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AUTHOR(S)

Victoria G Lyons, Natalie L Payne, Claire E McCoy

CITATION

Lyons, Victoria G; Payne, Natalie L; McCoy, Claire E (2018): Optimization Techniques for miRNA Expression in Low Frequency Immune Cell Populations.. Royal College of Surgeons in Ireland. Journal contribution. https://hdl.handle.net/10779/rcsi.10781804.v1

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Optimisation techniques for miRNA expression in low frequency immune cell populations

[1,2]Victoria G. Lyons, [3]Natalie L Payne and [2,4]Claire E. McCoy[*]

[1]School of Biochemistry and Immunology, Trinity Biomedical Sciences Institute, Trinity College Dublin, Dublin 2, Ireland.

[2]Hudson Institute of Medical Research, Department of Molecular and Translational Science, Monash University Faculty of Medicine, Nursing and Health Sciences, Clayton, VIC 3168, Australia.

[3]Australian Regenerative Medicine Institute, Monash University Clayton, VIC 3800, Australia.

[4]Molecular and Cellular Therapeutics, Royal College of Surgeons in Ireland, 123 St Stephen's Green, Dublin 2, Ireland.

[*]Corresponding author: clairemccoy@rcsi.ie

Running Title: miRNA Expression in Immune Cells

i. Summary

In this chapter we outline a RNA extraction method for very low immune cell populations isolated from the central nervous system of mice undergoing experimental autoimmune encephalomyelitis. We compare various normalisation and quantification techniques to examine miRNA expression analysis. Our data highlight that employing a mean normalisation procedure using a number of well-selected housekeeping miRNA genes. Followed by absolute quantification with a standard curve generated from a commercial miRNA oligo gave the most robust and reproducible miRNA expression results.

ii. Key Words

microRNA, experimental autoimmune encephalomyelitis, immune cells, RT-PCR, relative quantification, absolute quantification, mean normalisation, standard curve.

1. Introduction

microRNAs (miRNA) are small nucleotide (18-25 nt in length) non-coding RNA molecules that post-transcriptionally regulate coding mRNA molecules. They play an important role in almost all cellular functions to regulate differentiation, proliferation, activation status and effector functions in any given cell type. Due to their involvement in these processes, it is no surprise that their dysresgulation underpins various diseases (1). Thus, understanding how to reliably measure and quantify miRNAs has immense diagnostic potential, whilst monitoring the changes in expression in models of disease provides insight into complex regulatory networks and the identification of novel biological pathways that play a role in disease pathogenesis. In 2005, a successful real-time RT-PCR quantification technology was developed to detect miRNA molecules in cells and tissues (2). The technique involves a two-step process and remains the most widely used method for fast, accurate and sensitive miRNA measurement. The first step involves a stem-loop RT primer that hybridizes to the miRNA and is reverse transcribed using a Multi-Scribe reverse transcriptase. The second step measures the RT products using a conventional TaqMan PCR (2,3).

Although TaqMan RT-PCR has become the gold standard for miRNA detection in cells and tissues, there are numerous pre-requisite considerations. The first consideration is deciding on the method of RNA extraction. Many effective commercial kits and reagents are available for tissues and/or cells (Qiagen miRNeasy kit, Ambion miRVana PARIS, Analytik Jena kit, Trizol reagent). We have extensively illustrated robust miRNA analysis in RNA extracted from both primary macrophages and macrophage cell lines using a modified protocol from the Qiagen RNeasy kit (4). This method is ideal for extracting RNA when the starting material has a typical range of $1 \times 10^5 - 1 \times 10^6$ cells per sample. However this method was no longer appropriate when we performed experiments in vivo, with a need to detect miRNA

expression in immune cells with low numbers ranging from 1 x 10^2 – 1 x 10^4. Thus finding an alternative RNA extraction method became paramount to our studies. We needed to detect miR-155 expression in leukocytes isolated from the central nervous system (CNS) of mice undergoing experimental autoimmune encephalomyelitis (EAE). The CNS is a tissue with notoriously low immune cell numbers, particularly in naïve non-treated mice that are required for comparative analysis. Not only did we need to measure miR-155 in total CNS-isolated leukocytes but we wanted to measure miR-155 expression levels in sub-divided immune cell populations namely macrophages, T and B cells. We compared five methods of RNA extraction in FACS sorted immune cell populations from CNS-isolated leukocytes as follows: (i) Qiagen miRNeasy kit, (ii) modified Qiagen RNeasy kit (4), (iii) Analytik Jena kit, (iv) Trizol reagent and (v) Trizol reagent + carrier protein glycogen. Consistently in our hands, Trizol reagent alone extracted as low as 100 cells (1 x 10^2) cells, giving ranges of 20 – 200 ng/μl of RNA and robust detection of miR-155.

The second consideration is the normalization method. Traditionally, changes in miRNA expression is determined by normalizing Ct values to an internal control, often called a 'housekeeping gene' or in our case a 'housekeeping miRNA' (5). This miRNA should not vary in the tissues or cells under investigation, nor in response to the experimental treatment. Investigators often struggle to determine which housekeeping gene is most appropriate for their experiment in question, due to the divergence of choices that are available. For example, we ourselves have used a range of different housekeeping miRNAs such as RNU6B, snoRNA202, miR-191 and miR-16 depending on the cell type in question and the type of experiment performed (6-8). We have realized that although certain housekeeping miRNAs may be occasionally constant in one particular immune cell type or experimental condition, it may vary considerably in another type of immune cell. So we decided to

investigate methods to eliminate these discrepancies by comparing our data when normalizing to the average of three housekeeping genes (RNU6B, snoRNA202, miR-191) rather than the traditional use of just one (*see* **Figure 1**). We found that by averaging three housekeeping genes we got more consistent and reliable results between our experiments. This is not a surprise and in fact, this method of taking the average of multiple carefully selected housekeeping genes is validated as an accurate normalisation method (9,10). However, this method is often not sustainable, particularly when samples are precious and cost-burdens are a factor. Yet, by utilizing this method in our preliminary experiments, it gave us the confidence moving forward to choose just one housekeeping miRNA that could reliably reproduce the data obtained when three housekeeping genes were used.

Another important consideration is quantification methods. There are two methods; relative and absolute. Relative quantification describes the change in expression of the target gene (in our case, miR-155) relative to a reference group, often an untreated group or sample at time zero. This method is useful for determining how a given treatment increases the expression of a certain miRNA, where the data is represented as fold-change over non-treated control. The ΔΔCt calculation method is a convenient way to assess the relative expression of miRNAs from RT-PCR experiments (5). Although this method is popular, it does have drawbacks. The experiment must contain non-treated samples, the PCR efficiency of your target miRNA and housekeeping miRNA must be approximately equal, lastly it cannot accurately determine actual transcript copy numbers in your samples.

In contrast, absolute quantification determines the absolute copy number of the miRNA of interest, by relating the RT-PCR data to a standard curve with known amounts. This method is important for quantifying the actual amount of transcript between different cell types and

tissues for example. Using miR-155 as an example, we generated a standard curve using a commercially available miR-155 oligo. Graphing log concentration against Ct generates a slope where we can obtain an equation of the line. Using this equation, we can calculate the concentration of miR-155 in our samples. It also enables the calculation of samples irrespective of a non-treated reference sample that is particularly useful. In addition, we utilized a median normalization method, a method that has been increasing in popularity due to its success in reproducibility (11,12). This method removes the doubt that housekeeping genes themselves can be differentially expressed in experimental conditions, and instead relies on normalisation by calculating the mean Ct value from all the housekeeping miRNAs. This method is even more robust if three housekeeping genes have been used in the experimental design.

In summary, we outline relative and absolute quantification methods for determining miRNA expression in low number immune cells. The relative method is quick and fantastic for *in vitro* analysis when testing agonists and/or drug responses for example. However, we believe that the absolute quantification method is superior for *in vivo* analysis where the use of a standard curve and the mean normalisation technique ensures accuracy, consistency and reproducibility.

2. Materials

2.1. EAE Induction

1. 9-10 week old female C57BL/6J mice

2. Myelin oligodendrocyte glycoprotein (MOG) 35-55 peptide (lyophilized). Reconstitute lyophilized MOG35-55 peptide in sterile PBS to a concentration of 20mg/ml. Aliquot into sterile eppendorf tubes and store at -80°C.

3. Complete Freund's adjuvant (CFA).

4. *Mycobacterium tuberculosis* H37Ra (killed and desiccated). Reconstitute *M. tuberculosis* in 1ml sterile water to a concentration of 100mg/ml. Store at 4°C for up to 1 month.

5. Pertussis toxin from *Bordetella Pertussis* (lyophilized)

6. Sterile Phosphate buffered saline, no calcium or magnesium (PBS)

7. Sterile water

8. Glass Hamilton syringes (1 x 1ml, 2 x 10ml)

9. Three-way stopcock

10. 1ml syringe

11. 25-G needle

12. Insulin syringe

13. Sterile tubes- eppendorf, 50ml.

2.2. CNS Dissection

1. CO_2 induction chamber

2. Surgical instruments: straight dissecting scissors, small blunt curved serrated forceps, hemostats

3. 25G butterfly needle

4. 18G needle

5. 50ml syringe

6. 10ml syringe

7. PBS

8. 60mm petri dish

9. DMEM with high glucose

10. 80% ethanol

2.3. Leukocyte Isolation

1. 6 cm^2 tissue culture treated petri dishes to place CNS after dissection.
2. Sterile stainless steel scalpel blade.
3. Sterile Pasteur pipette.
4. Sterile 70 µm filters.
5. Sterile 15 ml and 50 ml Falcon tubes.
6. Complete Dulbecco's Modified Eagle Medium (DMEM): 500 ml DMEM supplemented with 10% Fetal Calf Serum (FCS) and 1% penicillin/streptomycin.
7. Percoll. diluted to stocks of 70% and 30% with PBS.
8. Trypan Blue to determine cell viability at a ratio of 1:1.
9. Dulbecco's Phosphate Buffered Saline (*PBS*).
10. Digestion buffer: 25 ng/ml DNase, 1 mg/ml Collagenase D in PBS. Digestion buffer needs to be as fresh as possible and prepared within 6 hours of carrying out experiment.
11. FACS buffer consisting of PBS, 5-10% FBS, 1 Mm Ethylenediaminetetraacetic acid (EDTA) and 0.1% NaN3 sodium azide.
12. Neubauer Haemocytometer.

2.4. Fluorescence activated cell sorting (FACS)

1. Corning™ Falcon™ Round-Bottom Polystyrene Tubes.
2. FACS buffer: 5-10% FBS, 1 Mm Ethylenediaminetetraacetic acid (EDTA), 0.1% sodium azide (NaN_3) in PBS.
3. CD16/CD32 (Fc antibody), freshly prepared at 1:150 in FACS buffer.
4. Compatible flow cytometry cell surface markers as highlighted in **Table 1**. Make a master mix by diluting all antibodies (1:200) in 100 µl FACS buffer per sample. Generate single stains for each antibody by diluting (1:500) in 50 µl FACS buffer.
5. BD Biosciences FACS CANTO II flow cytometry machine for sample acquisition and analysis.
6. Cell sorting machine; Beckman Coulter MoFlo XDP.
7. FlowJo Vx 10.0 software for phenotypic analysis.

2.5. RNA Extraction

1. 1.5 ml Eppendorf tubes.
2. TRIzol Reagent (1 ml/sample).
3. Chloroform (200 µl/sample).
4. Isopropanol (500 µl/ sample).
5. 80% Ethanol (1 ml/ sample).
6. DNase and RNase free H_2O (30 µl/ sample).
7. Fume hood (TRIzol contains phenol).
8. Heating block (set at 55-60°C).

2.6. RT-PCR

1. TaqMan® MicroRNA Reverse Transcription Kit (Applied Biosystems).

2. TaqMan MiRNA Assay (Applied Biosystems). Two components comprised within the assay: 5X primer and 20X FAM-labelled probe per specific miRNA.
3. SensiFAST™ SYBR® Hi-ROX
4. miR-155 synthetic oligonucleotide with ZEN modifications (5'- rUrUrA rArUrG rCrUrA rArUrU rGrUrG rArUrA GrGrG rGrU - 3') (IDT technologies). Reconstitute with DNA/RNase free H_2O to a 100 nM stock according to the material safety data sheet. Generate multiple smaller aliquots at 1nM and store at -80°C.
5. 1.5 Eppendorf tubes.
6. 8-Strip PCR tube caps (0.2 mL) with flat writing surface.
7. MicroAMP™ Optical 384-well or 96-well reaction plate (Applied Biosystems).
8. MicroAMP™ Optical Adhesive Film (Applied Biosystems).
9. 7900HT RT-PCR System with 384-well or 96-well block (Applied Biosystems).

2.7. Data Analysis

1. SDS v2.2 software (Applied Biosystems)

3. Methods

3.1. EAE Induction

EAE is induced by subcutaneous injection of 100µg of the encephalitogenic peptide MOG35-55 (also called antigen) in CFA supplemented with *M. tuberculosis* on day 0. An emulsion of MOG35-55 and CFA is prepared at a 1:1 ratio and each mouse receives two injections of 100µl (total of 200µl per mouse). 200ng of pertussis toxin is administered intraperitoneally in a volume of 200µl on day 0 and again on day 2 (*see* **Note 1**).

1. Dilute the 20mg/ml stock of MOG35-55 1:20 with sterile PBS to achieve a working concentration of 1mg/ml.
2. Thoroughly vortex the 100mg/ml stock of *M. tuberculosis* (from step 4) and add 400µl to a 10ml vial of CFA (*see* **Note 2**). This can be stored at 4°C for up to 1 month.
3. Calculate the volume of CFA/antigen emulsion required for immunization by multiplying the number of mice to be immunized by 200µl and then multiplying by 1.5 (*see* **Note 3**). Divide this number by half to give the volume of CFA and the volume of MOG35-55 required. For example: 10 mice x 200µl per mouse = 2000µl x 1.5 = 3000µl total volume required / 2 = 1500µl CFA + 1500µl MOG35-55.
4. To prepare the emulsion of CFA/antigen, connect 2x10ml glass Hamilton syringes (*see* **Note 4**) to a three-way stopcock and remove the plunger from syringe-1. Secure syringe-2 with tape so that syringe-1 remains upright.
5. Thoroughly vortex CFA prepared in step 2. To syringe-1, add equal volumes of CFA and MOG35-55 working solution (prepared in step 1) according to the volume calculated in step 3.
6. Slowly draw back the plunger of syringe-2 until the entire contents have been drawn into the syringe. Disconnect syringe-2 from the stopcock and expel any air bubbles within the

syringe (*see* **Note 5**). Replace the plunger in syringe-1 to the 0ml mark and then reconnect syringe-2 to the stopcock.

7. Thoroughly mix the contents between the two syringes by alternatively pressing down on the plungers to create an emulsion. Place on ice until required (*see* **Note 6**).
8. Calculate the total volume of Pertussis toxin required by multiplying the number of mice to be immunized by 200µl and then multiply by 1.2 (to account for loss during loading of syringes).
9. Reconstitute lyophilized Pertussis toxin to a concentration of 100µg/ml by slowly injecting sterile PBS through the lid with an insulin syringe. Mix gently and allow to dissolve for 5 minutes. This can be stored at 4°C for up to 1 month.
10. Dilute the 100ug/ml stock of Pertussis toxin (from step 11) 1:100 with sterile PBS in a 50ml tube to achieve a working concentration of 1µg/ml. Place on ice.
11. Immediately prior to immunization, place Pertussis toxin at room temperature and thoroughly mix the CFA/antigen emulsion between the two syringes.
12. Expel the entire emulsion into syringe-1, disconnect syringe-2 and connect a 1ml glass Hamilton syringe. Slowly load the 1ml syringe with the emulsion to the 1.2ml mark (*see* **Note 7**).
13. Disconnect the 1ml syringe, reconnect syringe-2 and place the emulsion on ice.
14. Attach a 25G needle to the 1ml syringe, tap gently and expel the emulsion to the 1ml mark to release any air bubbles.
15. Using the 1ml syringe, inject each mouse with 100µl of the CFA/antigen emulsion into each hind limb flank (total of 200µl per mouse) (*see* **Note 8**).
16. Inject each mouse with 200µl of Pertussis toxin (prepared in step 12) intraperitoneally. Repeat 48 hours later.

17. Monitor mice daily for clinical signs of disease from day 8 post immunization (*see* **Note 9**). Clinical scores are assigned according to the scale in **Table 2**.

3.2. CNS Dissection

The below protocol details dissection of the brain and spinal cord separately. Brain and spinal tissue can be processed together for isolation of leukocytes from the entire CNS, separately or if specific regions of the brain or spinal cord need to be examined.

1. Euthanize the mouse by CO_2 asphyxiation, secure the limbs and spray with 80% ethanol.
2. Open the chest cavity to expose the heart (*see* **Note 10**).
3. Insert a 25G butterfly needle attached to a 50ml syringe pre-filled with PBS into the left ventricle of the heart and cut the right atrium of the heart with scissors.
4. Confirm correct placement of the needle be pushing down on the plunger slowly and observing for efflux of blood from the right atrium.
5. Perfuse the mouse with PBS until the blood runs clear and the liver has lost its red colour (*see* **Note 11**).
6. To remove the brain, secure the limbs and spray the head and back of the mouse with 80% ethanol.
7. Make an incision through the skin coronally between the eyes and longitudinally to the base of the tail.
8. Hold the head at the base of the skull and cut the skull between the two olfactory bulbs by placing one blade of the dissecting scissors into each eye cavity and cutting coronally.
9. Make a longitudinal cut through the skull along the sagittal suture (*see* **Note 12**). Expose the brain by applying gentle tangential, lateral pressure to tilt back the frontal and parietal bones on each side with either the blade of the scissors or a pair of curved forceps

10. Carefully slide forceps under the anterior part of the brain and tilt the brain upwards to cut the cranial nerves with scissors.
11. Hold the interparietal bone with forceps and cut on either side to expose the area where the brain and spinal cord meet.
12. Make a cut at the base of the brain where it meets the spinal cord and carefully remove the brain. Place on ice in a 60mm petri dish with DMEM.
13. To remove the spinal cord, peel back the skin to the base of the tail in order to expose the spinal column.
14. Cut the spinal cord where the spinal column attaches to the pelvis and then cut along the spinal column on each side in order to remove the column.
15. Insert an 18G needle attached to a 10ml syringe pre-filled with PBS into the spinal canal at the caudal end of the spinal column, past the point of resistance.
16. Push down on the plunger to flush the spinal cord into a 60mm dish containing DMEM with high glucose.

3.3. Leukocyte Isolation

1. Transfer the intact CNS to new 6 cm^2 petri dishes containing freshly prepared digestion buffer.
2. Thoroughly dice the CNS using a carbon steel surgical blade.
3. Incubate the diced CNS for 30 minutes at 37˚C.
4. Add 2 ml of PBS to each sample to terminate the enzymatic reaction.
5. Within a biological safety cabinet, use a Pasteur pipette to flush the digested CNS through a 70 µm filter into a 15 ml Falcon tube. Continue flushing the CNS with PBS until there is a final volume of 15 ml within the Falcon tube.
6. Centrifuge samples at 4˚C for 5 minutes at 2,000 RPM.

7. Decant the supernatant and add 8 ml of 30% Percoll to resuspend the cell pellet.
8. Gently layer the cell suspension at a 45° angle onto the surface of 3 ml of 70% Percoll within a 15 ml Falcon tube.
9. Centrifuge the layered suspension at room temperature for 25 minutes at 2,000 RPM with no brake activation (*see* **Note 13**).
10. A white buffy coat layer will be visible between the will between the 30% and 70% percoll gradients. This layer contains CNS leukocytes. Using a Pasteur pipette, transfer the buffy coat to a clean 15 ml Falcon tube (*see* **Note 14**).
11. Add PBS to a final volume of 15 ml and centrifuge at 4˚C for 5 minutes at 1,500 RPM.
12. Re-suspend the pellet containing the CNS leukocytes in 1 ml of FACS buffer.
13. Count the cells using a Neubauer Haemocytometer in a dilution of 1:1 with Trypan Blue (*see* **Note 15**).

3.4. Fluorescence activated cell sorting (FACS)

1. Centrifuge the CNS leukocytes at 4˚C for 5 minutes at 1,200 RPM and re-suspend in 200 µl CD16/CD32 (1:150) for 30 minutes at 4°C (*see* **Note 16**).
2. Wash samples with 1 ml of FACS buffer. Remove 2 x 10^5 cells from each sample into a separate tube. These samples will be used for single stains (i.e. you will need 6 single stain samples if there are 6 antibodies in the master mix as highlighted in **Table 1**). The remaining cells in the original sample will be used for cell sorting.
3. Centrifuge all samples for 5 minutes at 4°C at 1,200 RPM.
4. Decant supernatants in one swift motion to reduce dead volume within the FACS tube.
5. Add 100 µl of antibody master mix to the cell sorting sample and 50 µl of single stain mix to the single stain samples (*see* **Note 17**).
6. Incubate samples for 30 minutes in the dark at 4°C.

7. Wash samples by pipetting 1 ml of FACS buffer into each FACS tube and centrifuging at 12,000 RPM for 5 minutes.
8. Decant supernatants and re-suspend cell pellet in 100-150 µl of FACS buffer.
9. Bring samples to the cell sorting machine on ice to prevent cell clumping and death. Most cell sorting machines i.e. FACS ARIA and MoFlo XDP Cell Sorters, can collect up to 4 cell populations in 4 separate fractions.
10. Determine the gating strategy to obtain the desired cell populations. Start by plotting a forward scatter (FS) and side scatter plot to determine live cells, then gate on $CD45^+$ cells to determine all leukocytes. $F4/80^+CD11b^+$ positive cells will represent macrophages. $CD3^+CD4^+$ positive cells will represent $CD4^+$ T cells and $B220^+$ will represent B cells.
11. Run each sample on the cell sorting machine until there is no sample remaining in the FACS tube.
12. Purified and enriched cell populations are collected into separate fractions within FACS tubes (*see* **Note 18**).
13. Purified populations should be placed on ice until downstream experiments are carried out i.e. RNA extraction.

3.5. RNA Extraction

The following RNA isolation protocol has been specifically modified to accommodate for low cell numbers using the chemical reagent TRIzol (*see* **Note 19**).

1. Lyse samples in 1 ml of TRIzol Reagent and incubate for 5 minutes at room temperature within Eppendorf tubes (*see* **Note 20**).
2. Add 200 µl of chloroform to samples and shake vigorously for 30 seconds to ensure complete mixing between cell lysates, TRIzol, and chloroform.
3. Incubate Eppendorf tubes for 10 minutes at room temperature.

4. Centrifuge cell lysates at 12,000 RPM for 15 minutes at 4°C.
5. After centrifugation samples are separated into 3 phases; an organic phase (containing proteins and lipids), an interphase (containing DNA), and **most importantly**; the desired upper aqueous phase (containing RNA).
6. Carefully pipette 400 µl of the aqueous phase into a clean Eppendorf tube containing 500 µl of Isopropanol. Samples can be placed at -20° C which has been suggested to increase the yield of RNA or you can proceed directly onto the next step.
7. Centrifuge Eppendorf tubes for 12,000 RPM for 10 minutes at 4°C and carefully decant supernatants (*see* **Note 21**).
8. Re-suspend the pellets in 1 ml of 80% Ethanol.
9. Centrifuge Eppendorf tubes at 7,500 RPM for 5 minutes at 4°C and decant supernatants.
10. Air dry samples by inverting Eppendorf tubes onto clean tissue and maintaining this inversion for 5-10 minutes.
11. Re-suspend samples in 30 µl of DNase and RNase free H_2O.
12. Place samples on a heat block set at 55-60°C.
13. Place samples immediately on ice and measure the RNA concentration by placing 1µl on a Nanodrop. Typical cell numbers and RNA concentrations from CNS-isolated immune cell populations in a WT naïve mouse are shown in **Table 3**. Store the RNA at -80°C indefinitely.

3.6. RT-PCR

3.6.1 Sample preparation

1. Prepare RNA at a stock concentration of 5-10 ng/µl for miRNA expression analysis using DNA/RNase free H_2O (*see* **Note 22**).
2. Thaw all reagents on ice from the TaqMan® MicroRNA Reverse Transcription Kit and the TaqMan® Primer from the TaqMan® MiRNA Assay Kit.

3. Briefly vortex and micro-centrifuge dNTPs, 10X buffer and TaqMan® MiRNA Primers.
4. In a microcentrifuge tube, prepare an RT reaction master mix as outlined in Table 4 comprising of: dNTP, 10X buffer, RNase inhibitor, RT enzyme and desired primers (*see* **Note 23**). It is essential that at least one housekeeping gene is included such as snoRNA202, RNU6B and/or miR-191.
5. Add 12 µl of RT reaction master mix into the correct number of thin wall 8-Strip PCR tubes (*see* **Note 24**).
6. Pipette 3 µl of each RNA sample to each labelled corresponding PCR tube and mix gently with a quick flick.
7. Microcentrifuge samples at a low speed for no longer than 5 seconds to ensure all liquid is gravitated down to the bottom of the PCR tubes.
8. Run the samples on a PCR machine using the following program: 16°C for 30 min, 42°C for 30 min, 85°C for 5 min and 15°C for ∞.
9. Samples may be kept overnight in a PCR machine at a holding temperature of 15°C, stored at 4°C for 1 week or stored at -20°C for up to 1 year before proceeding (*see* **Note 25**).
10. Thaw newly synthesized cDNA samples if frozen and keep on ice.
11. Prepare individual master mixes for each miRNA intended to be detected. Each master mix comprises of: 2X SensiFAST Probe HiROX buffer, 20X probe, and DNA/RNase free H_2O made up according to volumes outlined in Table 5 (*see* **Note 26**).
12. Vortex each master mix to ensure all reagents are thoroughly mixed.
13. Design the experimental layout on a 384 or 96-well RT-PCR plate (*see* **Note 27**).
14. Pipette 8.9 µl of each master mix to appropriate wells. (e.g. If there are 10 samples and 2 probes; each master mix would be pipetted into 20 wells (technical duplicates) resulting in a total of 40 wells being used (2 probes) in the 384 or 96-well plate).

15. Pipette 1 µl of each cDNA sample into appropriate wells. (2 µl of each cDNA sample should be used per probe due to duplicates) (*see* **Note 28**).

3.6.2 Synthetic miRNA standards preparation

A miR-155 synthetic oligonucleotide can be used as a method of determining absolute miR-155 expression levels by creating a miR-155 standard curve.

1. Thaw a miR-155 synthetic oligonucleotide (1 nM) aliquot on ice.
2. Generate a concentration of 32.8 pM from the 1 nM stock by diluting 4 µl of the 1nM stock into 118 µl of H_2O.
3. Create a 4-fold dilution by diluting 25 µl of the 32.8 pM stock into 75 µl of H_2O, this will be the top standard of the standard curve and contains a concentration of 8.2 pM or 8200 fM.
4. Create 5-7 more serial 4-fold dilutions, a final blank template control containing only H_2O must also be included to distinguish backround CT noise from the RT-PCR machine.
5. Run these serial dilutions in parallel with experiment samples. For example, following **steps 5-9**, **Section 3.6.1**, add 3 µl of each serial dilution to 12 µl of RT reaction mix. Following **steps 10-15**, **Section 3.6.1**, add 1µl of the cDNA sample generated onto the appropriate wells of a RT-PCR plate which contains the miR-155 probe.

3.6.3 Loading the plate

1. Seal the RT-PCR plate with an optical adhesive film. Once the film is sufficiently applied (using applicator provided), tear off the perforated edges.
2. Centrifuge the RT-PCR plate at 1000 RPM for 1 min. This is to ensure there are no droplets trapped at the edge of any wells (the laser within the RT-PCR machine may not identify these drops causing skewed results).

3. On the 7900HT System, start the SDS v2.2 software. In the main menu, select File – New. In the new document dialog box, select the following from the drop-down menu: Absolute Quantification (ΔΔCt) and 384-well format (or 96-well format depending on the plate used).
4. On the right-hand side of the screen, open the New Detector tab; name each miRNA being evaluated within the experiment. Ensure the FAM tab is also highlighted.
5. On the left-hand side of the screen, highlight the wells that contain samples from the experiment and label with the appropriate miRNA detector and sample number.
6. Save as an SDS 7900 Template (.sdt) file.
7. Load and run the RT-PCR plate using the standard default thermal-cycling conditions, changing the reaction volume to 10 µl (*see* **Note 29**). This RT-PCR run takes approximately 1 hour 20 minutes.
8. To retrieve data, go to File and Open saved .sdt file. Click analyse data by pressing the green triangle. Export data as a .txt file to a USB stick.

3.7. Data Analysis

3.7.1 ΔΔCT Method

1. Once the RT-PCR cycle containing all biological experimental samples has completed its run, using Microsoft Excel Spreadsheet, paste the Ct values of the miRNA of interest parallel to the housekeeping miRNA Ct values. The ΔΔCt method is shown in the following equation and described in the following steps.

$$Ct_{\text{miRNA sample}} - Ct_{\text{house-keeping miRNA}} = \Delta Ct$$

$$\Delta Ct_{\text{miRNA sample}} - \Delta Ct_{\text{reference sample}} = \Delta\Delta Ct$$

$$\text{Fold Induction} = 2^{-\Delta\Delta Ct}$$

2. Subtract the Ct values obtained from experimental miRNA samples away from the chosen housekeeping miRNA Ct values (termed ΔCt). Analysis is possible using one housekeeping gene or the average of multiple house-keeping genes i.e. snoRNA202, miR-191, U6. **Figure 1** illustrates the difference between normalizing the data to three housekeeping genes compared to individual housekeeping genes.
3. Determine the average ΔCt value from your reference group (non-treated group) only, and subtract this value from the ΔCt value of the miRNA sample.
4. The fold change ($2^{-\Delta\Delta Ct}$) is calculated by multiplying ΔΔCt values obtained in the previous step by the power of 2 i.e. POWER(2, ΔΔCt).

3.7.2 Absolute Quantification method

1. Once the RT-PCR cycle containing all the experimental and standard curve samples has completed its run, using Microsoft Excel Spreadsheet, paste all the Ct values onto an excel sheet.
2. Determine the standard curve first by plotting the Ct values from the serial dilutions against the corresponding concentration expressed in fM. Convert the fM value to a log value i.e. the top standard of 8200 fM has a log value of 3.9 (*see* **Figure 2**). This term is often denoted log copy number.
3. Next, calculate the mean Ct value from the entire set of houskeeping miRNAs used in the experiment. You can calculate this value from the average of one housekeeping miRNA or use the mean value from multiple housekeeping miRNAs.
4. Create a **Normalisation Factor** (NF) (12) by subtracting the mean housekeeping Ct value from the sample of interest from the mean Ct value generated in Step 2.

$$\text{Mean Ct}_{\text{entire house-keeping}} - \text{Mean Ct}_{\text{sample house-keeping}} = \text{NF}$$

5. Add the normalisation factor value to the raw Ct value generated for the experimental sample (ie. the Ct value generated for the miRNA of interest).

$$NF + Ct_{miRNA\ sample} = \text{normalized Ct value}$$

6. The normalized Ct value is then used to calculate the absolute copy number from the standard curve generated from the synthetic miRNA oligonucleotide (*see* **Figure 2**).
7. From the standard curve, determine the equation of the line i.e. **y = 1.11x + 22.22** (*see* **Note 30**).
8. To solve **"x"** (log copy number), subtract the numerical value at the end of the equation i.e. 22.22 from the normalised Ct values obtained in Step 5 and divide this by the numerical value adjacent to x i.e. 1.11. Therefore, this calculation would resemble the following: **(step 4 – 22.22) / 1.11 = x.** This gives you a value that equates to log copy number or log fM concentration.
9. Multiply the log value obtained in the previous step by the power of 10 i.e. POWER(10, 'x'). The value is often too low to represent on a graph and it is often necessary to multiply the value by the desired exponential factor to represent the data. **Figure 3** illustrates the exact same data from Section 3.7.1 calculated using the absolute quantification method instead of the ΔΔCt method.

4. Notes

1. Our immunization protocol is sufficient to achieve 100% disease incidence and clinical severity of score 3 (hind limb paralysis) at our facility. A number of factors can influence the incidence and severity of disease, including the age and source of mice, stress, the cleanliness of the animal facility and batch/source of MOG35-55 peptide, *M. tuberculosis* and Pertussis toxin. It is therefore recommended that mice are allowed to acclimatize for one week prior to immunization and that the dose of reagents is determined empirically by each investigator.
2. CFA contains 1mg/ml of heat-killed *M. tuberculosis*
3. Due to its consistency there can be a substantial loss of the CFA/antigen emulsion during loading of syringes and thus excess emulsion should be prepared.
4. Additional syringes may be required depending on the number of mice to be immunized.
5. It is critical that no air bubbles are introduced when preparing the emulsion.
6. An emulsion is formed if a drop discharged into room temperature water remains intact on the surface. If the drop disperses additional mixing is required.
7. This is only required the first time the 1ml syringe is loaded with the emulsion.
8. A bolus mass should form and persist under the skin for the duration of the experiment. Ensure the injection site is not located on the inguinal lymph nodes if they need to be harvested for analysis.
9. The tail can be marked to keep track of individual mice.
10. Hemostats can be used to hold the chest cavity open by clamping the sternum and placing the hemostat over the head.
11. 20ml is sufficient assuming correct placement of the needle.
12. Ensure the angle of the scissors is as superficial as possible to avoid damaging the underlying brain.

13. Room temperature is important to ensure complete phase separation between Percoll gradients and CNS leukocyte buffy coat layer.
14. It is wise to transfer about 2-3 ml of the buffy coat to maximise the amount of cells that are contained in this fraction.
15. A naïve mouse contains approximately 0.5 x 10^6 leukocytes in the CNS. This increases to approximately 1-2 x 10^6 in a mouse undergoing EAE.
16. Adding CD16/CD32 (Fc antibody) prevents non-specific binding of FACS antibodies.
17. It is also important to have one sample containing 2 x 10^5 cells that is unstained and contains no antibody.
18. Confirm with the FACS analyst prior to cell sorting which FACS tubes are compatible for obtaining and collecting samples as compatibilities between cell sorting machines differ.
19. It is possible to investigate both miRNA and gene expression with this optimised TRIzol method. However we only discuss miRNA expression analysis in this chapter.
20. This step must be carried out in a fume hood, due to the presence of phenol in the TRIzol.
21. RNA pellets can become easily dis-lodged in this step.
22. Keep the RNA samples on ice.
23. As multiple primers can be added to this master mix (maximum 8), the H_2O volume should be determined once all other reagents have been accounted for e.g. if there is a master mix prepared for 8 samples (+2 for pipetting error) with 2 primers: (10) 0.125 + (10) 1.5 + (10) 0.18 + (10) 1.0 + [10 (0.375 x 2)] = total volume of all other reagents. Required total master mix volume of 120 µl (12 µl x 10 samples): total volume of reagents – 120 µl = volume of H_2O.
24. We have found using 8 x strip tubes to be the most useful PCR tubes for assessing multiple samples and convenient storage.

25. We have experienced significant degradation and destabilisation of cDNA if kept longer than 1 year. It is better to repeat **Steps 1-8,** using stored RNA kept at -80°C rather than re-use stored cDNA for subsequent RT-PCR analysis.
26. Each sample must be run in technical duplicates for each miRNA being assessed.
27. The use of a 384 or 96-well RT-PCR plate depends on the block most frequently used by your gene expression facility and/or the amount of samples you have.
28. We have found that using the same pipette tip between duplicates gives tighter replicates. However, it is necessary to change the pipette tip between samples and when moving onto a new master mix to prevent cross- contamination.
29. It is recommended by the manufacturer's that RT-PCR is performed in a final 20 μl volume. However we have found a final volume of 10 μl works just as well, thus saving on overall costs.
30. Reliable equation of the lines are generated from standard curves with an R^2 value of 98 or above.

5. References

1. O'Connell RM, Rao DS, Chaudhuri AA, Baltimore D (2010). Physiological and pathological roles for microRNAs in the immune system. Nat Rev Immunol. 10:111-22.
2. Chen C, Ridzon DA, Broomer AJ, Zhou Z, Lee DH, Nguyen JT, Barbisin M, Xu NL, Mahuvakar VR, Andersen MR, Lao KQ, Livak KJ, Guegler KJ (2005). Real-time quantification of microRNAs by stem-loop RT-PCR. Nucleic Acids Res. 20:e179.
3. Schmittgen TD, Lee EJ, Jiang J, Sarkar A, Yang L, Elton TS, Chen C. (2008) Real-time PCR quantification of precursor and mature microRNA. Methods. 44:31-8.
4. Lyons, V and McCoy CE. (2016) Simple methods to analyse miRNA induction in response to Toll-like receptors. Toll-Like Receptors: Methods and Protocols.
5. Livak KJ, Schmittgen TD (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods. 25:402-8.
6. McCoy CE, Sheedy FJ, Qualls JE, Doyle SL, Quinn SR, Murray PJ, O'Neill LA. (2010) IL-10 inhibits miR-155 induction by toll-like receptors. J Biol Chem. 285:20492-8.
7. Gantier MP, Stunden HJ, McCoy CE, Behlke MA, Wang D, et al. (2012) A miR-19 regulon that controls NF-kappaB signaling. Nucleic Acids Res 40:8048-8058.
8. Fairfax KA, Gantier MP, Mackay F, Williams BR, McCoy CE (2015) IL-10 regulates Aicda expression through miR-155. J Leukoc Biol 97:71-78.
9. D'haene B, Mestdagh P, Hellemans J, Vandesompele J (2012) miRNA expression profiling: from reference genes to global mean normalization. Methods Mol Biol. 822:261-72.
10. Vandesompele J, De Preter K, Pattyn F, Poppe B, Van Roy N, De Paepe A, Speleman F (2002). Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biol. 2002 3:7
11. Qureshi R, Sacan A (2013). A novel method for the normalization of microRNA RT-PCR data. BMC Med Genomics. 6:S1-14

12. Kroh EM, Parkin RK, Mitchell PS, Tewari M (2010) Analysis of circulating microRNA biomarkers in plasma and serum using quantitative reverse transcription-PCR (qRT-PCR). Methods. 4:298-301.

Table Captions

Cell Marker	Fluorescent probe	Expression/Representative	Location
CD45	PerCpCy5.5	Leukocytes	Cell Surface
CD11b	APC	Myeloid Cells	Cell Surface
F480	FITC	Monocytes/macrophages/Eosinophils	Cell Surface
CD4	PE	$CD4^+$ cells	Cell Surface
CD3	eFluor450	T cells	Cell Surface
B220	PE/Cy7	B cells	Cell Surface

Table 1. Compatible flow cytometry cell surface antibodies

Score	Clinical Signs
0	Normal. Curling of the tail when the mouse is picked up.
0.5	Partial limp tail. The tip of the tail is limp when the mouse is picked up.
1	Limp tail. The tail is completely limp when the mouse is picked up.
2	Weak hind limbs. The mouse has an abnormal gait and struggles to right itself when placed on its back.
2.5	Paralysis of one hind limb. The mouse drags one hind limb and there is no response to toe pinch.
3	Paralysis of both hind limbs. There is complete loss of movement in both hind limbs and no response to toe pinch.
4	Paralyzed hind limbs and weak forelimbs. Mice are sacrificed for humane reasons.
5	Moribund. The mouse is cold to touch and not moving.

Table 2. Clinical grading system for assessment of EAE.

Immune cell population	Cell number	RNA concentration (ng/µl)
Macrophages	7600	205.5
$CD4^+$ T cells	745	75
B220 cells	1200	120

Table 3. Cell numbers from immune cell populations enriched by cell sorting with their corresponding RNA concentration yield (ng/µl) within the CNS of a WT naïve C57/Bl6 mouse.

Reagent	Volume (µl)
dNTP	0.125
10X buffer	1.5
RNase Inhibitor	0.18
RT enzyme	1.0
TaqMan primer (5X)	*0.375 (per each primer)
H_2O (DNA/ RNase free)	**dependent on primer number
Total Volume	**12**

Table 4. The reagents and volumes required to generate a 12 µl RT reaction mix for individual miRNA analysis. These volumes should be multiplied by the number of samples you have (+2 for pipetting error) to create an RT reaction master mix. *The reaction can allow multiple miRNA primers (maximum 8) to be accommodated in the one reaction mix. **The volume of H_2O will depend on the amount of primers included in the reaction mix.

Reagent	Volume (µl)
2X TaqMan Universal Master Mix	10.0
H_20 (DNA/RNase free)	8.0
20X probe	0.66

Table 5. The reagents and volumes required to generate a RT-PCR reaction mix for individual miRNA analysis. These volumes incorporate enough mix to measure one miRNA in duplicate as well as allowing extra for pipetting error. These volumes should be multiplied by the number of samples in your experiment to generate a master mix.

Figure Captions

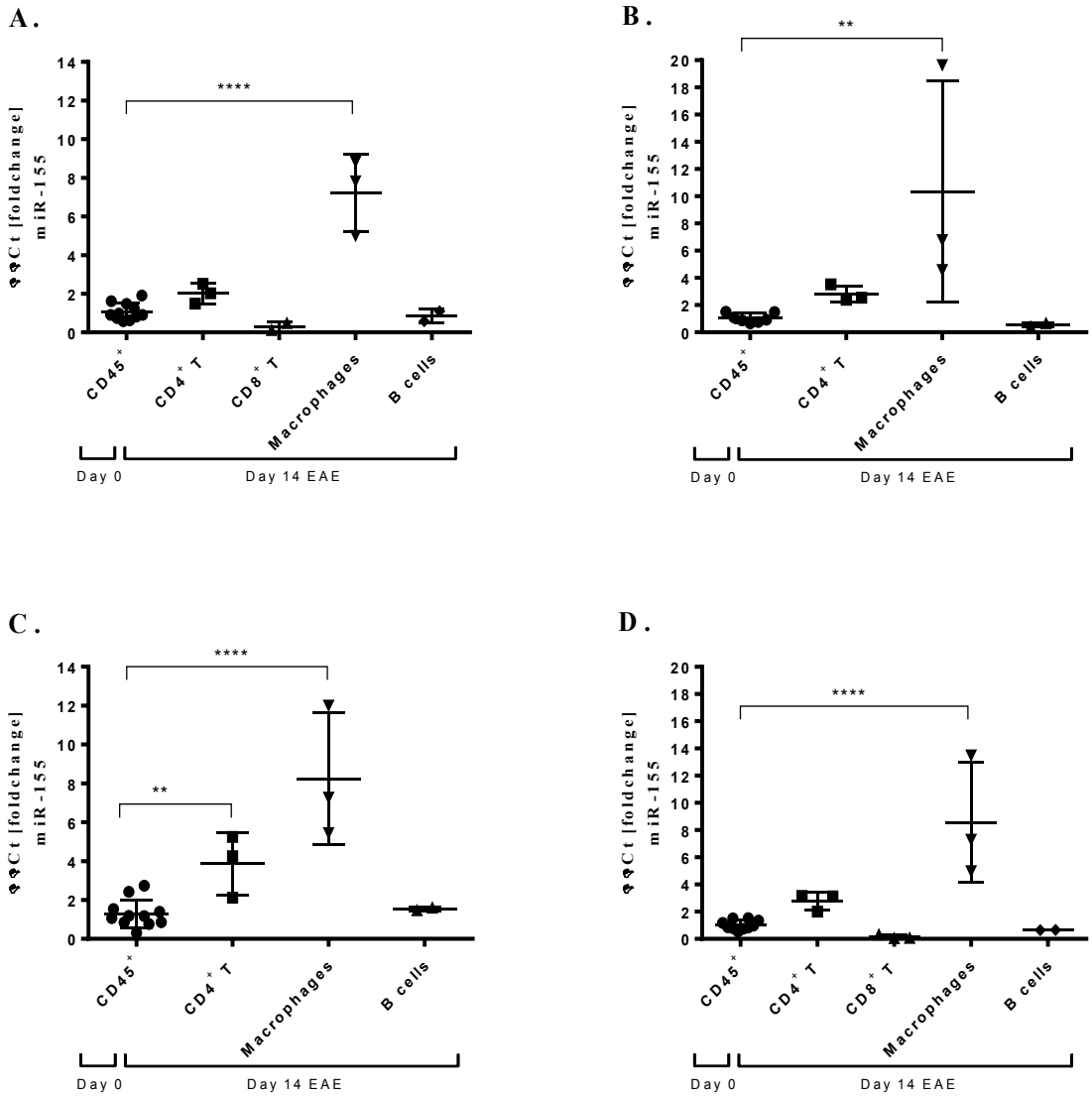

Figure 1 illustrates miR-155 fold induction using the ΔΔCt fold method within the CNS of EAE-induced mice at day 14 peak disease compared to naïve (Day 0) non-EAE induced mice. MiR-155 fold induction was analysed by normalizing data to individual house-keeping genes such as sno-202 (A) miR-191 (B), U6 (C), and the average of 3 house-keeping genes previously mentioned (D).

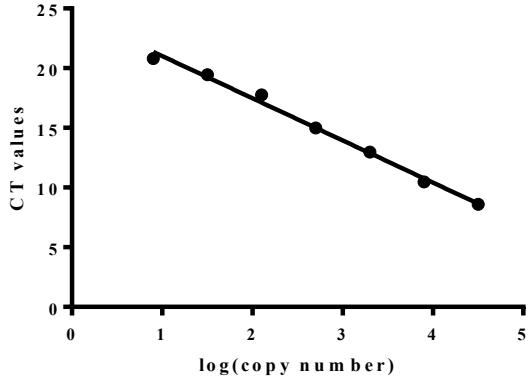

Figure 2. Standard curve demonstrating Ct values vs log copy number of miR-155 Oligo concentration (fM).

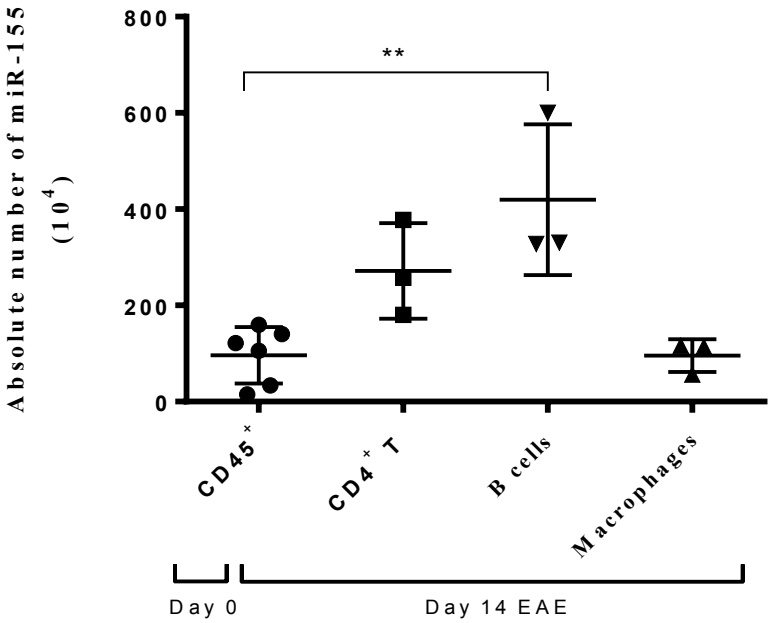

Figure 3 illustrates miR-155 copy numbers in different immune cells from the CNS of EAE induced mice. Data was calculated using the absolute quantification method and normalized using the mean normalization of three housekeeping miRNAs.