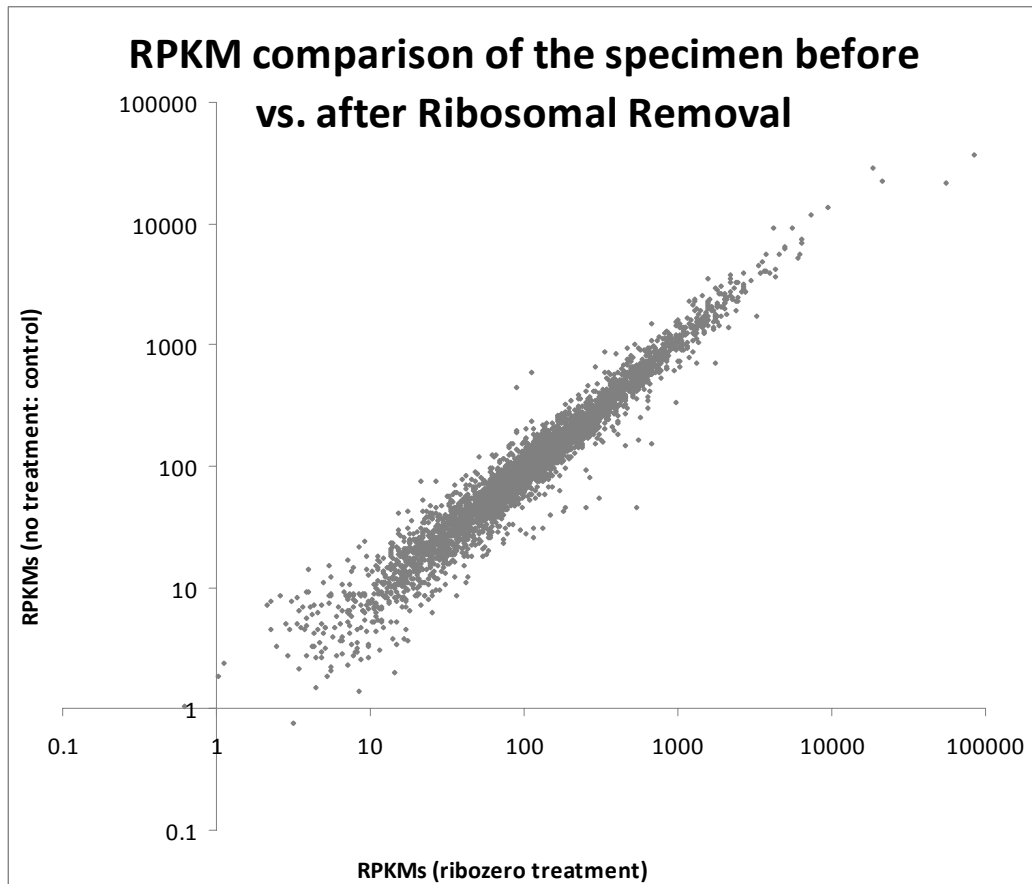


## Protocol: rRNA Removal from Total RNA Using the Epicentre Ribo-zero rRNA Removal Kit

Here we demonstrate that Epicentre Ribo-zero rRNA Removal Kit is an efficient tool for removal of 5S, 16S and 23S ribosomal RNAs from total MTB RNA. After subtraction of ribosomal structural RNAs 78.59% of all the sequence reads (35,482,011/45,147,506) could be successfully mapped back onto the reference genome of MT\_H37RV\_V2.bfa and 99.60% of the mapped reads (35,343,219/35,482,011) could be aligned to non-ribosomal regions of the sequenced sample. On another hand, a non-subtracted aliquot of the same specimen resulted in 87.92% of all reads (32,684,722/37,171,793) that were also successfully mapped back onto the reference genome of MT\_H37RV\_V2.bfa and 2.71% of the sequenced tags (888,265/32,684,722) aligned to non-ribosomal regions of the sample.

In Summary, the kit helps to reduce ribosomal RNA background which results in nearly 100% of the reads aligning to the H37Rv transcriptome. In RNASeq quantified transcript levels are expressed as reads per kilobase of coding sequence per million mapped reads (RPKM) [1, 2].

The RPKM measure of read density reflects the molar concentration of a transcript in the starting sample by normalizing for RNA length and for the total read number in the measurement. This facilitates transparent comparison of transcript levels both within and between samples.



This scatterplot with few exceptions confirms that treatment of the ribo-zero kit does not alter the relative abundance of the mRNAs significantly. Moreover, we get more genomic tag reads after the removal of M.tb 5S, 16S and 23S RNAs. The gene that is significantly off diagonal here (top-right point) corresponds to Rvns02--a structural RNA.

	#1 rRNA after rRNA removal 3.31.11	#2 Neg. control (No rRNA removal) 3.31.11	#3 Neg. control (No rRNA removal) 2.28.11	#4 Rv original
<b>16S</b>	<b>24.06</b>	<b>10.33</b>	<b>9.87</b>	<b>9.85</b>
<b>23S I</b>	<b>28.18</b>	<b>9.5</b>	<b>10.74</b>	<b>9.98</b>
<b>23S II</b>	<b>24.68</b>	<b>8.78</b>	<b>8.87</b>	<b>8.62</b>
<b>Rv0020c</b>	20.42	21.05	21.45	21.06
<b>Rv1449c</b>	21.85	22.29	22.97	22.2
<b>Rv1611</b>	20.57	22.03	22.09	21.35
<b>Rv1792</b>	19.16	19.37	19.81	19.33
<b>Rv0640</b>	20.24	20.9	21.44	20.75
<b>Rv0704</b>	21.62	21.96	22.66	22.14
<b>Rv0718</b>	19	19.76	20.02	19.49
<b>Rv1080c</b>	18.98	20.78	20.86	20.22
<b>Rv2890c</b>	19.55	20.47	20.78	20.11
<b>mean rRNA</b>	<b>25.64</b>	<b>9.54</b>	<b>9.83</b>	<b>9.48</b>
Mean Ct, 10 genes	20.15	20.96	21.34	20.74

**Table 1. Checking gene expression level of 11 genes in total H37Rv RNA preps from the in vitro culture before and after removal of rRNA with ribo Zero Kit via qRT-PCR (TaqMan). There was a 15 Ct drop in ribosomal RNA species after the procedure (could be more than 10,000.00 xfold reduction).**

Adapted from Epicentre Biotechnologies Ribo-zero Removal Kit (Gram-Positive Bacteria) manual <http://www.epibio.com/item.asp?id=580>

Epicentre's Ribo-zero Removal Kit will remove the 23S, 16S, and 5S rRNA from up to 5 µg of total RNA. Kits are available for various samples including gram-negative bacteria, gram-positive bacteria, and human/mouse/rat RNA preparations.

## 1. Total RNA Preparation

- 1.1 Total RNA is prepared according to standard operating procedures.
- 1.2 Qiagen RNeasy miniprep kits are used to purify total RNA. For use with the Ribo-zero rRNA removal kit, total RNA can either be dissolved in RNase-free water or TE buffer.
- 1.3 Table 1 – Volumes of Ribo-Zero Ribosomal RNA Removal Solution  
Reference: Ribo-zero rRNA Removal Kit (Gram-Positive Bacteria) manual

Amount of Input Total RNA	Maximum Volume of Total RNA that can be Added to Each Reaction	Volume of Ribo-zero rRNA removal Solution per Reaction
1 – 2.5 µg	28 µl	8 µl
> 2.5 – 5 µg	26 µl	10 µl

## 2. Ribo-zero Microspheres Preparation

- 2.1 Ribo-zero microspheres are stored at 4°C and warmed to room temperature prior to use.
- 2.2 Vigorously vortex the microspheres into a homogeneous slurry before dispensing into the provided 2-ml Wash Tubes.
- 2.3 For each reaction, pipette 65 µl of microspheres into a separate 2-ml Wash Tube. *It is important that microspheres are prepared separately for each RNA sample. Do not batch-wash microspheres for multiple samples.*
- 2.4 Centrifuge dispensed microspheres at 12,000 x g for 3 minutes. Carefully pipette off and discard the supernatant, without disturbing the microsphere pellet.
- 2.5 Wash microspheres by adding 130 µl of Microsphere Wash Solution to each tube. Mix by vortexing for 10 seconds. Centrifuge tube(s) at 12,000 x g for 3 minutes, and carefully pipette off and discard the supernatant.
- 2.6 Resuspend microspheres in 65 µl of Microsphere Resuspension Solution. Vortex for 10 seconds.
- 2.7 Add 1 µl of Riboguard RNase Inhibitor to each tube. Vortex briefly and store washed microspheres at room temperature for use in Step 5.

## 3. Hybridization of Total RNA to Ribo-zero rRNA Removal Solution

- 3.1 Prior to starting step 3, prepare a 50°C water bath for 2-ml tubes.
- 3.2 In a new tube, set-up the following reaction:
  - x µl RNase-free water
  - 4 µl Ribo-zero reaction buffer
  - 1-5 µg Total RNA (see Table 1 above)
  - x µl Ribo-zero rRNA removal solution (see Table 1 above)
Total reaction volume: 40 µl

- 3.3 Mix reaction then transfer the 40 µl mix into 2 Micro-Amp Fast Reaction PCR tubes (20 µl aliquots per sample). This step is necessary in order to use the

Applied Biosystems Fast Thermal Cycler, where the maximum reaction volume per tube is 30  $\mu$ l.

- 3.4 Incubate samples at 68°C for 10 minutes.
- 3.5 Remove the samples from the thermal cycler and incubate them at room temperature for 15 minutes.

#### 4. Binding of rRNA to Microspheres

- 4.1 Briefly mix by vortexing the washed microspheres in step 2 above. It is important to have a homogenous slurry before adding the RNA mix from step 3.
- 4.2 Using a pipet, add the hybridized RNA mix to the washed microspheres. Without changing the pipet tip, mix the RNA with the microspheres by rapidly pipetting the contents 15 times. Continue to the next sample.  
*Note: Always add the RNA to the microspheres and not the other way around!*
- 4.3 Incubate the tubes at room temperature for 10 minutes. Gently vortex mix the samples for 5 seconds every 3 minutes.
- 4.4 Briefly vortex the samples before placing them in the 50°C water bath. Incubate samples for 10 minutes.
- 4.5 Transfer the RNA-microsphere suspension to a provided Microsphere Removal Unit and centrifuge at 12,000 x g for 1 minute.  
*Note: **the eluate contains the rRNA-depleted sample** while the rRNA-microsphere complex remains on the filtration unit.*

#### 5. Purification of the rRNA-depleted Sample

- 5.1 Epicentre recommends two methods for purification of the rRNA-depleted from step 4 above. Purification can either be through an ethanol-precipitation procedure or through a commercial column-purification kit. We have chosen to use the Epicentre recommended Zymo Research RNA Clean & Concentrator-5 Column Kit.
- 5.2 Zymo Research Concentrator-5 Column Purification kit  
Reference: Zymo RNA Clean & Concentrator-5 manual for retention of RNAs > 17 nucleotides.
  - 5.2.1 Total volume of eluate is ~ 95  $\mu$ l.
  - 5.2.2 Add 2 volumes of the provided RNA Binding Buffer to each volume of eluate. i.e. 185  $\mu$ l RNA Binding Buffer added. Mix well.
  - 5.2.3 Add 1 volume of 100% ethanol to the mix. i.e. 280  $\mu$ l of 100% ethanol. Mix well.

- 5.2.4 Transfer the mixture to the Zymo-spin column.
- 5.2.5 Centrifuge at 12,000 x g for 1 minute.
- 5.2.6 Discard the flow-through. Add 400 µl of the RNA Prep Buffer to each column.
- 5.2.7 Centrifuge at 12,000 x g for 1 minute.
- 5.2.8 Discard the flow-through. Add 800 µl of the RNA Wash Buffer to each column.
- 5.2.9 Centrifuge at 12,000 x g for 1 minute.
- 5.2.10 Discard the flow-through. Add an additional 400 µl of the RNA Wash Buffer to the column.
- 5.2.11 Centrifuge at 12,000 x g for 1 minute.
- 5.2.12 Transfer the Zymo-spin column to a new waste tube.
- 5.2.13 Centrifuge at 12,000 x g for 2 minutes to dry the column.
- 5.2.14 Transfer the Zymo-spin column to a new collection tube.
- 5.2.15 Add **13 µl** RNase-free water to the center of the column. Let sit for 2 minutes.
- 5.2.16 Centrifuge at 10,000 x g for 1 minute.
- 5.2.17 Discard the Zymo spin-column. Sample tube(s) containing the purified rRNA-depleted RNA sample can be stored at -70°C or continue with for RNA-seq preparation.

## 6. Assessing the Yield and Quality of the rRNA-depleted Sample

- 6.1 Use a NanoDrop spectrophotometer to measure the concentration for each sample. Alternatively, an Agilent 2100 Bioanalyzer can be used.
- 6.2 Before proceeding with RNA-seq preparation, the samples were run on RT-PCR to qualitatively determine the rRNA removal efficiency.
- 6.3
  1. Pepke, S., B. Wold, and A. Mortazavi, *Computation for ChIP-seq and RNA-seq studies*. Nat Methods, 2009. **6**(11 Suppl): p. S22-32.
  2. Mortazavi, A., et al., *Mapping and quantifying mammalian transcriptomes by RNA-Seq*. Nat Methods, 2008. **5**(7): p. 621-8.