Appendix S1. Explanation and protocol for the qPCR assay for validation of target enrichment.

A. Assay strategy

The purpose of the qPCR enrichment validation is to verify that the MyBaits enrichment protocol was successful. This protocol assumes basic familiarity with the principle of qPCR. It is not necessary to determine absolute DNA quantities, which would require a more elaborate protocol; we simply aimed for ~10 cycles difference between assay samples. We used two types of assay, for which the input libraries and primers were different, but were identical in reagent recipe and amplification protocol. There could conceivably be underlying differences between samples that confound each assay (input libraries not representative of pre-enrichment pool, target and non-target loci have variable base frequencies in the genome), but congruent results between the two indicate strong evidence that the enrichment was successful.

The two assay types were:

1. Amplification of targeted loci for enriched pool vs. unenriched libraries.
   Target 1 and Target 2 loci (section B) are amplified in an enriched library pool and one or two of the unenriched input libraries. For instance, if libraries one through eight were multiplexed, one or two of these are arbitrarily chosen to compare to the enriched pool.
2. Amplification of target and non-target loci in an enriched pool.
   a. Target 1, Target 2, Off-target 1, and Off-target 2 loci (section B) are amplified in an enriched pool.

B. qPCR primers

Locus identification was based on BLAST results; primers were de-salted oligonucleotides synthesized by Sigma-Aldrich (St. Louis, Missouri, USA) to amplify exonic regions. We also tested additional loci, which are available upon request from the corresponding author.

Targeted loci:

Target 1 – locus 16 – Golgi complex protein. Expected product 117 bp.
T1-F: ATCCGGAACAGTGGTGGTAG
T1-R: AGACAAAGGGAGAACAGTCAGG

Target 2 – locus 1 – Uridine nucleosidase. Expected product 121 bp.
T2-F: CTTTCTGCGATTCCCTTCAG
T2-R: GTGGACATGGAAGAACAAGC

Off-target loci:

Off-target 1 – Transcriptional activator. Expected product 118 bp.
H1-F: CCGGGAAGCTGACAATAATC
H1-R: AAGTTTCTCCATGGGCATCC

Off-target 2 – (Cytosine-5)-methyltransferase DRM-2. Expected product 120 bp.
H2-F: CTTCGAGGAAGATCGCAAAG
H2-R: TGACGGCTGGTGATTTTAGG

C. Reaction mix

This is based on a protocol by the Faircloth Lab (Louisiana State University [LSU]), posted at http://ultraconserved.org/; suppliers and master mix strategy were modified. Additionally, we used pooled libraries for post-enrichment samples but individual libraries for pre-enrichment samples.

1. Prepare 2-µM aliquots of each primer (desalted [Sigma-Aldrich]).
2. Add to 1-µL library deionized water to adjust concentration of library (based on Qubit; Life Technologies, Carlsbad, California, USA) to 1 ng/µL.
3. For x libraries, prepare the following master mix:
   10 * (x + 1) µL iTaq Universal SYBR Green Supermix (reorder #172-5121; Bio-Rad Laboratories, Hercules, California, USA)
   6 * (x + 1) µL deionized water
   16 * (x + 1) µL total volume
4. Dispense 16-µL master mix into each well of a set of qPCR strip tubes.
5. Dispense 2-µL primer mix (2 µM) into each well of a set of qPCR strip tubes.
6. Add 2-µL library (1 ng/µL) into each well of a set of qPCR strip tubes.
7. Label orientation of strip tubes and spin down.
8. Transfer to thermocycler.

x = (# test loci) * (# assayed libraries/pools) * (# replicates),
where, for a single assay, # replicates = 2 in all assays, # assayed libraries/pools is 2–3 (assay a) or 1 (assay b), and # test loci is 2 (assay a) or 4 (assay b).

It is recommended to multiplex several assays, up to the capacity of the thermocycler, to avoid reagent waste and unnecessary freeze-thaw cycles.

D. Thermocycler profile

Derived from a protocol by the Faircloth Lab (LSU), posted at http://ultraconserved.org/; performed on a CFX96 Touch Real-Time PCR Detection System (Bio-Rad Laboratories). A melt curve has been omitted.

Lid temperature 105°C; volume 20 µL
1. 95°C, 5 min
2. 95°C, 10 s
3. 60°C, 20 s [Detection step]
4. 72°C, 30 s
5. Go to step 2 39×.

E. Result examples

These plots show a single qPCR run with both assay types multiplexed (enriched library vs. unenriched library for targeted loci; enriched library for target and non-target loci); the results are qualitatively indistinguishable and hence they are shown together. The group of red traces on the left is composed of enriched/target-locus samples; the group on the right contains unenriched/non-target-locus samples. The difference between the two groups is typically 10–12 cycles.

Linear Y-axis (fluorescence signal):