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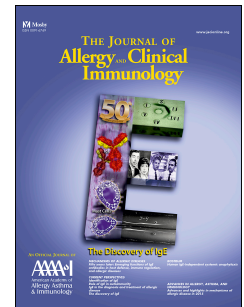
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Methods in Allergy and Immunology

MicroRNA

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28 List of Abbreviations:

29 RNA: ribonucleic acid

30 miRNA: microRNA

31 mRNA: messenger RNA

32 PCR: polymerase chain reaction

33 qPCR: quantitative polymerase chain reaction

34 CRISPR: clustered regularly interspaced short palindromic repeats

35 Cas9: CRISPR associated protein 9

36 AGO: Argonaute

37 GC-content: guanine-cytosine content

38

39 Key Words:

40 miRNA

41 microRNA

42 microRNA methods

43 Allergy

44 qPCR

45 in-situ hybridization

46 microarray

47 miRNA isolation

48 miRNA quantitation

49 miRNA profiling

50 miRNA expression

51 anti-miRs

52 antagomiRs

53 miRNA mimics

54 miRNA inhibitors

55 miRNA targets

56 CRISPR

57 CRISPR/Cas9

58 Gene-silencing

59 Stem-loop

60

Abstract

MicroRNAs (miRNAs) are small endogenous RNAs that regulate gene-expression post-transcriptionally. MiRNA research in allergy is expanding because miRNAs are crucial regulators of gene expression and promising candidates for biomarker development. MiRNA mimics and miRNA inhibitors currently in preclinical development have shown promise as novel therapeutic agents. Multiple technological platforms have been developed for miRNA isolation, miRNA quantitation, miRNA profiling, miRNA target detection and for modulating miRNA levels in vitro and in vivo. Here we will review the major technological platforms with consideration given for the advantages and disadvantages of each platform.

MicroRNAs (miRNAs) are short RNA molecules 19 to 25 nucleotides in size that regulate post-transcriptional silencing of target genes. A single miRNA can target hundreds of mRNAs and influence the expression of many genes often involved in a functional interacting pathway. MiRNAs has been shown to be involved in the pathogenesis of many allergic diseases including asthma, eosinophilic esophagitis, allergic rhinitis and eczema.¹⁻⁵ We refer our readers to references 6 - 9 for further readings on details of miRNA biology.⁶⁻⁹ This article will review methods used in miRNA isolation, expression level detection, target identification and potential ways to target miRNAs experimentally and therapeutically.

miRNA Isolation

MiRNA can be isolated from cells, tissues and body fluids such as serum, plasma, tears or urine.¹⁰ The early work published in the field used the traditional phenol-chloroform extraction followed by RNA precipitation (Figure 1A). A widely used reagent is Trizol. However, there is often the presence of high level of contaminants using this method. In addition, it has been found that miRNAs with a low GC-content are selectively lost during phenol-chloroform extraction from a small number of cells, and this is due to the inefficiency of precipitating small nucleic acids compared to long nucleic acids. Using a column based RNA adsorption method during miRNA isolation avoids these issues.¹¹ The initial column based method involves loading the aqueous phase from the Phenol-Chloroform extraction on to an RNA adsorption column followed by wash and elution of miRNA (Figure 1B). The mirVana and miRNEasy kits are two widely used kits for this method. Newer commercially available kits such as the Direct-zol kits skip the phase separation step. The phenol reagent containing miRNA can be directly loaded

onto an RNA adsorption column followed by wash and elution of miRNA (Figure 1B). Care should be taken to avoid overloading the columns, as overloading significantly reduces RNA yield and quality. Most commercial kits designed for miRNA isolation provides a protocol for isolation total RNA containing miRNA and an alternative protocol for separation of the small RNA (<200 nucleotides) enriched fraction and the large RNA (>200 nucleotide) fraction. The wash buffer RW1 included in the Qiagen RNEasy total RNA isolation kit wash away all small nucleic acids during the buffer wash step, and therefore this kit is not suitable for miRNA isolation. However, if samples were already lysed in the lysis buffer RLT included in the Qiagen RNEasy kit, modified protocols are available to re-capture the miRNA.¹² Isolation of miRNA from body fluids presents additional challenges. The yield of miRNA in body fluids is much lower compared to cells or tissues. A large amount of starting body fluid sample is often needed that exceeds the sample input volume limit of some commercially available kits.¹³ Both the miRNEasy serum/plasma kit and the miRCURY RNA isolation kit - Biofluids have a maximum fluid sample input of 200 μ l. The mirVana PARIS kit has been designed to isolate RNA from up to 625 μ L of liquid sample. For larger sample input volume, the QIAamp circulating nucleic acid kit can be used that enables miRNA isolation from up to 3 mL of starting sample.¹³ Care should be taken to specifically use the miRNA protocol and use the lysis buffer without the carrier RNA.

miRNA expression detection

MiRNA expression can be detected in both tissue samples as well as in cell-free biological fluids such as serum or plasma. Current methodologies used for detecting miRNAs include quantitative PCR (qPCR), in-situ hybridization, microarrays and RNA-sequencing. Because the

length of miRNA is typically only 21 to 23 base pairs, it is technically challenging to design PCR primers as the conventional PCR primer is about 20 base pairs long. The solution is to extend the length of miRNA during the reverse transcription step by either utilizing a miRNA specific stem loop primer for transcription (Figure 2A) or universal reverse transcription by adding a 3'poly A tail to the miRNA and then use a poly T primer with a universal sequence appended at the 3' end for reverse transcription (Figure 2B). Subsequently qPCR is performed with forward primer/probe that are specific to each miRNA and the reverse primer complementary to the stem-loop or the universal sequence of the poly T primer.^{14, 15} A universal adapter can also be added to the 5'end to allow an optional universal pre-amplification prior to qPCR for detection of very low abundance targets with primer/probe (Figure 2C). Compared to the universal reverse transcription, the stem-loop primer based method has a higher specificity but the reverse transcription step is limited to one miRNA at a time.¹⁶ Multiplex stem-loop primer pools are available to overcome this limitation.¹⁷ qPCR of specific template will typically give 10 to 100-fold higher amplification signal than template with a single nucleotide difference, although distinguishing miRNAs that differ by only 1-2 nucleotides with PCR remains a challenge.¹⁸ The microarray based methods include multiplex qPCR based arrays, and hybridization based arrays. The qPCR microarrays use pre-plated PCR primer/probes distributed across 96 or 384 well plates. For low amount of input material a microfluidic card is available that requires as low as 1 ng of total RNA and microfluidic systems are available that enable single cell miRNA profiling.¹⁶ A list of available PCR based arrays can be found in table 1. The hybridization based arrays have the advantage of allowing a large number of parallel measurements per sample at a relatively low cost. Due to limited specificity, findings from hybridization based arrays are typically

validated with a second method such as qPCR or in-situ hybridization.¹⁶ In-situ hybridization has the advantage of determining the tissue origin of the miRNA of interest and the relative expression level across different tissue compartments. Locked nucleic acid probes are frequently used to increase binding affinity and mismatch discrimination.^{19, 20} Using high throughput sequencing, a small RNA sequencing library can be constructed and sequenced to enable quantitative identification of all small RNA species in a particular sample, and to enable discovery of novel miRNAs and other small non-coding RNAs.¹⁶ A starting material of 10 to 50 ng of small RNA is required for the library construction.

Target Detection

MicroRNAs mediate post-transcriptional gene silencing of target genes by targeting the 3' untranslated region of mRNA, with the seed region (shown in Figure 3A) in nucleotide 2-7 in the 5' end of miRNA being the crucial sequence. From a transient double stranded miRNA duplex, the guide strand (mature miRNA strand) is incorporated into the RNA induced silencing complex to mediate gene silencing and the passenger strand is degraded.⁹ The in silico based target prediction methods utilize a variety of factors including complementarity to seed region of miRNA, and conservation through evolution, energetically favorable hybridization of miRNA/mRNA etc. Several algorithms are available through web interface including Targetscan.org²¹⁻²⁴ and microRNA.org²⁵⁻²⁸, which generate a large number of predicted targets, with many of them presumed to be false targets.²⁹ The in silico predicted targets can be verified by cloning 3'UTR of target genes into the 3'UTR region of a reporter vector, followed by co-transfection with targeting miRNA and demonstrating knock-down of reporter activity (Figure

3B). Specificity is demonstrated by mutating the region of target 3'UTR that binds the seed region of miRNA, and demonstrating that the targeting effect no longer exist after the seed region is mutated. Another method is to transiently transfect miRNA mimic or miRNA antagonist into the cell of interest, followed by whole transcriptome sequencing to identify both direct and indirect targets.^{30, 31} MiRNA mimics are double-stranded RNA molecules that imitate the endogenous miRNA duplexes. The transfection of miRNA mimics should be used with caution. It has been reported that transfection of miRNA mimics at low concentrations fails to suppress target gene expression and transfection of miRNA mimics at high concentrations leads to non-specific changes in gene expression.³² This may be due to the failure of the guide strand from the transfected miRNA mimics to be incorporated into the RNA induced silencing complex and the accumulation of high-molecular weight RNA species.^{32, 33} Significant incorporation of the passenger strand instead of the guide strand is another source for discrepancy.³⁴ Using plasmid transfection or lentiviral transduction appears to avoid these problems since the biogenesis of miRNA from these methods likely use the same cellular processing pathways as endogenous miRNAs.³² The transfection of miRNA inhibitors is less problematic since the miRNA inhibitors do not need to undergo cellular processing. Chemical modifications are incorporated into the miRNA inhibitor to increase its stability and protect it from degradation by endogenous nucleases.³⁵ MiRNA inhibitor cross reactivity for closely related miRNA family members has been reported where all family members with closely related sequences were inhibited by a particular miRNA inhibitor.³⁶ The recent development of CRISPR/Cas9 technology has enabled in vitro targeting of the genomic loci that encodes the miRNA of interest. Whole transcriptome sequencing can then be performed to identify both the

direct and indirect targets of the miRNA of interest (Figure 3C).³⁷ Advantages of this method include identifying targets in the cell of interest, identifying both direct and indirect targets, and stability of the targeting effect. Some mature miRNAs are generated from multiple separate loci. These miRNAs represent a challenge for genetic deletion using CRISPR/Cas9 technology, since multiple loci have to be targeted simultaneously. The binding of miRNA to mRNA requires a protein complex called RNA induced silencing complex with the core of the complex containing a miRNA guiding an Argonaute (AGO) protein to target mRNA. Another method to identify global miRNA/mRNA interaction is to cross-link the RNA/Protein complex followed by immunoprecipitation of the RNA induced silencing complex. The protein is then digested by proteinase K and the remaining RNA is sequenced to identify miRNA/target interaction (Figure 3D).³⁸

Modulating miRNA levels in vitro and in vivo

miRNA levels can be modulated in vitro by utilizing transient transfections of miRNA mimics or miRNA antagonists. The transfection efficiency and the transient nature of the transfection limits the type of functional study that can be performed. Stable clones can be generated in vitro by using lentiviral vectors that encode miRNA mimics or miRNA antagonists.^{39, 40} The promoters driving the expression of miRNAs needs to be carefully selected to make sure that the cellular miRNA processing machinery is not overwhelmed by the strong promoter-driven expression of the transduced miRNA. The CRISPR/Cas9 technology enables stable targeting of miRNA without the use of a lentiviral facility. However, clone selection is laborious and targeting miRNAs generated from multiple loci remains a challenge. Experimentally, gene

targeted mice where many individual miRNAs are either constitutively or conditionally knock-out have been developed.⁴¹ This enables the study of the function of miRNA in either the animal disease model of interest or the tissue of interest, provided that the miRNA and its binding sites within its target mRNAs are conserved between mice and humans. Another way to modulate miRNA levels in vivo is to use cholesterol conjugated anti-miRNAs that can be administered via an intravenous infusion using a weight based regimen, and the anti-miRNAs are efficiently up-taken by all tissues except the brain. The knock-down is stable for up to 21 days in vivo.⁴² Other methods to deliver miRNAs in vivo include liposomal mediated delivery or using polymer based nanoparticle delivery vehicles.⁴³ Topical delivery of the miRNA mimics/inhibitors intranasally that requires a much lower dose than systemic delivery has been described, and this can potentially be used in the study or treatment of allergic disease.⁴⁴

Summary

MiRNAs have been shown to regulate multiple allergic diseases and represent an exciting area of research. Several miRNAs have been showed to target key pathogenic pathways involved in allergic inflammation and have the potential to be developed into novel therapeutic targets. The methods reviewed above will aid researchers who are beginning to explore the field of miRNA in allergic inflammation, and help to avoid potential pitfalls involved in miRNA research.

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- 320

321

Figure Legends

Figure 1. miRNA Extraction methods. (A): Phenol-chloroform based phase separation followed by pelleting of RNA and re-dissolve in nuclease free water. (B): The Aqueous phase from the phenol-chloroform can be loaded onto an RNA adsorbing column. After wash steps, RNA is eluted in nuclease free water. Alternatively, newer miRNA extraction kits allow the user to skip the phase separation step. The samples homogenized in phenol can be loaded directly onto an RNA adsorbing column, followed by wash steps and elution of RNA. A proprietary lysis buffer may be provided by the manufacturer instead of the phenol based reagent.

Figure 2. Quantitative RT-PCR for miRNA detection. (A): Reverse transcription with miRNA specific stem-loop primer, followed by qPCR using a miRNA specific probe, a miRNA specific forward primer (Green) and reverse primer complementary to the stem-loop sequence (Orange). (B) Universal reverse transcription by adding polyA tails to 3'end of miRNA, followed by reverse transcription with a Poly T primer with a universal sequence (Tag) appended at the 3'end, then qPCR with miRNA specific forward primer (Green) and universal reverse primer (Orange). (C) Universal reverse transcription by adding a 5'adapter sequence and polyA tails to 3'end of miRNA, followed by reverse transcription with a Poly T primer with a universal sequence (Tag) appended at the 3'end, followed by optional universal amplification step with 5' primer binding to the adapter sequence (Blue) and 3'primer binding to the tagged sequence (orange), followed by qPCR with a miRNA specific probe, a miRNA specific forward primer (Green) and a universal reverse primer (Orange).

344

345 Figure 3. MiRNA target detection. (A) An illustration of a miRNA/mRNA base pair, highlighting
346 the critical seed region required for miRNA targeting. (B) Target verification by cloning the
347 3'UTR of the target mRNA into a luciferase reporter vector, followed by co-transfection of
348 miRNA mimics/inhibitors and reading the luciferase activity. (C) Specific miRNAs are targeted by
349 transfecting CRISPR/Cas9 targeting sequence, followed by puromycin selection and single clone
350 isolation. The clone with loss of miRNA expression (Red) is expanded and whole transcriptome
351 sequencing is performed to identify direct and indirect targets. (D). Protein/DNA in cells are UV
352 cross linked, followed by cell lysis and Immunoprecipitation of AGO protein in the RNA induced
353 silencing complex. A 3' linker is ligated, the RNA induced silencing complex is digested by
354 proteinase K and a 5' linker is ligated. This is followed by reverse transcription, PCR
355 amplification and high-throughput sequencing.

Table 1 Major qPCR array platforms used for miRNA profiling

Platform	Vendor	Recommended input amount	Reaction Volume
miScript miRNA PCR Arrays	Qiagen	125 – 250 ng of total RNA	25 µl for 96-well plate, 10 µl for 384-well plate
miProfile miRNA qPCR Arrays	Genecopoeia	500 – 1000 ng of small RNA	20 µl
TaqMan Array Human MicroRNA Cards	Thermo Fisher	1 – 350 ng of total RNA with pre-amplification, 350 – 1000 ng of total RNA without pre-amplification	1 µl
TaqMan Open Array microRNA Panels	Thermo Fisher	50 – 200 ng of total RNA	33 nl
microRNA Ready-to-Use PCR Panels	Exiqon	20 – 40 ng of total RNA	10 µl
miRNome microRNA Profilers	System Biosciences	100 – 800 ng of total RNA	5 µl
BioMark HD System	Fluidigm	20 ng of total RNA, Single cell as input when used with the C1 single cell prep-system	5 µl

Figure 1A

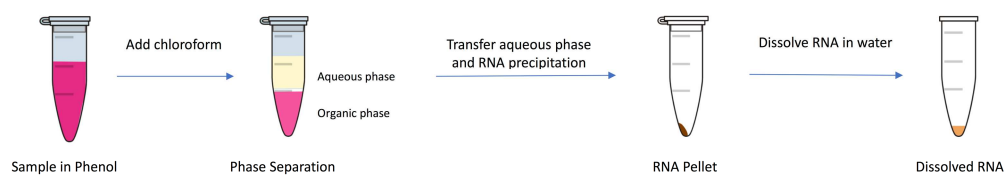


Figure 1B

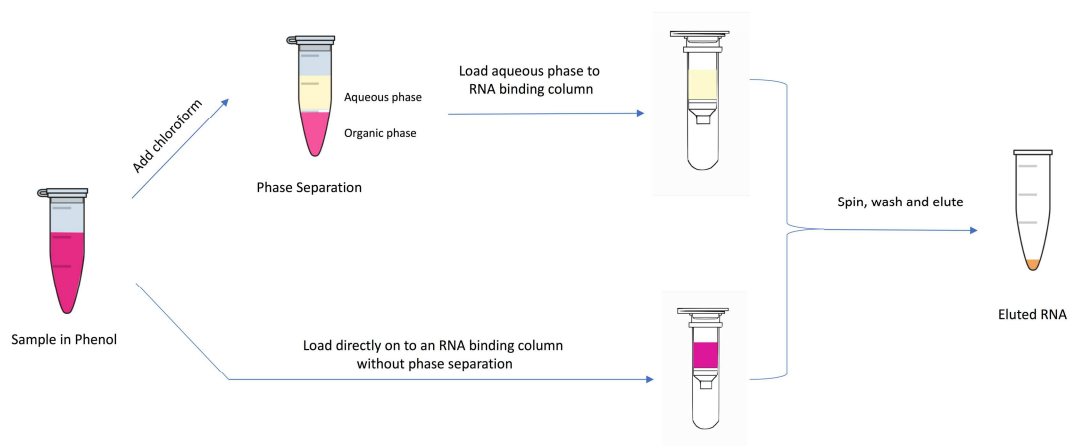


Figure 2A

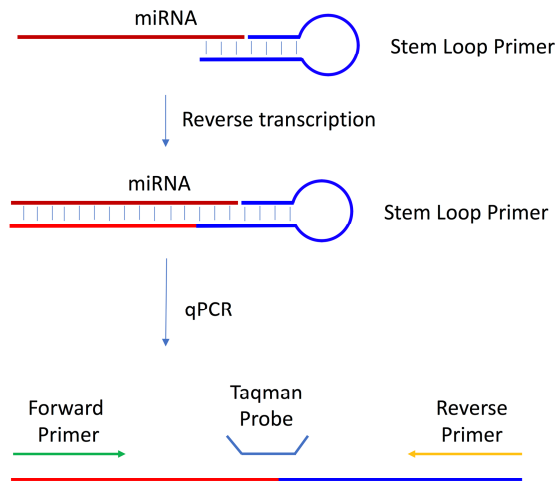


Figure 2B

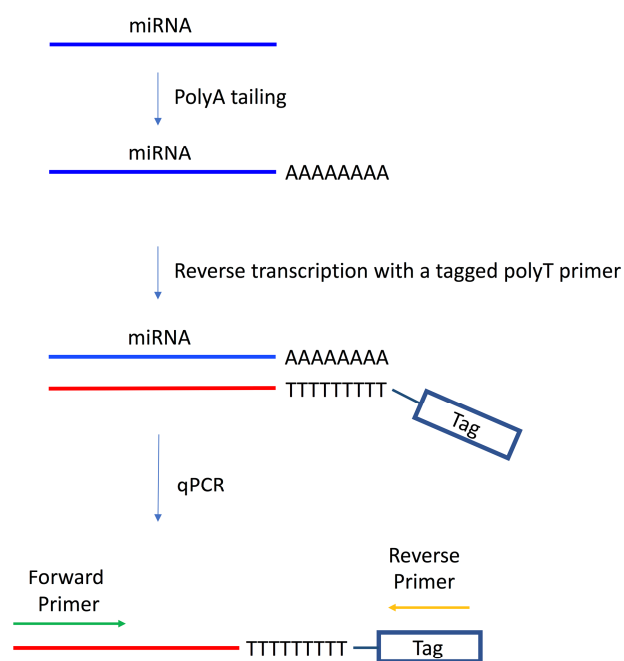


Figure 2C

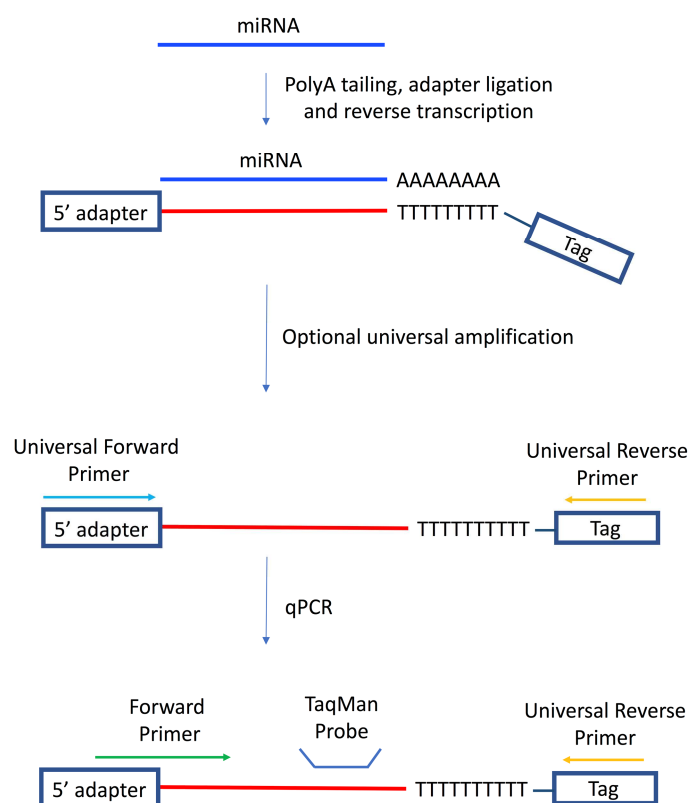


Figure 3A

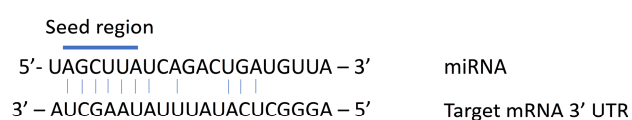


Figure 3B

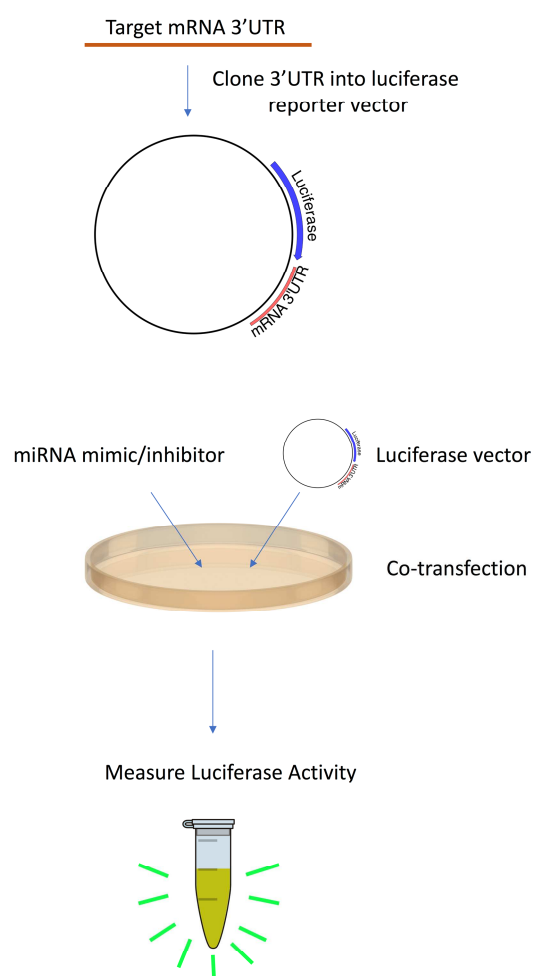


Figure 3C

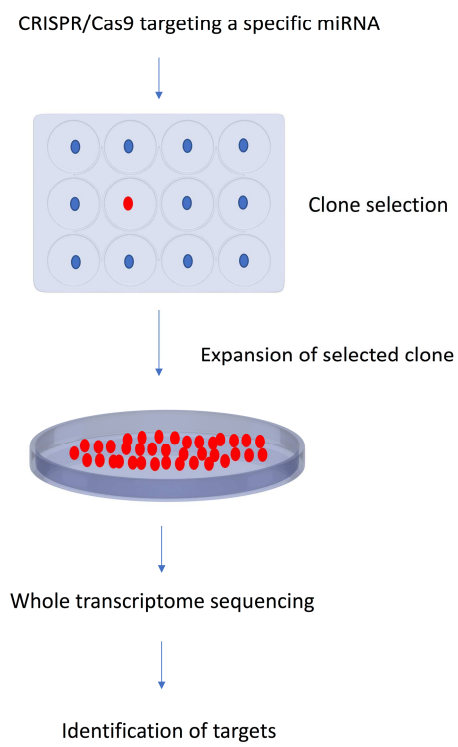


Figure 3D

