

V1.1 Post-hybridization, Post-amplification qPCR Check of Enriched UCE Libraries

After enrichment, but prior to pooling, quantifying, and sequencing target-enriched libraries, you may wish to validate that your libraries actually contain enriched product relative to the unenriched library or library pool. The easiest way to do this is to amplify a small portion of the target loci using qPCR of pre-enriched UCE libraries relative to post-enrichment, post-amplification (AKA PCR-recovery) libraries. This is “relative qPCR”.

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Materials

In the following, the SYBR Green master mix (or mix) that you use is really less important than putting the reactions together correctly, and running them on an appropriate qPCR platform that will compute (or allow you to compute) the C_p (“crossing point”) value of a given reaction. We use a Roche LightCycler 480 qPCR machine.

- PCR primers targeting UCE loci (*see below*)
- Roche SYBR Green I Master Mix (p/n 04 707 516 001)
- Molecular biology-grade water (for diluting samples and qPCR reaction mix setup)
- Optical plates (white) and optical sealing film
- Roche Light Cycler 480 (or similar)
- Qubit (BR or HS assay)

Primers

You can use the following primers to amplify targets that should have been enriched using either the 2,500 locus probe set used in Crawford et al. 2012 or the 5,000 locus probe set initially described in Faircloth et al. 2012.

If you have altered the probe set to reduce the number of loci captured (or for any other reason), you may have removed some loci targeted by these primers. You should double check any custom modifications to your probe set to ensure you find these priming sites within your probes.

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In the following, the primer name designates the UCE locus targeted and the genome name designates the genome-enabled taxa in which I have located the primers in their correct position and orientation. I designed primers and estimated T_m using Primer3 (Untergasser et al. 2012). You may also wish to include other primers, such as those targeting housekeeping genes as an additional control.

Primer sequences targeting UCE loci (5' – 3')

>uce-7567-upper (human, galgal3, anocar2, xentro3, chrpic1, latcha1) | Tm 60.8
TTATGAAGCTGAACCCAATG

>uce-7567-lower (human, galgal3, anocar2, chrpic1) | Tm 60.9
CCATAAGATTCTGTCGGAGTT

>uce-417-upper (human, galgal3, anocar2, chrpic1, latcha1) | Tm 59.7
ATCCAATTTGAAGTGATGACA

>uce-417-lower (human, galgal3, anocar2, xentro3, chrpic1, latcha1) | Tm 59.1
ATTAGTTCCATGTGCATTTAAT

>uce-4586-upper (hg19, galgal3, anocar2, chrpic1, latcha1) | Tm 61.6
GGCAGCTTTCATAAGACATTT

>uce-4586-lower (hg19, galgal3, anocar2, chrpic1, xentro3, latcha1) | Tm 62.3
TAATTCCTGTGACTTGCCTAA

>uce-277-upper (hg19, galgal3, anocar2, chrpic1) | Tm 62.3
AGTTTCCTGCGGTAAGTTCA

>uce-277-lower (hg19, galgal3, anocar2, chrpic1, xentro3, latcha1) | Tm 62.8
TGATAGAGCAGAACACCGTATG

>uce-1408-upper (hg19, galgal3, anocar2, xentro3, chrpic1) | Tm 63.1
GCCTCTGACAGCGAAACAC

>uce-1408-lower (hg19, galgal3, anocar2, xentro3, chrpic1, latcha1) | Tm 62.4
ATTTTAACCAGCAGCATGAGTA

>uce-551-upper (hg19, galgal3, anocar2, xentro3, chrpic1) | Tm 58.2
ATAACTGTTGGGGCTCTAAT

>uce-551-lower (hg19, galgal3, anocar2, xentro3, chrpic1, latcha1) | Tm 58.2
TGACAAATGGTGCTTAGAAG

> uce-729-upper (hg19, anocar2, xentro3, chrpic1, latcha1) | Tm 58.5
AATTGCAGCATTAAACATCCATT

> uce-729-lower (hg19, anocar2, xentro3, chrpic1) | Tm 57.8
CTTCATTTAGTCTTTAAGGTTGTGC

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Plate setup

You're going to setup what is known as a "relative qPCR", meaning that you are comparing the crossing point, or *C_p*, value of enriched versus unenriched libraries to compute a fold-enrichment for the enriched libraries relative to their unenriched counterparts. This is in contrast to *absolute* qPCR quantitation, which is what you do when you qPCR quantify enriched libraries prior to pooling and sequencing (you include known-concentration samples).

In order to do this, you need to amplify all 7 loci (+1 negative control) from each library (or a subset of enriched libraries) of both enriched and unenriched DNA. You can also run this qPCR against pooled enrichments (where you are enriching a pool of libraries versus a single library), but keep in mind that successful results from pooled libraries do not necessarily indicate successful enrichment of **ALL** libraries within the pool.

qPCR is generally setup in replicates of 3. So for each library, we would potentially prepare 3 replicates of each library amplified at each primer for both the unenriched and the enriched DNA (so, a total of 6 wells of a plate are needed to measure and compare both samples).

For the purpose of determining if UCE enrichment worked, I generally back off the standard 3-replicate approach and call 2 replicates of each sample "good enough". You are certainly encouraged to experiment and do things differently than I do - the following setup is only meant as a guide. Below, I've presented how you might setup 1/3rd of a 96-well PCR plate for qPCR verification of UCE enrichment. Note that using 2 replicates for each of the unenriched and enriched libraries requires 4 columns of a plate. As such, you can test 3 libraries (or library pools) per qPCR plate.

	Library 1 Unenriched Rep1	Library 1 Unenriched Rep2	Library 1 Enriched Rep1	Library 1 Enriched Rep2
	1	2	3	4
A	uce-7567-U-rep1	uce-7567-U-rep2	uce-7567-E-rep1	uce-7567-E-rep2
B	uce-417-U-rep1	uce-417-U-rep2	uce-417-E-rep1	uce-417-E-rep2
C	uce-4586-U-rep1	uce-4586-U-rep2	uce-4586-E-rep1	uce-4586-E-rep2
D	uce-277-U-rep1	uce-277-U-rep2	uce-277-E-rep1	uce-277-E-rep2
E	uce-1408-U-rep1	uce-1408-U-rep2	uce-1408-E-rep1	uce-1408-E-rep2
F	uce-551-U-rep1	uce-551-U-rep2	uce-551-E-rep1	uce-551-E-rep2
G	uce-729-U-rep1	uce-729-U-rep2	uce-729-E-rep1	uce-729-E-rep2
H	uce-729-U-Neg-rep1*	uce-729-U-Neg-rep2*	uce-729-E-Neg-rep1*	uce-729-E-Neg-rep2*

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* You may wish to have additional negative controls, but I typically use 1 primer pair to check the negative for amplification. If this concerns you, you can alternate the primer pair you use across the negative control samples to check all primer pairs and the reaction mix for contamination.

Steps

1. Prepare a mix of each primer (forward + reverse) at 2 uM **each primer**.
2. If you have not done so already, quantify your **unenriched** library or library pool on the Qubit.
3. Prepare a dilution of the unenriched library based on the quantification in Step 1. Generally, something like 40 uL of 1 ng/uL will suffice.
4. If you have not done so already, quantify your **enriched** library or library pool on the Qubit (following limited-cycle amplification and AMPure/Serapure cleanup).
5. Prepare a dilution of the enriched library based on the quantification in Step 3. Generally, something like 40 uL of 1 ng/uL will suffice.
6. Setup the following PCR reaction master mix (105X for a 96-well plate):

1050 uL Roche SYBR Green I Master Mix
630 uL Molecular biology-grade ddH₂O
1680 uL Total volume

7. Pipet the master mix into a strip tube and use a multichannel pipet to dispense 16 uL master mix into each well of a 96-well qPCR optical plate (these are usually different than standard PCR plates – use the plate type for your instrument).
8. Carefully pipet 30 uL primer mix for each primer into 1 well of an 8-well strip tube.
9. Using a multichannel pipet, add 2 uL of each primer to the corresponding well of a 96-well plate **according to your plate map from *Plate Setup, above***.
10. Using a single-channel or multichannel pipet, add 2 uL of the correct library (at 1 ng/uL) to the correct well of the plate **according to your plate map**. Be careful to place unenriched/enriched library in the correct well according to its replicate # and position.
11. Seal the plate with the **correct optical seal for your machine**. ALWAYS do this wearing gloves so you do not leave fingerprints on the seal.
12. Spin the plate down.

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13. Cycle using the following (or adjust for your qPCR machine):

- 95 C for 5 minutes (4.4 C ramp)
- => **40** cycles of:
 - 95 C for 10 seconds (4.4 C ramp)
 - 60 C for 20 seconds (2.2 C ramp)
 - 72 C for 30 seconds (4.4 C ramp)
- => Melt curve
 - 95 C for 5 seconds (4.4 C ramp)
 - 60 C for 1 minute (2.2 C ramp)
 - 97 C continuous (5-10 acquisitions per degree C)
- => Cooling
 - 40 C for 10 seconds (1.5 C ramp)

14. When the run is complete, you will need to determine how to compute the Cp value for each amplification curve. Most machines take a somewhat-easy-to-setup template that allows you to tell the machine which wells are replicates of other wells. Using this, the machine will average across replicate wells and report the average Cp value (and standard deviation) for each sample and give you some nifty output.
15. You can compute a rough-estimate of fold-enrichment by first computing delta-Cp between the unenriched and enriched average Cp values [$\text{delta-Cp} = \text{abs}(\text{enriched} - \text{unenriched})$] for each sample at each primer pair.
16. Then, you compute fold-enrichment as $1.78^{(\text{delta-Cp})}$. This is a bit of a kludge, because we actually don't exactly know the efficiency for these primers in all taxa, but it works reasonably well across pretty divergent taxa.
17. Successful enrichments will show anywhere from 100X to 400X (or more) fold-enrichment across **MOST** loci. You may see values as low as 50X. Values lower than 50X or negative values (meaning the enriched library contains less target DNA than the unenriched library) **across MOST primers** generally indicates a failed enrichment(s).
18. If you are satisfied with your enrichment success (I generally am satisfied if ~ 75% of loci show ~50-100X or greater enrichment), you should move on to quantifying your libraries and pooling them in preparation for sequencing.