**Supplementary materials**

**Suppl. 1: Library preparation for multiplexed second-generation sequencing**

We constructed low-cost multiplexed libraries for the Illumina platform. The Illumina library preparation protocol was derived from two sources (Meyer & Kircher, 2010; Rohland & Reich, 2012), is similar to another recently published (Dunham & Friesen, 2013). While our libraries were designed for 100 bp single-end single-index libraries for Illumina, they can easily be adapted to longer inserts as well as paired-end and double-indexing, by modifying the sonication parameters for DNA shearing and the indexed adapters (see below).

*1) DNA shearing and sizing:*

Long PCR amplicons for each specimen were purified using Millipore column and combined at equimolar ratios when necessary, i.e. when the mtDNA was amplified in more than one piece. Although this step is not strictly necessary, it allows for the removal of primer dimers, which can otherwise take up a significant portion of the libraries. Purified long-PCR amplicons were sheared to an average size of 600 bp for Illumina libraries and 200 bp for Ion Torrent libraries with a QSonica Q800R Sonicator (2 min or 3 min at 30% amplitude with 10 sec on/off for Illumina and Ion Torrent, respectively). The size window of sheared amplicons was verified on an Agarose gel. Sheared DNA was recovered with a homemade Serapure beads suspension (Rohland & Reich, 2012) and quantified using a BioTek Epoch Microplate Spectrophotometer.

We have found that sizing by a combination of sonication-Agarose gel-spectrophotometer yields satisfying results for library preparation on most platforms and is even preferable to more sophisticated and expensive sizing methods such as enzymatic shearing and use of the BioAnalyzer. They are also far less demanding in materials and therefore more environmentally friendly.

*2) Illumina library preparation:*

The library preparation consisted of the combination of several previously published massively parallel sequencing protocols (Meyer & Kircher, 2010; Rohland & Reich, 2012) into a double tagging approach: an internal tag attached to the 3’end of the universal adapters (we called barcode) and an external tag as part of the indexed adapter (we called index). This highly increased the number of samples to price ratio, but see below.

Each adapter was synthesized in two pieces (Table S4), with a 20 bp overlap between each half, and had an over-hanging T on the 3’end of the 3’half to increase the ligation efficiency (sticky-end ligation). We prepared an adapter mix containing a combination of the 3’half of a barcoded universal adapter and the 3’half of the index adapter. Sheared DNAs were blunt-end repaired using the NEB Quick Blunting Kit and A-tailed using Klenow Fragment (3’→5’ exo–). Adapter mixes (UniAdp\_barco1 & 2/UniComp\_barco1 & 2 and IndAdp\_short/IndCompAdp, Table S4) were ligated to individual samples using T4 Ligase, followed by adapter fill-in with *Bst* polymerase large fragments (barcoding). Adapters were completed by short PCR using a High-Fidelity DNA Polymerase and primers (UniAdp\_long and IndAdp\_long1 to 20) corresponding to the 5’half of the adapters with one indexed adapter per two samples (indexing). We quantified each library with Thermo Scientific SYBR Green/ROX qPCR Master Mix and pooled all samples in equimolar ratio.

*3) Quadruple tagging protocol for Illumina paired-end sequencing:*

Illumina has recently introduced a double indexing library method, where both adapters (i5 and i7) have indexes. As mentioned above, for paired-end sequencing it is possible to exponentially increase the multiplexing level by introducing a tag at the 3’end of both Illumina adapters. The library preparation would be similar to the one described above, with the adapter mix consisting of the combination of the 3’ half of an i5 adapter and the 3’ half of an i7 adapter. This will provide each sample with a combination of two indexes and two barcodes. In that way, it is possible to tag 600 samples with five indexes on i5 and four indexes on i7, six barcodes for i5 adapters and five barcodes for i7 adapters. Such an approach reduces the cost of multiplexing by reducing the number of tags, the main driver of high costs for multiplexed libraries when using standard kits. This approach will be most cost-effective for barcoding purposes, where the tags can be included at the 5’end of PCR primers, hence removing the substantial cost and potential errors associated with ligation.

**References**:

Dunham JP, Friesen ML. 2013. A cost-effective method for high-throughput construction of Illumina sequencing libraries. *Cold Spring Harbor Protocols* 2013.9: 820-834 DOI 10.1101/pdb.prot074187.

Meyer M, Kircher M. 2010. Illumina sequencing library preparation for highly multiplexed target capture and sequencing. *Cold Spring Harbor Protocols* 2010:1–8 DOI 10.1101/pdb.prot5448.

Rohland N, Reich D. 2012. Cost-effective, high-throughput DNA sequencing libraries for multiplexed target capture. *Genome Research* 22.5: 939-946 DOI 10.1101/gr.128124.111.