

Chapter 18

MicroRNA Function in Mast Cell Biology: Protocols to Characterize and Modulate MicroRNA Expression

Steven Maltby, Maximilian Plank, Catherine Ptaschinski,
Joerg Mattes, and Paul S. Foster

Abstract

MicroRNAs (miRNAs) are small noncoding RNA molecules that can modulate mRNA levels through RNA-induced silencing complex (RISC)-mediated degradation. Recognition of target mRNAs occurs through imperfect base pairing between an miRNA and its target, meaning that each miRNA can target a number of different mRNAs to modulate gene expression. miRNAs have been proposed as novel therapeutic targets and many studies are aimed at characterizing miRNA expression patterns and functions within a range of cell types. To date, limited research has focused on the function of miRNAs specifically in mast cells; however, this is an emerging field. In this chapter, we will briefly overview miRNA synthesis and function and the current understanding of miRNAs in hematopoietic development and immune function, emphasizing studies related to mast cell biology. The chapter will conclude with fundamental techniques used in miRNA studies, including RNA isolation, real-time PCR and microarray approaches for quantification of miRNA expression levels, and antagomir design to interfere with miRNA function.

Key words MicroRNA, RNA isolation, Microarray, Real-time PCR, Antagomir

1 Introduction

1.1 *MicroRNA Biogenesis and Structure*

More than 1,000 miRNAs have been identified in humans, and miRNAs predate early invertebrates, identifying a key role for miRNA function in biological processes [1, 2]. In fact, miRNAs may regulate the expression of >60 % of all genes in humans [3]. miRNAs are commonly encoded either within the introns of protein-coding genes or as independent genes and are generally transcribed by RNA polymerase II [4, 5]. Some miRNAs are grouped in clusters, which are transcribed as single transcripts and processed to generate multiple functional miRNAs [5].

After transcription, the primary transcript (pri-miRNA) is processed by the nuclear RNase III enzyme Drosha and DGCR8 (DiGeorge syndrome critical region gene 8), excising the pre-miRNA stem-loop structure [6]. The double-stranded RNA hairpin

pre-miRNA is exported from the nucleus by exportin 5 and further spliced by the cytoplasmic RNase III enzyme Dicer and TAR RNA-binding protein 2 (TRBP) into an miRNA-miRNA* duplex approximately 22 nucleotides (nt) in length [7, 8]. The miRNA duplex is finally unwound and one miRNA is incorporated into the RISC complex, which also contains proteins from the Argonaute family of RNA-binding RNA endonucleases and other proteins [9]. The RISC complex is finally directed to mRNA strands with sequence complementary to the mature miRNA and functions to reduce target mRNA expression [9, 10].

1.2 MicroRNA Function

miRNAs modulate target mRNA levels by binding to the 3' untranslated region (UTR) of mRNA transcripts [11]. Target recognition occurs through complementary binding of the highly specific, ~7 nt-spanning seed sequence at the 5' end of a miRNA to a target mRNA [12]. While seed sequence recognition is highly specific, sequence complementarity throughout the rest of the miRNA is often quite low, allowing individual miRNAs to target multiple mRNA targets and making it difficult to predict miRNA targets based on sequence information alone [12–15].

How miRNAs modulate mRNA translation is still not fully understood; however, three general mechanisms have been proposed. Initial studies suggested that miRNAs function through (1) direct suppression of mRNA translation: these studies demonstrate that miRNA expression decreases levels of the target mRNA-encoded proteins without affecting the levels or stability of the target mRNA itself [16]. miRNAs accomplish this by blocking the initiation of translation or by blocking access of the ribosome to the target mRNA, causing the ribosome to fall off the target mRNA during elongation [17–19]. More recent studies have also demonstrated (2) miRNA-mediated modulation of mRNA stability. Expression of certain miRNAs can result in decreased levels of target mRNAs through direct miRNA-mediated mRNA degradation [20]. This process occurs through de-adenylation of the target mRNA transcript, followed by removal of the mRNA 5' cap, resulting in degradation of the transcript in the cytoplasm [21, 22]. Additionally, miRNA recognition of target mRNAs can result in (3) sequestration of target mRNA transcripts into processing (P) bodies. P bodies are structures within the cytoplasm involved in mRNA storage and degradation. Some of the Argonaute family members (present within the RISC complex) localize to P bodies, and certain miRNAs move to P bodies after binding to target mRNAs [23]. Within the P bodies, mRNA target degradation can occur, or alternatively, sequestration within the P bodies alone can result in decreased mRNA translation, as P bodies are largely devoid of ribosomes. Each of these mechanisms likely occurs for different subsets of miRNAs and/or mRNA targets resulting in the observed fine-tuning of protein expression.

1.3 *MicroRNA in Hematopoietic Cells*

miRNA studies generally fall into two broad categories: (1) studies profiling miRNA expression across multiple cell types aimed at characterizing broad expression patterns and (2) functional studies of small numbers of miRNAs aimed at characterizing individual miRNA functions. To date, several studies profiling broad miRNA expression patterns in the hematopoietic lineage have included mast cells, and a growing number of studies have focused on the effects of specific miRNAs in mast cell development and function.

1.4 *MicroRNA Functions in Hematopoietic Development and Differentiation*

A number of studies have focused on roles for miRNAs in hematopoietic stem cell differentiation and self-renewal. Conditional loss of Dicer results in reduced hematopoietic contribution in a competitive bone marrow reconstitution model [24], while loss of Ars2 (a molecule required for miRNA-mediated repression) results in impaired cell proliferation and bone marrow failure in adult mice [25]. As Dicer and Ars2 are broadly required for miRNA processing and function, these findings suggest that one or more miRNAs are critical for long-term bone marrow maintenance. Several studies have identified mir-125a (along with other members of the cluster, mir-99b and let-7e) as preferentially expressed in long-term repopulating hematopoietic stem cells (LT-HSCs) [24, 26], and mir-125a plays a role in HSC expansion via targeting of Bak1 [24]. Overexpression of mir-125a results in reduced progenitor apoptosis and increased HSC numbers [24]. Mir-125a expression levels also correlate with HSC numbers across inbred mouse strains [24]. Further, forced overexpression of mir-125a confers an advantage in competitive reconstitution models, ultimately resulting in the development of myeloproliferative neoplasms [26]. The miR-125a homologue (miR-125b) is also upregulated in HSCs, and overexpression confers a similar advantage in competitive reconstitution assays, along with a dose-dependent progression to lethal myeloid leukemia [27]. MiR-155 expression is increased in human CD34⁺ hematopoietic stem-progenitor cells (HSPCs), and overexpression results in reduced myeloid and erythroid differentiation, suggesting that miR-155 helps maintain progenitor status and blocks maturation and/or differentiation [28]. Finally, miR-221 and miR-222 are highly expressed in CD34⁺ cord blood progenitor cells and dampen kit expression, a key molecule in the maintenance of stem cells [29]. Enforced overexpression of miR-221 and miR-222 results in impaired stem cell engraftment in transplant models, and downregulation is required for appropriate differentiation along the erythroid lineage [29].

miRNAs also play key roles in the regulation of more mature, multipotent progenitor populations. MiR-150 is preferentially expressed in megakaryocyte-erythrocyte progenitors (MEPs) and promotes megakaryocyte lineage commitment, at the expense of erythroid development, by targeting the transcription factor MYB [30]. In granulocyte progenitors, miR-223 negatively regulates

progenitor proliferation and differentiation by targeting the transcription factor Mef2c [31]. MiR-223 also affects mature granulocytes by dampening activation, and loss of miR-223 expression in mature cells results in increased sensitivity to activating stimuli and increased fungicidal activity [31].

In the lymphoid lineage, conditional deletion of Dicer results in impaired survival of immature $\alpha\beta$ T cells, with no effects on either $\gamma\delta$ T cells or CD4/CD8 lineage commitment [32]. MiR-181a expression is increased during T cell maturation, downregulating a number of phosphatases and resulting in increased T cell receptor sensitivity [33]. Conversely, inhibition of miR-181a results in reduced receptor sensitivity and impaired positive and negative selection during T cell maturation [33]. In B cell development, conditional loss of Dicer expression results in a differentiation block at the pro- to pre-B cell transition, likely through a miR-17-92-mediated mechanism targeting the proapoptotic molecule Bim [34]. In addition, loss of Argonaute 2 (Ago2) results in reduced miRNA levels and impaired B cell and erythroid development [35]. MiR-150 also plays a key role in the regulation of the transcription factor c-Myb in developing B cells, and premature expression blocks B cell maturation [36, 37]. Similarly, constitutive expression of miR-34a blocks B cell development, resulting in reduced B cell numbers by targeting the transcription factor Foxp1 [38].

In myeloid populations, miR-17-5p, miR-20a, and miR-106a are downregulated during monocyte differentiation, resulting in increased levels of the transcription factor acute myeloid leukemia-1 (AML1) [39]. Enforced expression of these miRNAs results in increased blast proliferation and inhibition of monocyte differentiation and maturation [39]. Further, miR-223 exhibits “myeloid gene” characteristics and is upregulated by the myeloid transcription factor PU.1 and the C/EBP transcription factors [40]. In human granulocyte precursors, miR-223 expression is regulated by competition between the transcription factors NFI-A and C/EBP α , and expression promotes granulocyte differentiation [41].

While miRNAs clearly have functional roles in normal hematopoietic differentiation, they are better understood for their role in malignancy, when expression patterns are altered. Some examples include miR-155, miR-29a, miR-15a, and miR-16-1. Levels of miR-155 accumulate in certain B cell lymphomas, and forced overexpression of miR-155 results in a preleukemic B cell proliferation in the spleen, followed by progression to B cell malignancy [42]. Similarly, miR-155 promotes normal myeloid differentiation but is overexpressed in patients with acute myeloid leukemia (AML) and contributes to malignancy following forced overexpression [43]. Ectopic expression of miR-29a in progenitor populations results in aberrant progenitor self-renewal capacity, myeloid differentiation, and ultimately progression to AML [44]. Finally, miR-15a and miR-16-1 deletion can lead to accelerated B cell proliferation by

modulation of cell cycle genes, and both miR-15a and miR-16-1 are often deleted in B cell chronic lymphocytic leukemias (B-CLL) [45, 46].

1.5 MicroRNA Functions in Innate and Adaptive Immunity

In addition to roles in cell differentiation and development, miRNAs play key roles in regulating immune cell function. In innate immune function, miRNA modulation of Toll-like receptor (TLR)-mediated signaling has been extensively studied. TLRs recognize pathogen-associated molecular patterns (PAMPs) and are key molecules required for initiation of the innate immune response to pathogens. TLR4 surface expression is regulated by let-7 [47]. MiR-9 is upregulated by TLR stimulation (as well as by TNF- α or IL-1 β) in both monocytes and neutrophils and dampens NF κ B1 expression to control inflammation [48]. TLR stimulation (or activation with IFN- β) of macrophages also stimulates increased miR-155 expression [49] and activates miR-147, which dampens inflammatory cytokine release [50]. Similarly, LPS stimulation of human peripheral blood mononuclear cells (PBMCs) increases miR-21 expression, which reduces levels of its target PDCD4, a molecule required for induced NF κ B activity in proinflammatory responses [51]. In human monocytes, miR-146a is induced in response to LPS treatment, in an NF κ B-dependent manner [52]. MiR-146a in turn targets TNF receptor-associated factor 6 (TRAF6) and IL-1 receptor-associated kinase 1 (IRAK), two molecules downstream of TLR signaling [52].

miRNAs also play roles in adaptive immune cell function. MiR-155 deficiency results in impaired B, T, and dendritic cell function, and miR-155 normally modulates a wide range of cytokines, chemokines, and transcription factors [53]. Further, miR-155 is critical for T cell-dependent antibody responses in germinal centers [54]. MiR-326 and MiR-181a both play key roles in modulating T cell function, with miR-326 expression promoting Th17 differentiation by targeting Ets-1 [55] and miR-181a modulating T cell signaling sensitivity [33].

Of particular interest for the mast cell field, two miRNAs have been identified that regulate allergic inflammation and airway disease. In a mouse model of house dust mite (HDM)-induced allergic disease, we identified a key role for miR-126 [56]. Following disease induction, miR-126 expression is upregulated in the lung, via a TLR4- and MyD88-dependent pathway [56]. Blocking miR-126 activity resulted in decreased disease pathology, including decreased eosinophil recruitment, reduced mucus production, suppressed cytokine levels, and abolished airway hyperresponsiveness [56]. In both ovalbumin (OVA) and aspergillus models of experimental allergic asthma, miR-21 expression is also increased, primarily in lung macrophages and dendritic cells [57]. MiR-21 targets IL-12p35, decreasing IL-12 production and increasing Th2 responses, eosinophilia and allergic airway inflammation [57].

MiR-21 is also upregulated in a bleomycin-induced lung fibrosis model and in patients with idiopathic pulmonary fibrosis, inducing TGF- β 1 from primary fibroblasts [58]. Mast cells have classically had a prominent role in these diseases, and while current studies have not addressed miRNA function in mast cells in these models, this will likely be a key area of future research.

1.6 MicroRNA in Mast Cell Biology

Numerous roles are emerging for miRNAs in hematopoietic immune cell function. However, to date, relatively little attention has been focused on roles in mast cell biology. Mast cells have only been included in a few of the broad miRNA profiling studies. In the first study, Monticelli et al. [59] assessed 181 miRNAs across a range of hematopoietic cell types, including cultured BMMCs (bone marrow-derived mast cells). BMMCs express high levels of miR-26a, miR-24, and miR-27a and low levels of miR-223 compared to other hematopoietic lineages. Interestingly, BMMC expression patterns are most similar to mature T cell subsets, which the authors attributed to the similar terminal maturation states of these cell lineages. In a more recent study, Kuchen et al. [60] profiled 600 miRNAs and identified a novel miRNA transcript “1073496_chr3” within the CPA3 gene, which was highly expressed in the mast cell and basophil lineages. These profiling studies provide a starting point for studying miRNA function in mast cells and a first step toward characterizing miRNA expression in mast cells.

In addition to profiling studies, a number of recent studies have explored functional roles for mast cell-expressed miRNAs. Several miRNAs have been implicated in the regulation of mast cell cycle, proliferation, and maturation. miR-221 and miR-222 are increased in bone marrow-derived mast cells (BMMCs) after activation, and overexpression dampens cell proliferation, with no effect on differentiation or cell survival [61]. During homeostasis, miR-381 and miR-539 targets microphthalmia-associated transcription factor (MITF) expression, maintaining appropriate mast cell proliferation [62]. In patients with mastocytosis, an activating kit receptor mutation results in decreased levels of miR-381 and miR-539, resulting in increased MITF expression and increased mast cell proliferation [62]. During mast cell maturation, miR-126 downregulation results in increased levels of its target Sprad1 (Sprouty-related Ena/VASP homology-1 domain-containing protein), regulating mast cell numbers and cytokine production [63]. Conversely, conditional knockout of Sprad1 results in increased mast cell numbers and increased cytokine production following activation [63].

Several miRNAs have also been implicated in the modulation of mast cell activation, degranulation, and migration. In addition to its role modulating cell cycle, miR-221 also modulates mast cell adhesion and migration. miR-221-overexpression results in

increased degranulation and cytokine production, decreased cell migration, and increased cell adhesion through a number of proposed target genes [64]. miR-132 levels in BMMCs increase following activation via IgE cross-linking, and miR-132 dampens heparin-binding EGF-like growth factor (HB-EGF) activation in a proposed negative feedback mechanism on mast cell activation [65].

1.7 Future Perspectives

miRNAs fine-tune expression of a large number of mRNA targets and clearly play key roles in immune cell development and function. Current data, although limited, indicates that miRNAs modulate many aspects of mast cell behavior, and this remains an emerging field of research. In the remaining portion of this chapter, we outline key techniques necessary to identify, quantify, and characterize miRNA expression for future studies of the role of miRNAs in mast cell biology. These techniques include microarray for broad surveys of miRNA expression, real-time PCR for miRNA validation and quantification, and antagomir development to target miRNAs in vitro and in vivo. These methods, and novel methods that continue to be developed, will underpin studies into understanding the role that miRNAs play in mast cell development and function.

2 Materials

2.1 Preparation of Samples

1. Cells of interest.
2. Microtubes, 1.5 mL or appropriate centrifuge tubes.
3. Phosphate-buffered saline (PBS).
4. TRI Reagent (TRIzol) at 4 °C. (NB: TRI Reagent is toxic and should only be used in a fume hood.)

2.2 Total RNA Isolation

1. Microtubes, 1.5 mL.
2. Chloroform—molecular grade (NB: Chloroform is harmful and should only be used in a fume hood).
3. Optional: Glycogen—working stock (5 µg/µL), store at –20 °C.
4. Isopropanol—molecular grade. (NB: Isopropanol is an irritant and is also highly flammable.)
5. Autoclaved Kimwipes.
6. 80 % ethanol: Add 80 mL of 100 % molecular-grade ethanol to 20 mL of nuclease-free water. (NB: Ethanol is highly flammable.)
7. Nuclease-free water.
8. Absorbance reader (e.g., NanoDrop).
9. Biological sample analyzer to determine RNA integrity (e.g., Agilent Bioanalyzer).

2.3 Reverse Transcription of miRNAs

1. Microtubes or microplates.
2. Nuclease-free water.
3. TaqMan miRNA reverse transcription kit (includes dNTPs (100 mM), MultiScribe Reverse Transcriptase (50 U/ μ L), RT buffer (10 $\times$), and RNase inhibitor (20 U/ μ L); Life Technologies), store at -20°C .
4. TaqMan RT primers, specific for individual miRNAs, from TaqMan miRNA Assays (Life Technologies), store at -20°C .
5. Thermocycler.

2.4 Real-Time PCR Detection of miRNAs

1. Microtubes.
2. Nuclease-free water.
3. TaqMan Universal PCR Master Mix (2 $\times$; Life Technologies), store at 4°C .
4. TaqMan miRNA Assays (containing TaqMan probe and PCR primer set, 20 $\times$) for each specific miRNA to be analyzed (Life Technologies), store at -20°C .
5. Optical microplates (96-well or 384-well).
6. Optical adhesive film.
7. Real-time PCR instrument.

2.5 TaqMan Low Density miRNA Arrays for miRNA Profiling

1. Microtubes.
2. Nuclease-free water.
3. Megaplex RT primers (10 $\times$, includes MgCl_2 (25 mM); Life Technologies), store at -20°C .
4. TaqMan miRNA reverse transcription kit (includes dNTPs (100 mM), MultiScribe Reverse Transcriptase (50 U/ μ L), RT buffer (10 $\times$), and RNase inhibitor (20 U/ μ L); Life Technologies), store at -20°C .
5. TaqMan Universal Master Mix II, No AmpErase UNG (Life Technologies), store at 4°C .
6. TaqMan miRNA Array Cards (Life Technologies), light sensitive, store at 4°C .
7. Real-time PCR instrument capable of holding TaqMan miRNA Array Cards (e.g., Applied Biosystems 7900HT, ViiA7, or QuantStudio 12K Flex machines).

2.6 Targeting miRNA Function with Antagomirs

1. Cells of interest (e.g., primary mast cells, BMMCs, or mast cell line).
2. Appropriate culture medium (dependent on cells of interest).
3. Nuclease-free water.
4. Antagomir stocks.

3 Methods

3.1 Preparation of Samples

The initial step in all experiments aimed at quantifying or assessing miRNA levels will be isolation of total RNA samples from your cells of interest.

1. Pellet cells of interest by centrifugation at $1,000\times g$ for 5 min.
2. Discard supernatant and wash cell pellet with 1 mL PBS.
3. Centrifuge at $1,000\times g$ for 5 min.
4. Repeat **steps 2 and 3** to wash cells.
5. Discard supernatant, resuspend cell pellet in 1 mL TRI Reagent, and homogenize by repeated pipetting.
6. Store lysate at -80°C until required.

3.2 Total RNA Isolation

In our experience, TRI Reagent gives a very good yield of total RNA at consistently high purity. This is crucial for downstream applications such as reverse transcription.

1. Allow samples to defrost completely before starting isolation (*see Note 1*).
2. Add 200 μL chloroform (per 1 mL of TRI Reagent), mix vigorously by hand for 15 s, let stand at RT for 5 min, and centrifuge at no more than $12,000\times g$ for 10 min at 4°C .
3. While samples are spinning prepare and label fresh tubes.
4. Optional: Add 2 μL of 5 $\mu\text{g}/\mu\text{L}$ glycogen to fresh tubes, for samples with low levels of RNA (*see Note 2*).
5. Carefully transfer the colorless, upper aqueous phase to the freshly prepared tubes. Use a 200 μL pipette tip to carefully remove this phase. Avoid disturbing the interphase (*see Note 3*).
6. Optional: When using glycogen, lightly vortex samples to ensure resuspension.
7. Add 500 μL isopropanol, mix thoroughly by inversion, let it stand at RT for 10 min, and centrifuge at no more than $12,000\times g$ for 10 min at 4°C (*see Note 4*).
8. Pour off supernatant and tap off remaining supernatant on a clean Kimwipe. Wash with 1 mL 80 % ethanol. Centrifuge at no more than $7,500\times g$ for 5 min at 4°C .
9. Very carefully remove the supernatant by using pipette tips of progressively smaller volume to avoid disturbing the pellet (the pellet is often loose). Wash with another 1 mL of 80 % ethanol. Centrifuge at no more than $7,500\times g$ for 5 min at 4°C .
10. Very carefully remove all the supernatant.
11. Air-dry pellets at RT for 5–10 min until all residual ethanol has evaporated (*see Note 5*).

12. Label a final set of fresh tubes. Carefully resuspend RNA pellet in required volume of pre-warmed 50 °C nuclease-free water (e.g., 10–15 µL for small quantities of RNA from ~100,000 initial cells) (*see* **Note 6**).
13. Once resuspended, transfer volume to a fresh tube. Quantify final RNA concentration and assess for impurities by measuring absorbance (e.g., NanoDrop) (*see* **Note 7**).
14. Optional: Analyze samples on a biological analyzer to confirm good RNA integrity (*see* **Note 8**).
15. Store at –80 °C.

3.3 Reverse Transcription of miRNAs

There are various approaches to assay miRNA expression levels via quantitative real-time PCR. We focus on the most commonly used approach in this protocol, the TaqMan miRNA Assay (Life Technologies). Reverse transcription (RT) is performed with miRNA-specific RT primers that contain a stem-loop structure, which enables the selective detection of mature miRNAs. In our experience, the use of TaqMan probes grants high specificity and sensitivity.

1. Dilute the isolated total RNA (as described in Subheading 3.2) to 0.2 to 2 ng/µL with nuclease-free water (5 µL volume required per miRNA to be assayed).
2. Prepare one RT master mix per miRNA as follows (volume for one sample, allow for pipetting error) (*see* **Notes 9–11**):

Nuclease-free water	4.16 µL
Reverse transcription (RT) buffer (10×)	1.50 µL
dNTPs (100 mM)	0.15 µL
RNase inhibitor (20 U/µL)	0.19 µL
MultiScribe Reverse Transcriptase (50 U/µL)	1.00 µL
TaqMan miRNA-specific RT primer (5×)	3.00 µL
Total	10.00 µL

3. Mix RT master mix by pipetting and add 10 µL per PCR well. Use multiple tubes/wells, if multiple miRNAs are to be reverse transcribed from the same sample.
4. Add 5 µL of diluted RNA from **step 1** per well. Seal wells with strips.
5. Gently vortex and lightly centrifuge samples to bring volume to the bottom of wells.
6. Place samples in thermocycler and run the following cycle:

(a) 16 °C for 30 min
(b) 42 °C for 30 min
(c) 85 °C for 5 min

7. Store miRNA cDNA at –20 °C until required.

3.4 Real-Time PCR
Detection of miRNAs

Real-time PCR allows for detection of specific miRNAs within a starting sample as well as relative or absolute quantification of miRNA levels. Quantification is based on comparison to endogenous controls and other experimental samples (relative) or comparison to known standards (absolute).

1. Prepare PCR master mix as follows (volume per well, allow for pipetting error) (*see* **Note 12**):

TaqMan Universal PCR Master Mix (2×)	10.00 μL
Nuclease-free water	7.67 μL
TaqMan miRNA Assay (20×)	1.00 μL
Total	18.67 μL

2. Mix PCR master mix by pipetting and add 18.67 μL per well in optical microplates compatible with your real-time PCR machine. Use separate wells if multiple miRNAs are to be amplified from the same sample (i.e., multiple cDNAs per sample). Run each miRNA in triplicates. Also include controls lacking template (*see* **Note 13**).
3. Transfer 1.33 μL of the RT product generated in Subheading 3.3 above to each well.
4. Seal the plate with an optical adhesive cover.
5. Briefly centrifuge microplate to bring solution to bottom of wells.
6. Run plate at the following cycle parameters on a real-time PCR instrument:

(a) 95 °C for 10 min
(b) 95 °C for 15 s
(c) 60 °C for 60 s
(d) Run 40 cycles of steps (b) and (c)

7. Analyze real-time PCR relative quantification data, comparing cycle number to endogenous control genes or different treatment groups, depending on the experimental setup.

**3.5 TaqMan Low
Density miRNA Arrays
for miRNA Profiling**

miRNA array profiling allows the assessment of a broad number of miRNAs within a single isolated sample. Individual miRNA expression levels should be confirmed and validated by subsequent real-time PCR experiments.

1. Dilute RNA (if necessary) to a concentration of 350–1,000 ng of total RNA in 3 µL RNA per reaction. Keep the total amount of RNA consistent for all samples within one experiment (*see Note 14*).
2. Prepare Megaplex RT master mix as follows (volume per sample):

Nuclease-free water	0.2 µL
RT buffer (10×)	0.8 µL
dNTPs (100 mM)	0.2 µL
Megaplex RT primers (10×)	0.8 µL
MgCl ₂ (25 mM)	0.9 µL
RNase inhibitor (20 U/µL)	0.1 µL
MultiScribe Reverse Transcriptase (50 U/µL)	1.5 µL
Total	4.5 µL

3. Mix gently and centrifuge to bring solution to the bottom of tube.
4. For each sample add 3 µL of RNA per 4.5 µL Megaplex RT master mix.
5. Run sample on thermocycler at the following parameters (*see Notes 15 and 16*):

(a) 16 °C for 2 min
(b) 42 °C for 1 min
(c) 50 °C for 1 s
(d) Repeat steps (a)–(c) for 40 cycles
(e) 85 °C for 5 min

6. Allow TaqMan miRNA Array Card to warm to room temperature (*see Note 17*).
7. Prepare PCR reaction mix as follows (volume for one array):

TaqMan Universal PCR Master Mix	450 µL
Megaplex RT product	6 µL
Nuclease-free water	444 µL
Total	900 µL

8. Manually shake tube to mix reagents well.
9. Dispense 100 μ L of PCR reaction mix into each of the eight ports on the TaqMan miRNA Array Card.
10. Centrifuge TaqMan miRNA Array Cards at $300\times g$ for two consecutive 1 min spins (*see Note 18*).
11. Seal TaqMan miRNA Array Cards using the array card stacker (*see Note 19*).
12. Load and run TaqMan miRNA Array Cards at the following cycling parameters in standard mode:

(a)	50 °C for 2 min
(b)	95 °C for 10 min
(c)	95 °C for 15 s
(d)	60 °C for 1 min
(e)	Repeat steps (c) and (d) for 40 cycles

13. Analyze quantification data.

3.6 Targeting miRNA Function with Antagomirs

Antagomirs are chemically engineered nucleotides designed with complete sequence complementarity to specific miRNAs. The use of antagomirs in vitro or in vivo results in specific blockade of miRNA function, allowing for assessment of miRNA function in an assay of interest.

Antagomir Design

1. Obtain the mature miRNA sequences for the miRNAs of interest from miRBase: the microRNA database, Faculty of Life Sciences, University of Manchester, United Kingdom (<http://www.mirbase.org/>).
2. Generate the complementary RNA sequence for the miRNAs of interest. Annotate in 5'- to 3' orientation.
3. Add the following sequence modifications:
 - 2'-O-methyl-modified phosphoramidites for all nucleotides (annotated as m, before each nucleotide).
 - Phosphorothioate linkages for the first 2 and the last 4 nucleotides (annotated as *, after appropriate nucleotides).
 - Hydroxyprolinol-linked cholesterol at the 3'-end (annotated as -Chol).
 - Optional: Addition of a fluorescent modification at the 5'-end (e.g., fluorescein) allows for the visualization and tracking of antagomir-treated cells.
4. Order the antagomir from a custom oligo synthesis service provider (*see Note 20*). An example of a design for a specific antagomir targeting mmu-miR-21-5p:

Step	Description	Sequence
1	Mature mmu-miR-21-5p	5'UAGCUUAUCAGACUGAUGUUGA-3'
2	Complementary sequence to mmu-miR-21-5p	5'UCAACAUCAGUCUGAUAAAGCUA-3'
3	Antagomir for mmu-miR-21-5p	5'mU.*.mC.*.mA.mA.mC.mA.mU.mC.mA.mG.mU.mC.mU.mG.mA.mU.mA.mA.mG.*.mC.*.mU.*.mA.*.3'-Chol

Antagomir Application

5. Select cells of interest and culture under appropriate conditions.
6. Resuspend lyophilized antagomirs in nuclease-free water at a concentration of 100 μM.
7. Add specific antagomir or control (scrambled antagomir) at varying concentrations. Start with concentrations in the range of 0.5–5 μM.
8. Assay effect of the antagomir treatment by monitoring cell numbers, morphology, or performing your functional assays of interest (*see* **Note 21**).
9. Assess effectiveness of miRNA knockdown via real-time PCR (*see* **Note 22**).

4 Notes

1. Wear lab coat, safety goggles, and blue nitrile gloves over a pair of latex gloves during all extraction procedures. Work in the fume hood. Work with sterile technique and filtered pipette tips to avoid contamination. Use RNase-free solutions kept aside for RNA work only.
2. If using samples with low levels of RNA, glycogen addition can help increase the precipitation of RNA for better yields.
3. Following centrifugation, the mixture will separate into a lower red, phenol chloroform phase (containing protein), and a colorless upper aqueous phase (containing RNA) separated by an interphase (containing DNA). Care should be taken when pipetting the aqueous phase to avoid transfer of contaminating DNA from the interphase section. We recommend leaving some of the upper aqueous phase to minimize carry-over of DNA contamination.
4. Volumes are based on an expected aqueous phase volume of 500 μL, from the previous step. If the actual amount differs, adjust the volume of isopropanol added to make a 1:1 ratio. If using samples with low levels of RNA, samples can be incubated overnight at –20 °C at this step to maximize RNA precipitation. When loading tubes into the centrifuge, it helps

to have all tubes in the same orientation. This ensures RNA pellets are in the same position in each tube, making it easier to identify the pellets. The pellet is slightly white in color and should be visible at this stage. It is easier to identify against a dark background, e.g., blue gloves.

5. Do not allow pellet to air-dry completely, as this will decrease its solubility. Once pellet begins to change from a white to transparent color, add RNase-free water to resuspend.
6. Do not vortex or mix vigorously. Pipette sample up and down thoroughly to avoid leaving residues on the side of the tube.
7. RNA purity is assessed by determining the ratio of absorbance at 260 versus 230 and 280 nm. A ratio of approximately 2.0 indicates good quality RNA sample. A lower ratio may indicate the presence of protein, phenol, salt, or other contaminants, potentially carried over from the isolation procedure.
8. Determining the purity of RNA is important for downstream applications. However, it does not give any indication on the integrity of the purified RNA. One approach to determine RNA integrity is using an Agilent Bioanalyzer. This will also allow for the identification of DNA contamination. In the case of DNA contamination, we strongly recommend DNase treatment of RNA samples prior to further processing.
9. There are a number of different manufacturer's products and approaches for miRNA reverse transcription and real-time PCR experiments. For the protocols in this chapter, we will present approaches using Applied Biosystems TaqMan kits (Life Technologies).
10. When preparing the RT master mix, multiply each reagent by the number of samples to be analyzed. Make one RT master mix for each miRNA to be analyzed. Always include ~10 % excess to allow for pipetting error.
11. Before opening a tube of TaqMan RT primer, centrifuge to bring solution to the bottom of the tube to prevent loss of liquid trapped in the lid.
12. When preparing the PCR master mix, multiply reagents by the number of samples to be analyzed. Make one PCR master mix for each miRNA to be analyzed. Always include ~10 % reagent excess to allow for pipetting error. Allow for triplicate wells for each sample and each miRNA.
13. Always perform endogenous control assays for each sample used on the plate to maintain experiment-to-experiment reproducibility. "No template" controls should also be performed for each different miRNA assay on the assay plate to confirm reaction specificity.
14. We suggest adding the PCR master mix to the wells first, followed by addition of the RT product.

15. If using a PCR master mix containing AmpErase UNG, add a 50 °C for 2 min step before step (a) in the PCR protocol to induce activity of AmpErase UNG.
16. Samples with a total RNA concentration of 115 ng/μL or above can be reverse transcribed into cDNA for direct use in Taqman Low Density MicroRNA Arrays. For samples with a lower concentration it is recommended to perform a pre-amplification before performing Taqman Low Density MicroRNA Arrays.
17. Array cards A and B are available for both human and mouse samples. Each panel A and B must be used in separate RT reactions using Megaplex primer pools A or B. We recommend performing the reaction for each panel for all samples at the same time.
18. Visually confirm that the PCR master mix is loaded on the array card. The level for all eight loading ports should be equal; individual cells should all contain PCR master mix and no bubbles should be present within the cells.
19. Take care that the stacker and TaqMan miRNA Array Card are in the correct orientation before sealing the card, as sealing in the wrong orientation will damage the backing foil and destroy the array.
20. Prior to ordering antagomirs, confirm the proper annotations with each synthesis provider and their capability to integrate each of the required modifications to ensure desired products are produced.
21. If using fluorescently labeled antagomir, cells can be assessed under the fluorescent microscope or via flow cytometry to assess antagomir uptake.
22. Knockdown of mature miRNA levels is often observed at these suggested antagomir concentrations, but the detection, confirmation, and degree of knockdown may vary by cell type, experimental setup, and initial miRNA expression levels in the cells.

References

1. Lewis BP, Burge CB, Bartel DP (2005) Conserved seed pairing, often flanked by adenosines, indicates that thousands of human genes are microRNA targets. *Cell* 120(1):15–20
2. Berezikov E (2011) Evolution of microRNA diversity and regulation in animals. *Nat Rev Genet* 12(12):846–860
3. Friedman RC et al (2009) Most mammalian mRNAs are conserved targets of microRNAs. *Genome Res* 19(1):92–105
4. Lee Y et al (2004) MicroRNA genes are transcribed by RNA polymerase II. *EMBO J* 23(20):4051–4060
5. Lee Y et al (2002) MicroRNA maturation: stepwise processing and subcellular localization. *EMBO J* 21(17):4663–4670
6. Denli AM et al (2004) Processing of primary microRNAs by the Microprocessor complex. *Nature* 432(7014):231–235
7. Hutvagner G et al (2001) A cellular function for the RNA-interference enzyme Dicer in the maturation of the let-7 small temporal RNA. *Science* 293(5531):834–838
8. Ketting RF et al (2001) Dicer functions in RNA interference and in synthesis of small RNA involved in developmental timing in *C. elegans*. *Genes Dev* 15(20):2654–2659

9. Bartel DP (2004) MicroRNAs: genomics, biogenesis, mechanism, and function. *Cell* 116(2):281–297
10. Weinmann L et al (2009) Importin 8 is a gene silencing factor that targets argonaute proteins to distinct mRNAs. *Cell* 136(3):496–507
11. Hammond SM et al (2001) Argonaute2, a link between genetic and biochemical analyses of RNAi. *Science* 293(5532):1146–1150
12. Mallory AC et al (2004) MicroRNA control of PHABULOSA in leaf development: importance of pairing to the microRNA 5' region. *EMBO J* 23(16):3356–3364
13. Liu J (2008) Control of protein synthesis and mRNA degradation by microRNAs. *Curr Opin Cell Biol* 20(2):214–221
14. Lewis BP et al (2003) Prediction of mammalian microRNA targets. *Cell* 115(7):787–798
15. Lim LP et al (2005) Microarray analysis shows that some microRNAs downregulate large numbers of target mRNAs. *Nature* 433(7027):769–773
16. Wightman B, Ha I, Ruvkun G (1993) Posttranscriptional regulation of the heterochronic gene *lin-14* by *lin-4* mediates temporal pattern formation in *C. elegans*. *Cell* 75(5):855–862
17. Pillai RS et al (2005) Inhibition of translational initiation by Let-7 MicroRNA in human cells. *Science* 309(5740):1573–1576
18. Nottrott S, Simard MJ, Richter JD (2006) Human let-7a miRNA blocks protein production on actively translating polyribosomes. *Nat Struct Mol Biol* 13(12):1108–1114
19. Petersen CP et al (2006) Short RNAs repress translation after initiation in mammalian cells. *Mol Cell* 21(4):533–542
20. Bagga S et al (2005) Regulation by let-7 and lin-4 miRNAs results in target mRNA degradation. *Cell* 122(4):553–563
21. Giraldez AJ et al (2006) Zebrafish MiR-430 promotes deadenylation and clearance of maternal mRNAs. *Science* 312(5770):75–79
22. Wu L, Fan J, Belasco JG (2006) MicroRNAs direct rapid deadenylation of mRNA. *Proc Natl Acad Sci U S A* 103(11):4034–4039
23. Liu J et al (2005) MicroRNA-dependent localization of targeted mRNAs to mammalian P-bodies. *Nat Cell Biol* 7(7):719–723
24. Guo S et al (2010) MicroRNA miR-125a controls hematopoietic stem cell number. *Proc Natl Acad Sci U S A* 107(32):14229–14234
25. Gruber JJ et al (2009) Ars2 links the nuclear cap-binding complex to RNA interference and cell proliferation. *Cell* 138(2):328–339
26. Gerrits A et al (2012) Genetic screen identifies microRNA cluster 99b/let-7e/125a as a regulator of primitive hematopoietic cells. *Blood* 119(2):377–387
27. O'Connell RM et al (2010) MicroRNAs enriched in hematopoietic stem cells differentially regulate long-term hematopoietic output. *Proc Natl Acad Sci U S A* 107(32):14235–14240
28. Georgantas RW 3rd et al (2007) CD34+ hematopoietic stem-progenitor cell microRNA expression and function: a circuit diagram of differentiation control. *Proc Natl Acad Sci U S A* 104(8):2750–2755
29. Felli N et al (2005) MicroRNAs 221 and 222 inhibit normal erythropoiesis and erythroleukemic cell growth via kit receptor downmodulation. *Proc Natl Acad Sci U S A* 102(50):18081–18086
30. Lu J et al (2008) MicroRNA-mediated control of cell fate in megakaryocyte-erythrocyte progenitors. *Dev Cell* 14(6):843–853
31. Johnnidis JB et al (2008) Regulation of progenitor cell proliferation and granulocyte function by microRNA-223. *Nature* 451(7182):1125–1129
32. Cobb BS et al (2005) T cell lineage choice and differentiation in the absence of the RNase III enzyme Dicer. *J Exp Med* 201(9):1367–1373
33. Li QJ et al (2007) miR-181a is an intrinsic modulator of T cell sensitivity and selection. *Cell* 129(1):147–161
34. Koralov SB et al (2008) Dicer ablation affects antibody diversity and cell survival in the B lymphocyte lineage. *Cell* 132(5):860–874
35. O'Carroll D et al (2007) A Slicer-independent role for Argonaute 2 in hematopoiesis and the microRNA pathway. *Genes Dev* 21(16):1999–2004
36. Xiao C et al (2007) MiR-150 controls B cell differentiation by targeting the transcription factor c-Myb. *Cell* 131(1):146–159
37. Zhou B et al (2007) miR-150, a microRNA expressed in mature B and T cells, blocks early B cell development when expressed prematurely. *Proc Natl Acad Sci U S A* 104(17):7080–7085
38. Rao DS et al (2010) MicroRNA-34a perturbs B lymphocyte development by repressing the forkhead box transcription factor Foxp1. *Immunity* 33(1):48–59
39. Fontana L et al (2007) MicroRNAs 17-5p-20a-106a control monocytopoiesis through AML1 targeting and M-CSF receptor upregulation. *Nat Cell Biol* 9(7):775–787
40. Fukao T et al (2007) An evolutionarily conserved mechanism for microRNA-223 expression revealed by microRNA gene profiling. *Cell* 129(3):617–631

41. Fazi F et al (2005) A minicircuitry comprised of microRNA-223 and transcription factors NFI-A and C/EBPalpha regulates human granulopoiesis. *Cell* 123(5):819–831
42. Costinean S et al (2006) Pre-B cell proliferation and lymphoblastic leukemia/high-grade lymphoma in E(mu)-miR155 transgenic mice. *Proc Natl Acad Sci U S A* 103(18):7024–7029
43. O'Connell RM et al (2008) Sustained expression of microRNA-155 in hematopoietic stem cells causes a myeloproliferative disorder. *J Exp Med* 205(3):585–594
44. Han YC et al (2010) microRNA-29a induces aberrant self-renewal capacity in hematopoietic progenitors, biased myeloid development, and acute myeloid leukemia. *J Exp Med* 207(3):475–489
45. Calin GA et al (2002) Frequent deletions and down-regulation of micro-RNA genes miR15 and miR16 at 13q14 in chronic lymphocytic leukemia. *Proc Natl Acad Sci U S A* 99(24):15524–15529
46. Klein U et al (2010) The DLEU2/miR-15a/16-1 cluster controls B cell proliferation and its deletion leads to chronic lymphocytic leukemia. *Cancer Cell* 17(1):28–40
47. Chen XM et al (2007) A cellular micro-RNA, let-7i, regulates Toll-like receptor 4 expression and contributes to cholangiocyte immune responses against *Cryptosporidium parvum* infection. *J Biol Chem* 282(39):28929–28938
48. Bazzoni F et al (2009) Induction and regulatory function of miR-9 in human monocytes and neutrophils exposed to proinflammatory signals. *Proc Natl Acad Sci U S A* 106(13):5282–5287
49. O'Connell RM et al (2007) MicroRNA-155 is induced during the macrophage inflammatory response. *Proc Natl Acad Sci U S A* 104(5):1604–1609
50. Liu G et al (2009) miR-147, a microRNA that is induced upon Toll-like receptor stimulation, regulates murine macrophage inflammatory responses. *Proc Natl Acad Sci U S A* 106(37):15819–15824
51. Sheedy FJ et al (2010) Negative regulation of TLR4 via targeting of the proinflammatory tumor suppressor PDCD4 by the microRNA miR-21. *Nat Immunol* 11(2):141–147
52. Taganov KD et al (2006) NF-kappaB-dependent induction of microRNA miR-146, an inhibitor targeted to signaling proteins of innate immune responses. *Proc Natl Acad Sci U S A* 103(33):12481–12486
53. Rodriguez A et al (2007) Requirement of bic/microRNA-155 for normal immune function. *Science* 316(5824):608–611
54. Thai TH et al (2007) Regulation of the germinal center response by microRNA-155. *Science* 316(5824):604–608
55. Du C et al (2009) MicroRNA miR-326 regulates TH-17 differentiation and is associated with the pathogenesis of multiple sclerosis. *Nat Immunol* 10(12):1252–1259
56. Mattes J et al (2009) Antagonism of microRNA-126 suppresses the effector function of TH2 cells and the development of allergic airways disease. *Proc Natl Acad Sci U S A* 106(44):18704–18709
57. Lu TX, Munitz A, Rothenberg ME (2009) MicroRNA-21 is up-regulated in allergic airway inflammation and regulates IL-12p35 expression. *J Immunol* 182(8):4994–5002
58. Liu G et al (2010) miR-21 mediates fibrogenic activation of pulmonary fibroblasts and lung fibrosis. *J Exp Med* 207(8):1589–1597
59. Monticelli S et al (2005) MicroRNA profiling of the murine hematopoietic system. *Genome Biol* 6(8):R71
60. Kuchen S et al (2010) Regulation of microRNA expression and abundance during lymphopoiesis. *Immunity* 32(6):828–839
61. Mayoral RJ et al (2009) MicroRNA-221-222 regulate the cell cycle in mast cells. *J Immunol* 182(1):433–445
62. Lee YN et al (2011) KIT signaling regulates MITF expression through miRNAs in normal and malignant mast cell proliferation. *Blood* 117(13):3629–3640
63. Ishizaki T et al (2011) miR126 positively regulates mast cell proliferation and cytokine production through suppressing *Spred1*. *Genes Cells* 16(7):803–814
64. Mayoral RJ et al (2011) MiR-221 influences effector functions and actin cytoskeleton in mast cells. *PLoS One* 6(10):e26133
65. Molnar V et al (2012) MicroRNA-132 targets HB-EGF upon IgE-mediated activation in murine and human mast cells. *Cell Mol Life Sci* 69(5):793–808