Development of an Identity Assay for In Process and Release testing of Exosomes derived from proliferating and differentiating CTX cells

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Background

Stem-cell derived **exosomes** are **a rapidly emerging therapeutic field**, demonstrating preclinical efficacy in a wide range of applications from wound repair to immune modulation and oncology. ReNeuron's allogeneic human neural CTX stem cell line has previously demonstrated therapeutic relevance in a Phase II efficacy study in patients with stable paresis of the arm following an ischemic stroke. **CTX cells are highly efficient producers of exosomes** and because they are a clonal population they have the potential of producing homogenous exosome populations **at a scale essential for clinical translation**. However, the challenge is how to characterise the exosomes to ensure consistency and control during manufacture.

This study demonstrates an approach to optimise an In Process and Release RT-qPCR assay for miRNA targets found **in exosomes** produced by proliferating and differentiating CTX cells. The miRNA targets were identified by Next Generation Sequencing. The characterisation of both these exosome populations will support future development of therapeutic products with efficacy across multiple disease indications.

Identification of miRNA targets in exosomes

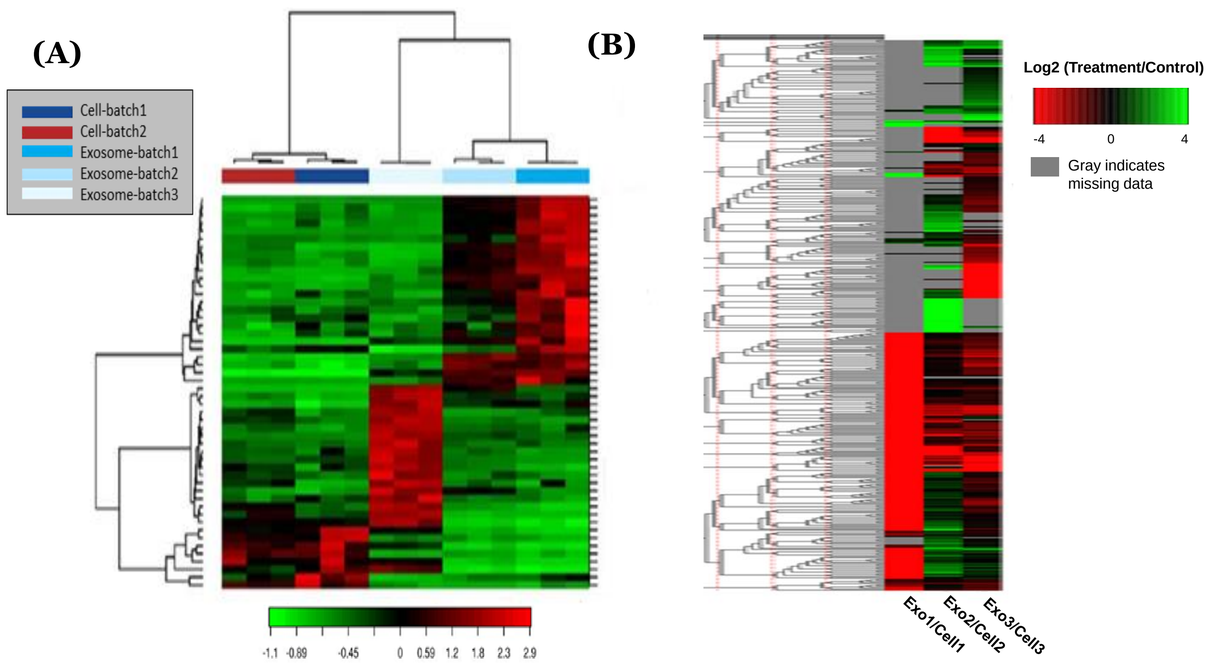


Figure 1. Heat maps generated from miRNA NGS analysis on exosomes derived from proliferating and differentiating CTX cells. Unsupervised hierarchical clustering was performed on exosomes derived from proliferating CTX cells and on the top 50 microRNAs with highest CV based on TMM normalized reads **(A)**. Fold change in miRNA reads in differentiated CTX exosomes compared to differentiated CTX cells **(B)**.

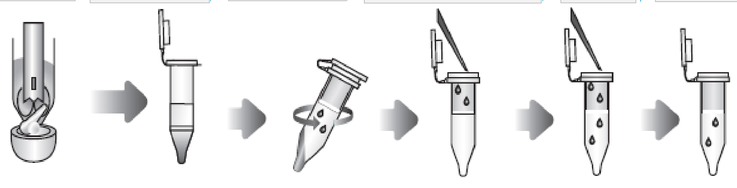
Target	NGS sample	Batch 1 (TPM)	Batch 2 (TPM)	Batch 3 (TPM)
hsa-miR-1	ExoPro	1.45E+02	7.87E+02	1.07E+03
	ExoDiff	2.25E+06	2.87E+05	1.96E+06
hsa-miR-2	ExoPro	1.58E+02	3.98E+02	3.01E+02
	ExoDiff	7.01E+09	1.36E+08	8.28E+07
hsa-miR-3	ExoPro	1.31E+02	2.42E+02	1.18E+02
	ExoDiff	3.63E+06	2.33E+06	3.92E+06
hsa-miR-4	ExoPro	2.09E+03	1.90E+03	2.96E+02
	ExoDiff	1.00E+06	6.36E+05	8.45E+06
hsa-miR-5	ExoPro	1.76E+04	2.75E+04	4.37E+03
	ExoDiff	2.75E+06	5.97E+06	3.49E+06
hsa-miR-6	ExoPro	1.52E+04	2.04E+04	4.15E+04
	ExoDiff	1.52E+09	1.41E+07	1.22E+07

Table 1. Identification of miRNA targets. miRNA targets were selected by differential TPM (transcripts per million) read. Namely low TPM was selected in exosomes derived from proliferating CTX cells and high TPM reads in exosomes derived from differentiated CTX cells.

Optimisation of miRNA extraction

mirVana™ miRNA Isolation (Ambion)

Lysis & Disruption → Organic Extraction → Removal of large RNA → Immobilisation of small RNA to filter → Wash → Elute Small RNAs (<200 nt)



Direct-zol™RNA MiniPrep (Zymo Research)

Trizol-Add binding agent → Bind RNA directly to a spin-column → Elute RNA

Extraction Method	(ng/µl)	Total RNA (ng)	260/280	260/230
mirVana™ kit	1.20	42	2.26	0.30
Direct-zol™ kit	40.5	2.025	1.62	0.73

Table 2.Quantity & Quality of RNA. A 48-fold increase in total RNA quantity was obtained when using the Direct-zol™ extraction method compared to the mirVana ™ extraction method

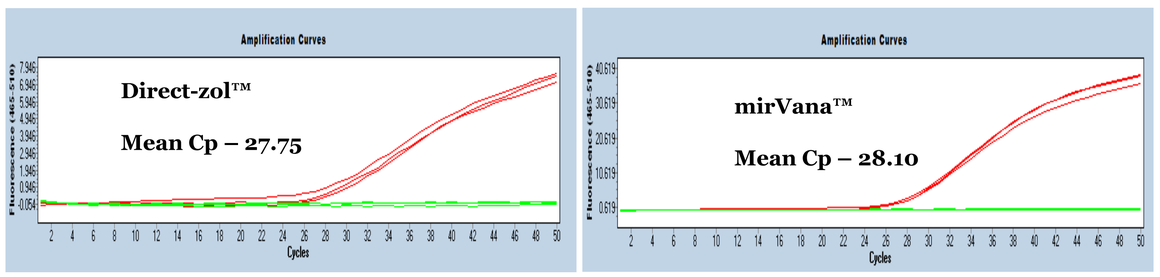


Figure 2. Comparison of miRNA detection in exosomes using mirVana™ versus Direct-zol™ extraction methods. miRNA RT-qPCR was performed on exosome RNA extracted using both methods. Comparable Cp values were obtained for a reference hsa-miR indicating that miRNA target quantification was consistent across the methods.

miRNA target detection - TaqMan Advanced™

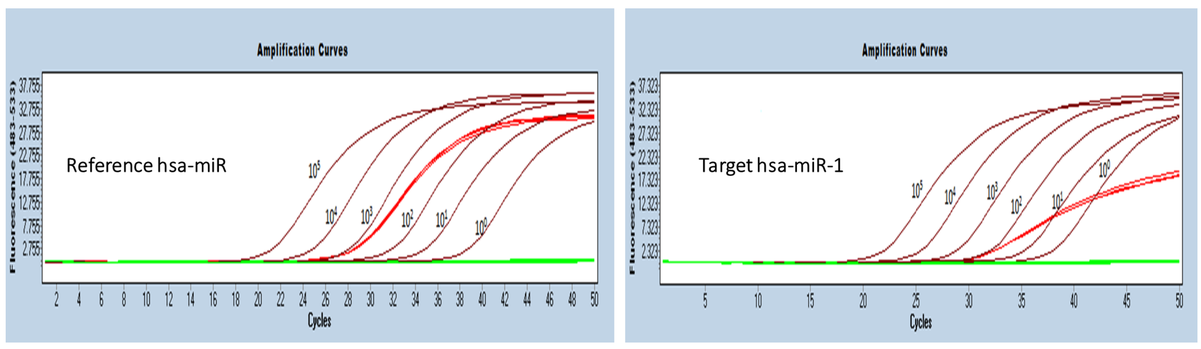


Figure 3. Detection of reference hsa-miR & hsa-miR-1 in exosomes derived from proliferating CTX cells. hsa-miR-1 amplification curves display a lower plateau phase than the reference hsa-miR. The hsa-miR-1 probe has sequence homology to a snRNA. The lower plateau phase suggests that the probe may be competitively binding to the snRNA sequence. The TaqMan Advanced™ miRNA RT-qPCR method is not specific in detecting certain miRNA targets.

miRCURY LNA™ – An alternative miRNA detection method

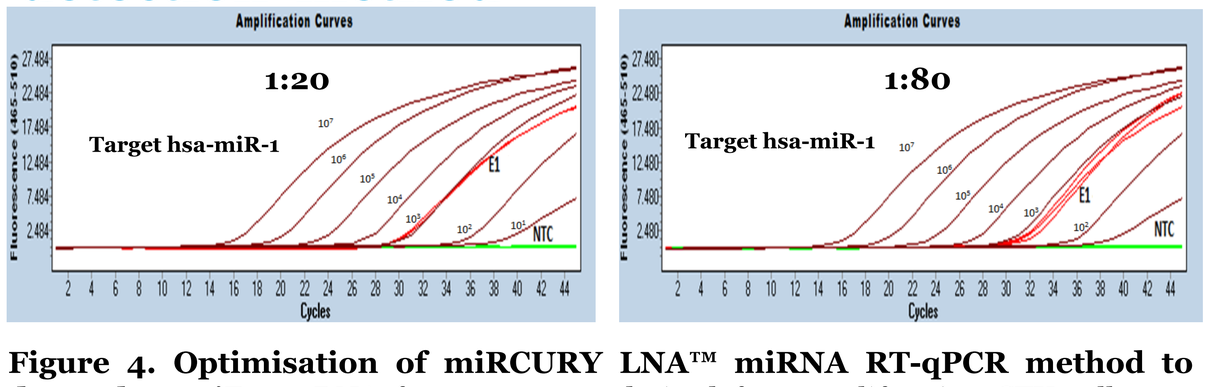


Figure 4. Optimisation of miRCURY LNA™ miRNA RT-qPCR method to detect hsa-miR-1. cDNA from exosomes derived from proliferating CTX cells was diluted 1:20 and 1:80 prior to RT-qPCR. hsa-miR-1 was detected at 4.4 x 10^2 copies in the 1:20 diluted exosome cDNA and 1.1 x 10^2 copies in the 1:80 diluted exosome cDNA.

miRNA detection in differentiating Exosomes

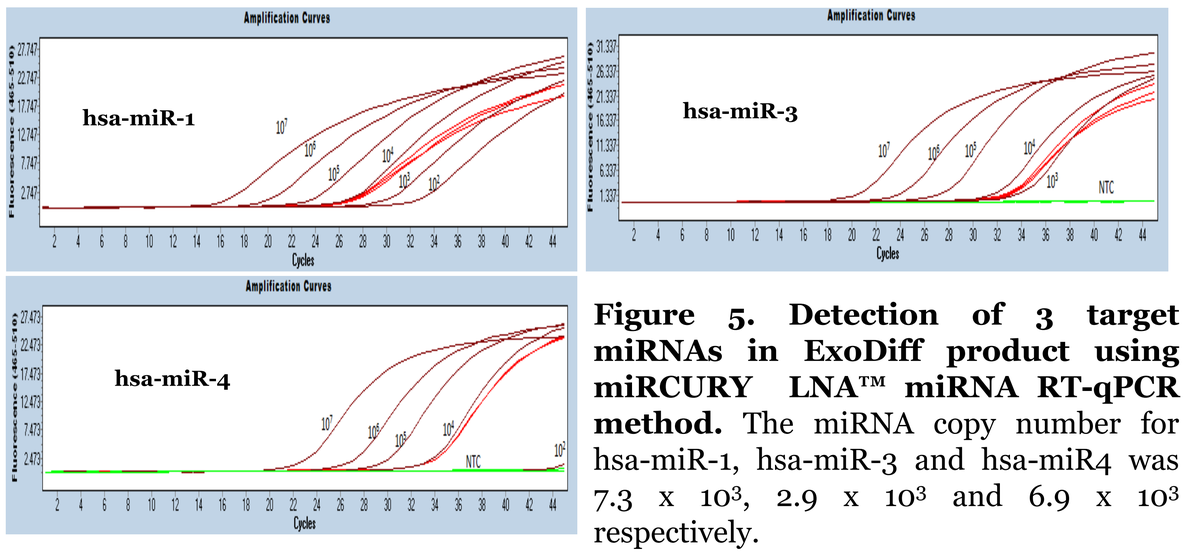


Figure 5. Detection of 3 target miRNAs in ExoDiff product using miRCURY LNA™ miRNA RT-qPCR method. The miRNA copy number for hsa-miR-1, hsa-miR-3 and hsa-miR4 was 7.3 x 10^3, 2.9 x 10^3 and 6.9 x 10^3 respectively.

Conclusion & Significance

Stem cell derived exosomes can exert biological function and relevance in therapeutic treatments. Characterisation of exosome populations however, still remains a challenge. Here **we presented our approach for development of an RT-qPCR assay which can be applied during in process and release testing of exosomes.** We have demonstrated the importance of chemistry and extraction method selection for detection of targets of interest. **Outcomes of this work lead to a fully validated Identity assay which will support drug product characterisation**.

We work with Innovate UK

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