNA concentration are all parameters to be optimized for each PCR reaction
- Optimizations are best done at 10-20 mL and run on a 2% agarose gel

This protocol is written for either gDNA amplification or plasmid DNA amplification.

- Differences will be noted in **bold**

Materials

Materials

- Micropipettes and tips: P2, P20, P200, P1000
- 0.2 mL 8-strip cap PCR tubes (clear) with lids
- 0.2 mL 8-strip cap PCR tubes (white) with lids (flat, clear)
- Primers
- SYBR Green I
- Q5 2x Master Mix
- Nuclease free water
- 70% ethanol (freshly made)
- AmPure XP magnetic beads
- Magnetic plate (for 0.2 mL PCR tubes or for 1.7 mL Eppendorf tubes)
- 1.7 mL Eppendorf tubes
- Agarose
- SYBR Safe (10,000x)
- 100 mL glass beaker
- 1x TBE (from carboy)
- 1kb+ DNA ladder
- 6x orange dye
- New straight-edge razor
- Freeze n Squeeze columns
- Lo-bind DNA tubes
- Qubit dsDNA HS Assay kit
- Qubit assay tubes

Equipment

- PCR thermocycler
- qPCR thermocycler (MiniOpticon)
- GelDoc
- Blue light gel box
- Orange protective eyewear
- Microwave
- Gel casting tray and gaskets
- Agarose gel electrophoresis box and power supply
- Benchtop microcentrifuge

PCR 1: Adding adapters and/or crossing the recombination junction

For gDNA, set up 8 identical PCR reactions as follows:

	A	B	C	D
	Reagent	**Volume for 50 uL reaction**	**Volume for 8 reactions**	**Final concentration**
	10 mM forward primer (NP0492)	2.5 uL	20 uL	0.5 mM
	10 mM reverse primer (NP0546)	2.5 uL	20 uL	0.5 mM
	2x Q5 master mix	25 uL	200 uL	1x
	gDNA template	2500 ng (varies)	varies	50 ng/mL
	Nuclease free water	varies	varies	n/a

For plasmid DNA, set up 1 reaction as follows:

	A	B	C
	Reagent	**Volume for 50 uL reaction**	**Final concentration**
	10 mM forward primer (NP0492)	2.5 uL	0.5 mM
	10 mM reverse primer (NP0493)	2.5 uL	0.5 mM
	2x Q5 master mix	25 uL	1x
	Plasmid DNA template	10 ng (varies)	0.2 ng/uL
	Nuclease free water	varies	n/a

PCR 2: Nested PCR to add indices and cluster generators

For gDNA: set up 1 PCR reaction as follows in white PCR tubes with clear caps.

	A	B	C
	Reagent	**Volume for 50 uL reaction**	**Final concentration**
	10 mM forward primer (e.g. JS1000)	2.5 uL	0.5 mM
	10 mM reverse primer (e.g. NP0547)	2.5 uL	0.5 mM
	2x Q5 master mix	25 uL	1x
	gDNA template	8 uL (40% of elution)	varies
	100x SYBR Green I	0.25 uL	0.5x
	Nuclease free water	11.75 uL	n/a

For plasmid DNA: set up 1 PCR reaction as follows in white PCR tubes with clear caps.

	A	B	C
	Reagent	**Volume for 50 uL reaction**	**Final concentration**

	A	B	C
	10 mM forward primer (e.g. JS1000)	2.5 uL	0.5 mM
	10 mM reverse primer (e.g. JS391)	2.5 uL	0.5 mM
	2x Q5 master mix	25 uL	1x
	Plasmid DNA template	15 uL	varies
	100x SYBR Green I	0.25 uL	0.5x
	Nuclease free water	4.75 uL	n/a

Before start

Notes about PCR 1:

The first PCR in the gDNA amplification process is designed to both add adapter sequences to one end of the amplicon as well as cross the recombination junction.

- The design of these primers should be such that the forward primer sits within the recombining plasmid and has a standard adapter sequence attached.
- The reverse primer should sit within the landing pad (usually at the start of BFP).
- The use of these two primers, in these two locations, ensures that the only gDNA that gets amplified is gDNA from cells that have successfully recombined.
    - Unrecombined cells will not be able to bind the forward primer, and leftover plasmid will not bind the second primer.

For this step, we run 8 identical reactions for two reasons:

- To reduce “jackpotting” where a random polymerase error gets propagated through the sequencing run.
- To ensure we use enough gDNA to cover our library, since gDNA is extremely large (>6 billion bp) and thus has very few copies even in thousands of ng of gDNA.

The process for plasmid DNA is identical, but we use 10 ng plasmid DNA instead of 2500 ng gDNA in the reaction and we only do 1 reaction instead of 8.

- The reverse primer for plasmid DNA, since it does not need to span a recombination junction, uses a different reverse primer that contains an adapter sequence (just like the forward primer) – use NP0493.

Notes about AmPure XP cleanup:

The amount of AmPure XP beads to use is dependent on the size of the amplicon that is being purified. Generally, AmPure XP cleans up smaller fragments, leaving larger ones to be eluted and saved.

- More beads allows smaller fragments to be retained.

Notes about PCR 2:

The second PCR in the gDNA amplification process is designed to reduce the size of the final amplicon to one that will cluster well on Illumina (smaller amplicons perform better) as well as add the sample indices and cluster generator sequences that will be used by Illumina to sequence and demultiplex samples.

- Each individual sample will require a separate pair of indices, one on the forward and one on the reverse primer.
    - We usually have 14 distinct indices for the forward primer and 14 distinct indices for the reverse primer.

- The indexes can be combined to create up to 196 unique index combinations (14 forward x 14 reverse) to multiplex up to 196 samples on the same Illumina run.
- In practice, we usually use less than 20 on a single Illumina run, which is entirely dependent on how many sequencing reads we need per sample compared to the number of sequencing reads produced by the Illumina kit.

○ The reverse primer will bind directly to the amplicon sequence and will contain a unique index and a cluster generator sequence (P7).

- This primer binds 5’ to the reverse primer in PCR1, since we need to reduce the size of the amplicon, usually to about 250-350 bases, for good Illumina sequencing.
- If the location of the barcode changes for whichever reason, it’s necessary to change these primers to match that new location.

○ The forward primer will bind to the adapter sequence added in PCR1 and will contain a unique index and the other cluster generator sequence (P5).

- The adapter sequence is designed such that we do not get amplification of any gDNA other than that which was amplified in PCR1. This ensures that we continue to not amplify random plasmid or gDNA that does not contain our barcoded region.

- For plasmid DNA, you will need to alter the reverse primer, since it has an adapter sequence to bind to.

PCR 1: Adding adapters and/or crossing the recombination junction

1 **For gDNA**, set up 8 identical PCR reactions as follows:

	A	B	C	D
	Reagent	**Volume for 50 uL reaction**	**Volume for 8 reactions**	**Final concentration**
	10 mM forward primer (NP0492)	2.5 uL	20 uL	0.5 mM
	10 mM reverse primer (NP0546)	2.5 uL	20 uL	0.5 mM
	2x Q5 master mix	25 uL	200 uL	1x
	gDNA template	2500 ng (varies)	varies	50 ng/mL
	Nuclease free water	varies	varies	n/a

2 **For plasmid DNA**, set up 1 reaction as follows:

	A	B	C
	Reagent	**Volume for 50 uL reaction**	**Final concentration**
	10 mM forward primer (NP0492)	2.5 uL	0.5 mM
	10 mM reverse primer (NP0493)	2.5 uL	0.5 mM
	2x Q5 master mix	25 uL	1x
	Plasmid DNA template	10 ng (varies)	0.2 ng/uL
	Nuclease free water	varies	n/a

3 Run 5 cycles of PCR on a standard thermocycler with the following parameters:

	A	B	C
	Step	**Temperature**	**Time**
	1	98°C	30 s
	2	98°C	10 s
	3	61°C	30 s
	4	72°C	30 s (1 min/kb)
	Go to 2		4x
	5	72°C	2 min
	6	4°C	hold

AmPure XP cleanup

13m

4 Pool all 8 PCR reactions into a single 1.7 mL Eppendorf tube

5 **For gDNA**, add 0.8 mL AmPure XP beads for every 1 mL of product and mix by pipetting.

- Approximately 320 µL of beads to 400 µL of product, not accounting for evaporation.

6 **For plasmid DNA**, add 1 mL AmPure XP beads for every 1 mL of product.

- Approximately 50 µL of beads to 50 µL of product, not accounting for evaporation.

7 Incubate at Room temperature for 00:05:00 .

5m

8 Add tube to magnetic stand and let sit for 00:02:00 to pull beads to the side.

2m

9 Carefully aspirate with a pipette without disturbing the beads.

10 Wash with 2x volume of 70% ethanol.

> **Note**
>
> Approximately 800 µL of 70% ethanol.

11 Incubate at Room temperature for 00:02:00 on magnetic plate.

2m

12 Carefully aspirate with a pipette without disturbing the beads.

13 Repeat wash with 2x volume of 70% ethanol.

14 Allow any leftover ethanol to fully evaporate by leaving the lid off.

15 **For gDNA:** Add 21 µL nuclease free water to the beads and mix by vortex or pipetting.

16 **For plasmid DNA:** add 16 µL nuclease free water to the beads and mix by vortex or pipetting.

17 Incubate at Room temperature for 00:02:00 .

2m

18 Incubate at Room temperature for 00:02:00 on magnetic plate.

2m

19 Transfer the eluted DNA (~20 µL) to a new PCR tube.

PCR 2: Nested PCR to add indices and cluster generators

20 **For gDNA:** set up 1 PCR reaction as follows in white PCR tubes with clear caps.

	A	B	C
	Reagent	**Volume for 50 uL reaction**	**Final concentration**
	10 mM forward primer (e.g. JS1000)	2.5 uL	0.5 mM
	10 mM reverse primer (e.g. NP0547)	2.5 uL	0.5 mM
	2x Q5 master mix	25 uL	1x
	gDNA template	8 uL (40% of elution)	varies
	100x SYBR Green I	0.25 uL	0.5x
	Nuclease free water	11.75 uL	n/a

21 **For plasmid DNA:** set up 1 PCR reaction as follows in white PCR tubes with clear caps.

	A	B	C
	Reagent	**Volume for 50 uL reaction**	**Final concentration**
	10 mM forward primer (e.g. JS1000)	2.5 uL	0.5 mM
	10 mM reverse primer (e.g. JS391)	2.5 uL	0.5 mM
	2x Q5 master mix	25 uL	1x
	Plasmid DNA template	15 uL	varies
	100x SYBR Green I	0.25 uL	0.5x
	Nuclease free water	4.75 uL	n/a

22 **For gDNA:** run 15-20 cycles of qPCR on the MiniOpticon or CFX with the following parameters:

	A	B	C
	Step	**Temperature**	**Time**
	1	98°C	30 s
	2	98°C	10 s
	3	65°C	30 s
	4	72°C	30 s (1 min/kb)
	Go to 2		19x
	5	72°C	2 min
	6	4°C	hold

23 **For plasmid DNA:** run 9-12 cycles of qPCR instead.

	A	B	C
	Step	**Temperature**	**Time**
	1	98°C	30 s
	2	98°C	10 s
	3	68°C	30 s
	4	72°C	30 s (1 min/kb)
	Go to 2		19-24x
	5	72°C	2 min
	6	4°C	hold

Note

IMPORTANT: stop the qPCR reaction when the relative fluorescence units (RFU) for all samples has risen to or above 0.3 (MiniOpticon) or 3000 (CFX). It does not need to complete the final extension or hold steps

- **For gDNA**: this is often around 15-20 cycles.
- **For plasmid DNA**: this is often around 9-12 cycles.

Gel extraction of amplicon

1h 17m

24 Make a 2% agarose gel in 1x TBE.

7m

a. Melt in microwave at 30% power for 00:03:00 - 00:04:00, watch for over-boiling.

b. Mix in 12 µL SYBR Safe by swirling gently.

c. Pour into gel casting tray (make sure you have the gaskets in tightly!) after ensuring that the gel is level.

d. Insert a 10 well thick comb.

e. Cover with foil to protect from light until solidified.

25 Fill gel box to maximum level with 1x TBE.

26 Insert gel (remove gaskets but keep plastic tray) into gel box vertically.

27 When adding DNA to the gel, skip every other lane to ensure less bleedover between samples.

28 Add 20 µL 1kb+ ladder to the first well.

29 Add all 50 µL of qPCR product to 10 uL 6x orange dye and load the entire volume into the gel.

30 Add 20 µL 1kb+ ladder to the last well.

31 Run the gel at 120V for 01:00:00 . 1h

32 Visualize gel on a blue light gel box.

> **Note**
>
> Wear orange safety glasses!

32.1 Using a brand new straight razor, carefully cut out the band of expected size.

32.2 Trim the edges of the gel so only illuminated sample is present in the final gel piece.

> **Note**
>
> Rotate in all directions to make sure.

32.3 Discard any extra gel, leaving the extracted piece with amplicon on the illuminated gel box.

Note

If running multiple samples to extract, move the gel to the side/back into the gel tray until the next step is complete.

32.4 Finely chop the gel containing the amplicon using the razor. Add the cubes to a labeled freeze n squeeze column.

Note

If running multiple samples, repeat these steps to cut out each band and place in a new freeze n squeeze column.

33 Place freeze n squeeze column(s) into -20 °C freezer for 00:05:00 . 5m

34 Spin sample at maximum speed in the tabletop centrifuge (> 13.000 x g, 00:05:00). 5m

35 Collect DNA amplicon(s) from collection tube(s) and place in lo-bind DNA tube(s).

Qubit amplicon to determine DNA concentration 2m

36 Collect one Qubit assay tube (0.5 mL) for each sample plus two tubes for standard DNAs.

- Label the lids, not the sides. Qubit measures through the sides of the tubes.

37 Make working solution by diluting dsDNA HS reagent 1:200 in dsDNA HS buffer in plastic tubes and mix by vortexing.

37.1 This comes out to 1 µL dsDNA HS reagent for every 199 uL dsDNA HS buffer.

37.2 You'll need 200 µL for each sample + 400 µL for standards and pipetting error.

- For example, if you have 2 samples, you'll need to make 800 µL of working solution (2x200 uL + 400 uL) by mixing 8 µL of dsDNA HS reagent to 792 µL dsDNA HS buffer.

38 Add 190 µL working solution to each of the two standards tubes.

39 Add 10 µL of each standard to the appropriate tube and mix by vortexing.

40 Add 198 µL working solution to each sample tube.

41 Add 2 µL of sample to the appropriate tube and mix by vortexing.

42 Incubate samples and standards at Room temperature for 00:02:00 . 2m

43 Take samples to Qubit reader (2.0).

- Ensure Qubit is set to DNA – dsDNA high sensitivity.

44 On the standards screen, select yes to read standards.

45 Read standard #1 by placing into reader, closing lid, and pressing read.

46 Remove standard #1 and repeat with standard #2.

47 Once the sample screen appears (should appear automatically), remove standard #2 and place in sample.

48 Close lid and press read.

- Record the concentration reported by Qubit (in ng/mL).

49 Repeat with each sample until all samples are read.

50 Back-calculate the actual sample concentration(s)

- ng/mL
- Qubit measures the concentration without accounting for the dilution into working solution.

51 Then, calculate the nM concentration of the amplicon.

51.1 Estimate the dsDNA molecular weight using this tool by copying the amplicon sequence from benchling: https://www.bioinformatics.org/sms2/dna_mw.html

- **Make sure you have it selected as double stranded linear DNA**
- Since the amplicon has N nucleotides, it will give you a range of values. Take the average of these two numbers

51.2 Use “molarity from mass and volume” from this tool to calculate a molar concentration: https://www.graphpad.com/quickcalcs/Molarityform.cfm

- Another option is to use the following equation if this tool no longer works for whatever reason: where [sample] is the sample concentration in ng/mL and MW is the molecular weight from the previous step.

52 Store DNA amplicon at -20 °C until ready for use.

- Requantify using Qubit at the time of use to account for evaporation or binding to tubes.

Sequencing

53 Pool the prepared libraries by molarity in proportions appropriate for the sequencing kit.

54 Sequence using an appropriate Illumina NGS sequencing kit.