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Library construction for human placenta bulk RNAseq

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This protocol describes the generation of stranded RNA-seq libraries from placenta total RNA. Since RNA quality can be an issue with total RNA isolated from placental tissue, it is advisable to enrich mRNA using a ribodepletion method rather than polyA selection.

Prior to ribodepletion, it is especially important to ensure that the input RNA is free of contaminating DNA. This protocol, therefore, begins with DNase treatment of samples, using Ambion's DNA-*free* DNase Treatment and Removal Reagents. Next, ribodepletion and library construction are performed using the KAPA RNA HyperPrep Kit with RiboErase (HMR). Libraries are indexed with KAPA Unique Dual-Indexed Adapters to enable multiplexed sequencing on an Illumina instrument.

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Veriti 96-Well Thermal Cycler
Applied Biosystems **4375786**

Qubit
Flurometer

Invitrogen **Q33228**

Centrifuge
Benchtop Centrifuge

Eppendorf **5405000441**

Any benchtop centrifuge will suffice

Set of micropipettes with rack: 100-1000 µl, 20-200 µl, 2-20 µl, and 0.5-10 µl
Pipettor set

Pipetman **QP-1001-07**

Can use equivalent Pipettors

Vortex mixer

Any **xx**

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Magnetic Stand
Magnetic Stand

Thermo Scientific **MR02**

Any magnetic rack that fits your tubes will suffice.

Bioanalyzer
Bioanalyzer

Agilent **G2991AA**

Any bioanalyzer will suffice.

Nuclease-free Water **Contributed by users**

1M Tris pH 8.0 **Sigma Aldrich Catalog #T2694**

200 Proof Ethanol pure **Sigma Aldrich Catalog #E7023**

Qubit RNA BR Assay Kit **Thermo Fisher Scientific Catalog #Q10211**

Qubit dsDNA HS Assay kit **Thermo Fisher Scientific Catalog #Q32854**

Agilent High Sensitivity DNA Kit **Agilent Technologies Catalog #5067-4626**

DNA-free DNA Removal kit **Invitrogen Catalog #AM1906**

KAPA RNA HyperPrep Kit with RiboErase (HMR) **Kapa Biosystems Catalog #KK8560 (24 libraries) or KK8561**

KAPA Unique Dual-Indexed Adapter Kit **Kapa Biosystems Catalog #KK8727**

DNA removal

1 To remove any possible contaminating DNA prior to ribodepletion, follow Ambion's protocol (Publication # 1906M, Revision E) for their DNA-*free* DNase Treatment and Removal Reagents.

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DNA-free™ Kit DNase Treatment and Removal Reagents User Guide.pdf

Follow instructions for routine DNase treatment.

Tip: when transferring supernatant during the final step, be sure not to draw up any of the DNase Inactivation Reagent. Take off a volume that you are comfortable with. There is practically zero loss of RNA using this method, only what you leave behind. If you need to maximize your RNA yield you can always increase the starting volume by adding nuclease-free water, but be sure that the RNA will still be at least **100 ng/ul** for library construction in the next step.

Tip: The DNase Inactivation Reagent settles very quickly, so it is important to follow the instruction to resuspend it immediately before use, and several times during incubation with the reaction mix.

Quantitate DNase-treated total RNA using Qubit RNA Broad Range Assay.

Ribodepletion and library construction 10m 30s

2 Follow KAPA's protocol (KR1351 - v4.21) for their KAPA RNA HyperPrep Kit with RiboErase (HMR). In our hands, this protocol yields great results with partially degraded total RNA samples from placenta, some with RIN scores as low as 2. 10m 30s

KAPA RNA HyperPrep Kit with RiboErase (HMR) KR1351 - v4.21.pdf

For HuBMAP samples, the following parameters were used:

Input total RNA: **1000 ng**

Fragmentation: tailored to RIN score (RIN score 6.0 and above, **00:04:00** **85 °C** ; RIN score 5.0-5.9, **00:03:30** **85 °C** ; RIN score 4.9 and below, **00:03:00** **85 °C**)

Library amplification: 8 cycles

For indexing, and to enable efficient multiplexed sequencing, use the KAPA Unique Dual-Indexed Adapter Kit , and dilute adapters to **7 micromolar (µM)** . Using UDIs reduces "index hopping" which can be an issue when using combinatorial dual indexes, especially on patterned flow cells like the NovaSeq.

KAPA Unique Dual-Indexed Adapter Kit KR1736 - v3.20.pdf

Be extremely careful when pipetting out of the adapter plate - you do not want to cross-contaminate wells. Make sure to centrifuge the adapter plate before carefully peeling off the foil cover. Promptly replace with a fresh foil cover when done. Use provided KAPA Adapter Dilution Buffer to dilute adapters from **15 micromolar (µM)** to **7 micromolar (µM)** .

If using a thermocycler to incubate the adapter ligation reaction at **20 °C** , do not pre-heat the lid as this may cause the tube/plate temperature to be too warm, allowing DNA ends to breathe and reducing adapter ligation efficiency.

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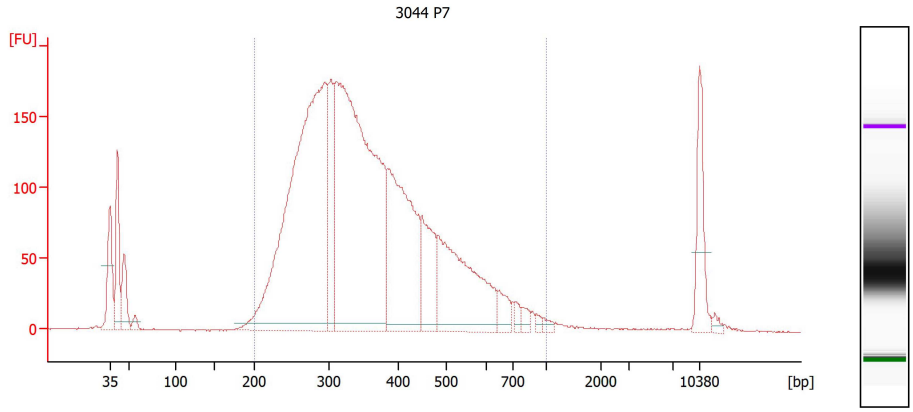
Quality control

3 Quantitate libraries using the Qubit DNA High Sensitivity Assay, and check library distribution by running the DNA High Sensitivity Assay on an Agilent Bioanalyzer. A typical trace is shown below.



Note the appearance of several primer dimer peaks at ~40-60bp which are present due to a combination of the high concentration of primers in the 10x Library Amplification Primer Mix, and the low number of amplification cycles performed (low number of PCR cycles is preferable to reduce amplification bias). These primer dimers are not an issue since they cannot cluster on the flow cell.

Pay attention to the presence of any adapter dimers, which will appear at ~120bp. These will cluster on the flow cell and reduce the number of reads obtained in a sequencing run. If adapter dimers are present, perform another 1x bead-based cleanup using KAPA Pure Beads.

4 If multiplexing samples, first perform a balancing run to ensure equal representation of all samples in the pool. For the balancing run, prepare an "equal volume" pool by combining **2 µL** each library together. Run the pool on a MiSeq instrument using a MiSeq Reagent Kit Nano. Based on the proportion of reads assigned to each index during the Nano run, prepare a balanced pool that will yield an equal read depth for all samples in the pool.

5 Prior to submitting for sequencing, quantitate the pool using the Qubit High Sensitivity DNA assay. Determine the average fragment size for each library from the Bioanalyzer traces.

Tip: set a region from 200bp to 1000bp in the region table tab (fragments over ~1kb don't cluster efficiently on the flow cell) and the Agilent software will calculate the average fragment size.

Determine the average fragment size in the balanced pool, and use the following formula to determine the nM concentration:

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$$\frac{(\text{concentration in ng}/\mu\text{l})}{(660\ \text{g/mol} \times \text{average library size in bp})} \times 10^6 = \text{concentration in nM}$$

Submit the pool to your sequencing facility, noting the nM concentration.

For HuBMAP bulk RNA-seq samples, the multiplexed pool was sequenced on a NovaSeq 6000 S4 lane using a 100bp paired-end run configuration.

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