Supplementary Methods

RNAseq library construction

Strand-specific, rRNA-depleted RNAseq sequencing libraries were constructed using a method adapted from (Giannoukos *et al.*, 2012). First, DNA was removed from the total RNA sample by treating the sample with 4U of Turbo DNase (Ambion) (37 °C, 30 min, in a 50 µL final reaction volume). DNA-free RNA was purified using RNAClean XP beads (Agencourt/Beckman, Brea, CA, USA; beads added at 1.8x reaction volume) following the manufacturer's instructions. RNA fragmentation was carried out by magnesium hydrolysis (40 mM Tris-acetate, pH 8.1, 100 mM KOAc, 30 mM MgOAc final concentrations) for 4 minutes at 83^{o}C to yield fragments with a mean peak size of 300 - 350nt; these were purified using RNAClean XP beads (2.0x reaction volume).

Strand-specific RNAseq libraries were prepared using the dUTP second-strand approach (Giannoukos *et al.*, 2012). Fragmented RNA (~100 ng) was mixed with 3 µg of random hexamer primers in a 7 µL reaction and incubated at 70 °C for 10 minutes, then placed on ice. To complete the first-strand DNA synthesis reaction, the following reagents were added to the RNA in a 20 µL final reaction volume: 1x first strand reaction buffer (Invitrogen, Carlsbad, CA, USA), 10 mM DTT, 0.5 mM dNTP mix, 4 µg Actinomycin D (USB/Affymetrix, Santa Clara, CA, USA), 20 U SUPERase-In (Ambion), and 200 U SuperScript III (Invitrogen) (all final concentrations). This reaction was heated to 25 °C for 10 minutes, then placed at 55 °C for 1 hour (with a gradual ramping from 25 °C to 55 °C at a rate of +0.1 °C / sec). First strand cDNA products were purified using a 2.0x volume of RNAClean XP beads (incubating beads with DNA at room temperature for 20 minutes to improve recovery of single-strands) and eluted in 30 µL of water. To synthesize dUTP-labeled second strands, the following components were added to the entire 30 µL of purified first strand product: 0.1x first strand buffer, 1 mM DTT, 267 nM dNTPs (containing dUTP instead of dTTP), 1x second strand buffer (Invitrogen), 12 U *E. coli* DNA polymerase (Invitrogen), 3 U *E. coli* DNA ligase (Enzymatics, Beverly, MA, USA) and 1.5 U RNase H (Enzymatics) (all final concentrations) in a 45 µL final reaction. This reaction was incubated at 16 °C for 2 hours then stopped by adding 6 µL of 0.5 M EDTA and placing the reaction on ice. Double-stranded cDNA was purified using AMPure XP beads (Agencourt/Beckman) at a 2x reaction volume.

Next, the double-stranded cDNA was prepared for library enrichment by treatment with end-repair mix (Enzymatics) according to the manufacturer's instructions, and purified with AMPure XP beads (1.8x reaction volume). Terminal A-base addition was performed by incubating 10 µL of end-repaired DNA with 1x Klenow buffer (Enzymatics), 6 U Klenow (Enzymatics) and 0.2 mM dATP in a 20 µL reaction at 37 °C for 30 minutes; the reaction was placed immediately on ice afterwards. Y-adapter oligonucleotides were then ligated onto these A-tailed fragments (5' – ACACTCTTTCCCTACACGACGCTCTTCCGATC*T – 3' and 5' – /5Phos/GATCGGAAGAGCGGTTCAGCAGGAATGCCGAG – 3' were annealed prior to ligation by slowly cooling an euqimolar mixture of the two oligos from 90 °C to 4 °C in a 10 mM Tris pH 8, 10 mM NaCl buffer). These adapters were added using T4 DNA rapid ligase (Enzymatics) in a 50 µL reaction with 0.4 µM oligos and 1200 U of ligase for 10 minutes at 25 °C, then purified using AMPure XP beads at a 0.8x ratio and eluting in 10 µL water. The dUTP-containing strands were degraded using the Uracil cleavage system (Enzymatics) according to the manufacturer's instructions, incubating at 37 °C for 30 minutes followed by a 95 °C inactivation for 5 minutes.

Initial library amplification was carried out by amplifying Y-adapter ligated fragments with shortened enrichment primers (F: 5'-TCTTTCCCTACACGACGCTC – 3'; R: 5'-GGCATTCCTGCTGAACCGCT-3'; 0.5 µM each) in a 50 µL reaction containing 2 µL of Uracil-cleaved cDNA, 1.0x Phusion HF buffer (ThermoFisher, Waltham, MA, USA), 0.8 U Phusion HS II enzyme (ThermoFisher), 0.2 mM dNTPs, and 0.25X SYBR Green I (Qiagen, Valencia, CA, USA). Reactions were split into two independent wells of 25 µL each, denatured at 98 °C for 3 min, then followed in real-time on a CFX96 (Bio-Rad, Hercules, CA, USA) with cycling parameters: 98 °C, 30 seconds; 60 °C, 30 seconds, 72 °C, 1 min. Amplification was halted once the reactions were exponentially amplifying and before the amplification profile began to level off, typically 12-15 cycles. Reactions were extended at 72 °C for 10 minutes and purified with AMPure XP beads (at a 0.8x ratio).

The ribosomal RNA abundance in each library was reduced using a duplex-specific normalization approach (Yi *et al.*, 2011; Giannoukos *et al.*, 2012). 100 ng of amplified cDNA was mixed with hybridization buffer (50 mM HEPES pH 7.5, 0.5 M NaCl) and incubated at 98 °C for 10 minutes, followed by a gradual cooling to 68 °C, in 0.1 °C increments, over the span of 4 hours. A mixture of DSN buffer (1x final concentration) and 4 U DSN enzyme (Evrogen, Moscow, Russia) was preheated at 68 °C for 10 minutes then added to the cDNA. This reaction was held at 68 °C for 25 minutes, then stopped with 1x DSN stop solution and purified with AMPure XP beads (1.6x reaction volume). The final library was constructed by enriching the DSN-treated mixture in a PCR reaction as before using Phusion HF II with Illumina-compatible forward primers and barcoded reverse primers (0.5 µM each; (Rodrigue *et al.*, 2009)).

References

Giannoukos G, Ciulla DM, Huang K, Haas BJ, Izard J, Levin JZ, *et al.* (2012). Efficient and robust RNA-seq process for cultured bacteria and complex community transcriptomes. *Genome Biol* **13**: R23.

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