**miRNA Library preparation**

Total RNA was isolated from whole cells or from exosome pellets using the Ambion mirVana miRNA Isolation Kit (cat# AM1560). The instructions were followed for total RNA isolation from cell pellets. RNA concentration was measured using the Nanodrop spectrophotometer. At least 30ug protein (70ug was a lot better though) of exosome is needed to produce enough RNA for a good quality library.

Approximately 10ug total RNA was used to prepare the miRNA library for whole cell library. An entire prep of RNA from 30-70ug protein exosomes was used to prepare the exosome miRNA library. RNA was then DNaseI treated before proceeding with library preparation. (see p.97 book 3 for DNaseI reactions).

The protocol requires a starting volume of 6ul RNA. If necessary, RNA was concentrated in a speedivac until the volume was reduced. From 50ul of RNA, it would take 30-50min to concentrate to 5-6ul.

Ligation of the various adapters was achieved by following the protocol for the NEBNext Multiplex Small RNA Library Prep Set for Illumina (1-12) (Cat# E7300S) pages 4-7. After PCR amplification, products were purified with the High Pure PCR product purification kit, eluted in 25ul RNase free water and stored at -20⁰C until the next day.

The product then needs to be purified on a PAGE gel. 6% acrylamide with TAE:

1.8ml acrylamide (40%)

7.59ml dH2O

2.4ml 5x TAE

200ul 10% APS

10ul TEMED

Pour a 1mm gel – may need to use bulldog clips to make sure comb is tightly held between glass plates otherwise the wells don’t form cleanly.

Load gel as described in the manual – page 8. Once the gel has run, post stain in 50ml 1xTAE with 0.5ul ethidium bromide for 2-3min.

Cut out the two bands at 160 and 147 as they are too close to just get the 147bp one (the one we’re interested in). Crush the gel slices with a yellow tip – breaks up easily. After 2 hrs incubation in elution buffer, the mixture can be spun straight through a high pure column (keep flow through!) – flow through can be processed as described in the manual (step 11 onwards).

Once the library is ready, 1ul can be run on the bioanalyser (DNA 1000 chip) to check the concentration and size of the library you’ve isolated. (the High sensitivity DNA chip can also be used, just dilute your library 1:10 before supplying the 1ul sample). At the very least, 10ng of library product is required for sequencing. (Deb’s lab book – starts page 97 book 3)

Methods - summary

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Ligation of the various adapters was achieved by following the protocol for the NEBNext Multiplex Small RNA Library Prep Set for Illumina (1-12) (Cat# E7300S) pages 4-7. After PCR amplification, products were purified with the High Pure PCR product purification kit (Roche, Cat# 11732668001), eluted in 25ul RNase free water and stored at -20⁰C until the next day.

The product was then purified on a 6% TAE PAGE gel. Run at 100-120V for 40-60min, not letting dye front run off the end. Post stain in ethidium bromide/TAE before cutting out the 140-160bp bands, purifying and precipitating as per NEBNext kit manual. Evaluate total ng of library collected using a DNA 1000 chip or a high sensitivity chip on the bioanalyser before submission for sequencing. At least 10ng in total for each sample is required for sequencing.