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Chapter 2

C0010 # microRNA Profiling: An Overview of Current Technologies and Applications

Sinead M. Smith* and David W. Murray†

*Department of Clinical Medicine, School of Pharmacy and Pharmaceutical Sciences, Trinity College Dublin, Dublin 2, Ireland

†Department of Physiology and Medical Physics, Royal College of Surgeons in Ireland, Dublin 2, Ireland

Chapter Outline

s0005 1 INTRODUCTION

p0005 Recent insight into the transcriptional landscape of mammalian genomes gained from high-throughput next-generation sequencing (NGS) technologies has revealed that although 80% of the genome is transcribed, less than 2% is subsequently translated into protein, resulting in the generation of a large number of ncRNA transcripts *(1–5)*. Although originally considered to be nonfunctional, recent data have highlighted that noncoding RNAs (ncRNAs) play key roles in gene regulation by influencing transcription and translation of target genes, thus regulating a diverse range of biological processes under normal physiological conditions and in disease settings *(6)*. Broadly speaking,

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ncRNAs may be subdivided according to their size. First, long ncRNAs (lncRNAs) range in size from a few hundred nucleotides (nt) to multiple kilobases in length represent the largest class of ncRNAs and account for much of the transcribed genome *(6)*. In contrast to lncRNAs, small ncRNAs are less than 300 nt in length and include microRNAs (miRNAs), piwi-interacting RNAs, small-interfering RNAs, and small nuclear RNAs among others *(7)*. The miRNAs (18–25 nt) are the most widely studied family of the small ncRNAs. Since their discovery in 1993 *(8,9)*, the diversity and significance of this class of regulatory molecule has become increasingly appreciated. miRNAs posttranscriptionally decrease the expression of thousands of target genes by binding to specific messenger RNA (mRNA) targets and promoting their degradation and/or inhibiting their translation *(10–12)*. Although a relatively limited number of miRNAs (approximately 1000) have been identified in humans compared with the number of mRNAs and proteins (approximately 30,000), a single miRNA may regulate hundreds of mRNAs and thus has the potential to greatly impact gene-expression networks *(10)*.

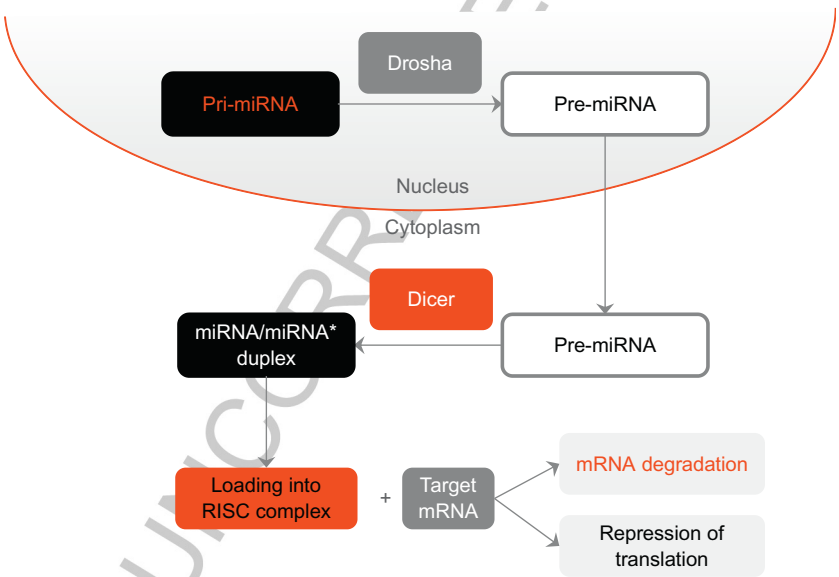
p0010 The importance of miRNAs in biological processes is demonstrated by their high levels of evolutionary conservation *(13)*. Accumulating evidence supports a role for miRNAs in normal cellular physiology, where they act as key regulators of development *(14)*, differentiation *(15,16)*, cell proliferation, and apoptosis *(17,18)*. For example, both specific miRNAs and proteins involved in miRNA processing play fundamental roles in the development and function of B- and T-cells within the immune system *(19–23)*. Additionally, miRNAs coordinate cellular responses in innate immune cells *(24)* and play key roles in the regulation of host–pathogen interactions during infection *(25)*. miRNA expression is also regulated during inflammation *(25–27)* and as a consequence perturbed miRNA expression is associated with a number of autoimmune diseases, including multiple sclerosis *(28)*, rheumatoid arthritis *(29,30)*, and systemic lupus erythematosus *(31,32)*. Furthermore, expression profiling has implicated miRNAs in numerous cancers, including B-cell chronic lymphocytic leukemia *(33)* and cancer of the breast *(34–37)*, colon *(35,38,39)*, liver *(40,41)*, and lung *(35,42–47)*, among others. miRNAs function as both tumor suppressors and oncogenes *(48,49)*. Given the key roles of miRNAs in normal cellular homeostasis and their dysregulation under disease conditions, investigation of miRNA expression profiles and regulation of their mRNA targets is essential for a complete understanding of cell signaling mechanisms that mediate cell function in health and disease, with the potential to identify new therapeutic agents or drug targets. Moreover, as miRNAs are stable in a variety of clinical specimens, including formalin-fixed paraffin-embedded (FFPE) tissue, blood, and urine, there is substantial interest in their development as biomarkers for diagnostic applications. Indeed, profiling of circulating miRNAs has already shown promise for detection of cancers *(36,44,45,50–53)*.

Comp. by: DElayaraja Stage: Proof Chapter No.: 2 Title Name: COACChennai
Date:4/3/14 Time:15:11:28 Page Number: 22

B978-0-444-62650-9.00002-6, 00002

s0010 2 miRNA BIOGENESIS AND NOMENCLATURE

p0015 An understanding of the multistep processes involved in miRNA processing and biogenesis is required for the design and application of analytical techniques for miRNA detection and quantitation. Primary miRNA (pri-miRNA) transcripts are either transcribed by RNA polymerase II from independent genes or represent introns of protein-coding genes *(54–56)*. A pri-miRNA transcript can contain a single miRNA or multiple miRNAs that are processed from the same transcript *(25)*. The pri-miRNA folds into a hairpin structure, which acts as a substrate for cleavage by the endonuclease Drosha resulting in an approximately 70–100 nt long precursor miRNA (pre-miRNA) (Figure 1). Following the Drosha cleavage, the pre-miRNA is exported to the cytoplasm by Exportin, where it is further processed by the endonuclease Dicer to produce an miRNA duplex comprised of miRNA strands derived from the 5′ and 3′ regions of the precursor duplex *(7,10,55)*. One strand of this duplex, representing a mature miRNA, is incorporated into the RNA-induced silencing complex (RISC), while the other passenger strand is usually degraded. As part of the RISC, miRNAs base pair with complete or partial



f0005 **FIGURE 1** miRNA Biogenesis. In the nucleus, pri-miRNA transcripts are either transcribed by RNA polymerase II from independent genes or represent introns of protein-coding genes. Drosha mediates the processing of a pri-miRNA to a pre-miRNA. Following Drosha cleavage, the pre-miRNA is transported to the cytoplasm. Once in the cytoplasm, Dicer processes the pre-miRNA to produce a miRNA duplex derived from the 5′ and 3′ regions of the precursor. Mature miRNAs are loaded into the RISC complex to target specific mRNA molecules for degradation or inhibition of translation.

Comp. by: DElayaraja Stage: Proof Chapter No.: 2 Title Name: COACChennai
Date:4/3/14 Time:15:11:28 Page Number: 23

B978-0-444-62650-9.00002-6, 00002

complementarity to sequences in the 3′ untranslated region (3′-UTR) of target mRNAs and induce mRNA translational repression or instability by deadenylation and degradation *(55)*.

p0020 Different mature miRNA species can be produced from a single pre-miRNA molecule, as distinct miRNAs are generated from the 3′ and 5′ arms of the pre-miRNA duplex. In addition, a given mature miRNA may comprise a distribution of sizes centered around 22 nt rather than a discrete length. The variation in mature miRNA length is due to 3′ or 5′ end posttranscriptional modifications that include addition or deletion of nucleotides *(10,57,58)*, which have been shown to affect miRNA stability and function. Addition or deletion of nucleotides to the 5′ end of the mature miRNA can have significant effects on miRNA function by shifting the sequence of the seed region, which is the +2 to +8 nucleotide position from the 5′ end of the miRNA that determines the mRNA target within the RISC *(59)*. Profiling approaches therefore need to distinguish among pri-miRNAs, pre-miRNAs, and mature miRNAs and take into account mature miRNA sequence variations.

p0025 miRNAs are usually designated with a three letter species prefix (e.g., hsa-miR-X for *Homo sapiens*; mmu-miR-X for *Mus musculus*) and a number that designates the specific miRNA *(56)*. Prefixes may also be added to reflect the stage of miRNA biogenesis of a given transcript (e.g., pri-mir-X for a pri-miRNA; pre-mir-X for a precursory miRNA) *(10)*. Furthermore, suffixes are also added to indicate whether a mature miRNA arose from the 3′ (miR-X-3p) or 5′ arm (miR-X-5p) of the pre-miRNA hairpin, and a capital "R" is used in mature miRNA nomenclature. miRNAs with related sequences belonging to the same family often have lower case letters following the name (e.g., miR-Xa; miR-Xb, miR-Xc) *(56)*. Mature miRNAs with identical sequences that are derived from transcripts encoded by multiple loci are differentiated by a numerical suffix (e.g., miR-X-1, miR-X-2, miR-X-3) *(10)*. miRNA nomenclature allows for distinction between the two single-stranded mature sequences that originate from the two strands of the double-stranded miRNA molecule, for example, miR-X and miR-X*, which indicate the major and minor strands, respectively *(10,56)*. Since recent NGS analysis has shown that the relative quantities of miRNA strands can vary in different cell types and tissues, this form of strand differentiation may soon be replaced by solely describing the strands using the -5p and -3p suffixes.

s0015 3 CONSIDERATIONS FOR miRNA PROFILING

p0030 Properties specific to miRNA molecules must be taken into account during miRNA profiling. The short length of mature miRNAs (approximately 22 nt) is not sufficient for annealing of traditional primers commonly used to generate complementary DNA (cDNA) during reverse transcription (RT) for subsequent downstream profiling. Mature miRNAs also lack a poly(A) tail

Comp. by: DElayaraja Stage: Proof Chapter No.: 2 Title Name: COACChennai
Date:4/3/14 Time:15:11:29 Page Number: 24

B978-0-444-62650-9.00002-6, 00002

for use as a universal primer binding site or in selective enrichment processes. This has implications, given that miRNAs must be selectively detected in a background of diverse RNA molecules, including pri-miRNAs and pre-miRNAs that contain the same sequence as the mature miRNA *(10,58)*. miRNAs are heterogeneous in their GC content, resulting in variations in their melting temperatures (Tm). miRNAs within a family can differ by a single nucleotide. Additionally, as mentioned in Section 2, posttranscriptional modifications can result in mature miRNAs of different lengths centered around 22 nt *(10,57,58)*.

p0035 There has been a rapid advancement in the analysis platforms available for miRNA profiling and analysis in recent years. The three major approaches to detect miRNA expression are RT quantitative PCR (qPCR), hybridization-based microarrays, and RNA-sequencing (RNA-seq) (Table 1). The method of choice depends on the specific aims of a given experiment and local expertise, as well as the budget and access to specific equipment. qPCR methods allow for detection and quantitation of miRNAs through methods that are sensitive and specific, and allow for small quantities of starting material, which is particularly important in situations where samples are limited. Hybridization-based methods involve incubating labeled miRNAs with custom-made microarrays or commercial high-density oligonucleotide microarrays *(60)*. Microarray technology allows for the detection and quantitation of thousands of miRNAs simultaneously but is limited by high background levels due to cross-hybridization, a limited dynamic range due to both background and saturation of signals, and a reliance upon existing knowledge about genome sequences *(7,60,61)*. Moreover, miRNAs that display sequence length variations or base substitution differences go undetected by these approaches *(7,57)*. Recently developed RNA-seq technologies have revolutionized our ability to characterize and quantify transcriptomes by enabling RNA analysis through cDNA sequencing on a massive scale *(61)*. RNA-seq approaches provide a more precise measurement of transcript levels and their isoforms than microarray technology *(60,61)*. RNA-seq technologies provide single-base resolution and have limited background signal compared with microarrays, as the cDNA sequence reads are unambiguously mapped to unique regions of the reference genome. RNA-seq does not have an upper quantification limit, resulting in a large dynamic range of expression levels *(60)*. RNA-seq results correlate well with qPCR assays and demonstrate high levels of reproducibility for both technical and biological replicates *(60)*. Perhaps, the most exciting aspect of next-generation RNA-seq technology is the detection of both known and novel transcripts, which is particularly important in an era when novel genes and their splice variants are continually being described. Additionally, RNA-seq is particularly useful when working with poorly characterized genomes from different species. The extensive volume of data generated by RNA-seq, however, requires substantial data storage systems and computational expertise and support.

Comp. by: DElayaraja Stage: Proof Chapter No.: 2 Title Name: COACChennai
Date:4/3/14 Time:15:11:30 Page Number: 25

B978-0-444-62650-9.00002-6, 00002

t0005 **TABLE 1** Examples of miRNA Profiling Platforms

Method	Comments	Source	Platform
qPCR	Highly specific and sensitive Low quantities of starting material required Broad dynamic range of detection Customizable Cost effective Low throughput	Qiagen Exiqon Life Technologies	miScript miRNA PCR system miRCURY LNA Universal RT microRNA PCR system Taqman small RNA assays
qPCR arrays	Highly specific Low quantities of starting material required Broad dynamic range of detection Customizable Cost effective Medium throughput	Qiagen Exiqon Life Technologies	miScript miRNA PCR arrays miRNA qPCR panels Taqman low-density miRNA arrays
miRNA microarray	Less specific than qPCR or RNA-seq Limited dynamic range of detection High background Cost effective High throughput Relies on existing knowledge on genome sequence	Agilent Life Technologies Affymetrix Exiqon	SurePrint microRNA microarray NCode miRNA microarrays GeneChip microRNA array miRCURY LNA microRNA arrays
RNA-seq	Highly specific and sensitive Broad dynamic range Low background Expensive Extremely high throughput Identifies known and novel miRNAs Bioinformatically challenging	Illumina Roche Life Technologies	TruSeq small RNA sequencing 454 Sequencing SOLID next-generation sequencing

s0020 4 miRNA EXTRACTION

p0040 High-quality miRNA isolation is a fundamental step in the analytical process. miRNAs can be successfully extracted and purified from cell lines and a variety of tissue specimens and biofluids, including blood products and urine

Comp. by: DElayaraja Stage: Proof Chapter No.: 2 Title Name: COACChennai
Date:4/3/14 Time:15:11:31 Page Number: 26

B978-0-444-62650-9.00002-6, 00002

(62,63). Cell lines and tissue samples yield far greater amounts of miRNA than plasma or urine, which contain high levels of endogenous ribonuclease (RNase) enzymes that degrade RNA molecules even in very small quantities *(10,64)*. Generally speaking, the principles for miRNA isolation are similar to those for total RNA isolation, except that miRNA purification protocols are modified to retain or enrich the fraction of small RNA molecules less than 200 nt. As with extracting total RNA, extreme care must be taken to avoid degradation of the RNA sample by exposure to RNase enzymes *(65)*. Samples for RNA processing should be harvested as rapidly as possible to protect against RNase activity and to prevent unwanted changes in gene expression. Samples should be frozen immediately at −80 °C, placed in a suitable RNA stabilization solution such as RNAlater for tissue samples (Qiagen, Life Technologies, and Sigma Aldrich) and RNAprotect Cell Reagent for cultured cells (Qiagen), or lysed and homogenized immediately upon harvesting in the presence of RNase-denaturing buffers. RNA work should be performed in a designated area of the laboratory using dedicated equipment and pipettors with nuclease-free aerosol-resistant tips. RNase decontamination solutions, such as RNase Zap (Life Technologies) or RNaseKiller (Qiagen) should be used to remove RNase contamination from bench surfaces and laboratory equipment. Aseptic technique is recommended when working with RNA samples, and powder-free latex or vinyl gloves should be worn and changed frequently to prevent the introduction of RNase contamination. Samples and reagents should be prepared on ice to inhibit RNase activity and sterile, disposable, certified RNase-free plasticware should be used.

p0045 A variety of isolation kits are commercially available that involve solvent-based miRNA extraction, followed by solid-phase purification on columns *(62)*. The miRVana miRNA Isolation kit (Life Technologies) utilizes an organic extraction procedure followed by purification on a glass fiber filter to isolate total RNA ranging from 10 nt to multiple kilobases from cells and fresh or frozen tissue samples. The miRNeasy range of miRNA isolation kits (Qiagen) employ a combined phenol/guanidine-based lysis procedure, followed by a silica-membrane-based purification of total RNA from 18 nt upward. The miRNeasy mini Kit isolates total RNA including the small RNA fraction from cultured cells, laser capture microdissected specimens, and fresh or frozen tissue samples, while the miRNeasy Serum/Plasma Kit copurifies total RNA and small RNAs from serum/plasma or urine samples. The lysis buffer provided with these kits enables sample lysis, RNase activity inhibition, and cellular DNA and protein removal by organic extraction.

p0050 Histopathology archives of FFPE tissue samples are valuable resources for retrospective studies of disease. The miRNeasy FFPE kit is available from Qiagen to purify total RNA together with the small RNA fraction from FFPE tissue specimens. As nucleic acids in FFPE samples are usually heavily fragmented and chemically modified by formaldehyde, they are often a lower molecular weight than those obtained from fresh or frozen tissue samples. Prior

Comp. by: DElayaraja Stage: Proof Chapter No.: 2 Title Name: COACChennai
Date:4/3/14 Time:15:11:32 Page Number: 27

B978-0-444-62650-9.00002-6, 00002

to miRNA isolation using the miRNeasy FFPE kit, the FFPE tissue specimens are treated with a solution provided with the kit that reverses formaldehyde modification as much as possible without further RNA degradation. Proteinase K is contained within the lysis buffer to release RNA from the tissue sections. A short high-temperature incubation step partially reverses formalin cross-linking of the released nucleic acids and is followed by a DNase treatment to eliminate genomic DNA, and subsequent ethanol precipitation and purification on columns. Using either the miRVana miRNA Isolation kit or the miRNeasy range, further enrichment of the small RNA species (<200 nt) may be performed to increase the sensitivity of downstream analyses.

p0055 The concentration of the RNA sample is determined by measuring the absorbance at 260 nm in a spectrophotometer. Because larger RNA species will dominate the absorbance measurement, it is not recommended to quantify purified small RNA fractions by spectrophotometry. RNA concentration and quality should be assessed using the total RNA preparation before enriching for the small RNA fraction. Based on the fact that a 40-μg/mL sample of pure RNA has an absorbance of 1 at 260 nm, the concentration of the sample can be calculated *(65)*. The ratio of the absorbance at 260 and 280 nm is used to determine the quality of the preparation. A ratio of between 1.8 and 2.1 is indicative of high-quality RNA *(65)*. Recently developed NanoDrop spectrophotometers (Thermo Scientific) are useful for the measurement of limited sample preparations as they accurately determine RNA concentration and quality in samples volumes as small as 0.5 μL. The integrity and size distribution of total RNA can be determined using the Bioanalyzer 2100 (Agilent Technologies) or by agarose gel electrophoresis and ethidium bromide staining. In a high-quality RNA sample, the 28s ribosomal RNA (rRNA) and 18s rRNA bands should appear as sharp peaks on the Bioanalyzer or sharp bands on a gel. The desirable ratio of 28s rRNA to 18s rRNA is approximately 2:1. The Bioanalyser 2100 also provides an RNA Integrity Number that should be close to 10, especially if the RNA samples are to be considered for use in downstream applications such as microarrays or RNA-seq.

s0025 5 qPCR ANALYSIS OF miRNAs

p0060 Quantitation of the mature miRNA in its functionally active form is often performed rather than that of the pri-miRNA or pre-miRNA. However, it is sometimes of interest to investigate pri-miRNA expression in order to elucidate transcriptional control of miRNA expression. In addition, combined differential expression analysis of pri-miRNAs, pre-miRNAs, and mature miRNAs may determine whether altered mature miRNA expression is a result of regulation in miRNA biogenesis. Many commercially available assays for qPCR analysis of pri- and pre-miRNAs are available. The first step in qPCR analysis of miRNAs involves the RT of RNA to generate a cDNA sequence. Because of the small size of mature miRNAs and their lack of a poly(A) tail,

Comp. by: DElayaraja Stage: Proof Chapter No.: 2 Title Name: COACChennai
Date:4/3/14 Time:15:11:33 Page Number: 28

B978-0-444-62650-9.00002-6, 00002

certain considerations need to be taken into account for primer design for mature miRNA analysis by qPCR. One method involves tailing of miRNAs with a common sequence and performing RT using a universal primer. One such approach involves the addition of a poly(A) tail to the mature miRNA molecule, for example, Qiagen's miScript PCR System *(66)*. Total RNA containing the small RNA fraction is used as a starting material. Mature miRNAs are polyadenylated by poly(A) polymerase and reverse transcribed into cDNA in a first-strand cDNA synthesis reaction using oligo-dT primers containing a universal tag on the 5′ end. miRNA abundance is then detected by SYBR green qPCR using a miRNA-specific forward primer and a universal reverse primer. As the oligo-dT primer detects all poly(A)-tailed miRNA molecules during the RT reaction, this assay enables detection of multiple miRNAs within a single cDNA preparation. The miRCURY LNA Universal RT microRNA PCR system (Exiqon) also employs a universal RT approach involving the addition of a 5′ universal tagged poly(A) tail, followed by qPCR with miRNA-specific locked nucleic acid (LNA)-enhanced forward and reverse primers. LNA is a synthetic RNA/DNA analog characterized by increased thermostability of nucleic acid duplexes. As each incorporated LNA monomer increases the Tm of a given primer *(58)*, this technology overcomes PCR specificity and sensitivity issues due to Tm variations resulting from miRNAs that are heterogeneous in their GC content.

p0065 Another approach for detecting miRNA levels by qPCR is through the use of stem-loop RT primers that are specific for the 3′ end of miRNAs *(67)*. Taqman Small RNA assays (Life Technologies) are based on this approach. Following first-strand cDNA synthesis using a stem-loop primer, the RT cDNA product is quantified by Taqman PCR using an miRNA-sequence-specific forward primer, a specific reverse primer, and a Taqman probe. Because this approach utilizes a sequence-specific stem-loop RT primer, a separate RT reaction is required for quantitation of each miRNA of interest. However, there are advantages to using the stem-loop RT primer. By annealing a short RT priming sequence to the 3′ end of the miRNA, better specificity for discriminating similar mature miRNA sequences is achieved *(58,67,68)*. In addition, the double-stranded stem-loop structure inhibits hybridization of the RT primer to pre-miRNAs and other long RNAs. Further, the base stacking of the stem-loop enhances the stability of miRNA and DNA duplexes, improving the efficiency of the RT reaction. Lastly, the stem-loop structure when unfolded adds sequence downstream of the miRNA after RT, resulting in a longer RT template more suitable for TaqMan assay design *(68)*.

p0070 qPCR-based platforms that allow simultaneous profiling of hundreds of miRNAs from the one sample using customizable or predesigned microfluidic cards/plates are also available (Table 1). Examples of these include miScript miRNA PCR Arrays (Qiagen), miRNA PCR panels (Exiqon), and TaqMan Low Density miRNA Arrays (Life Technologies). These PCR-based arrays enable quantification of up to 752 human miRNAs from small quantities of

Comp. by: DElayaraja Stage: Proof Chapter No.: 2 Title Name: COACChennai
Date:4/3/14 Time:15:11:35 Page Number: 29

B978-0-444-62650-9.00002-6, 00002

starting total RNA. Following RT, the resulting cDNA templates are mixed with a PCR master mix and added to the microfluidic array cards or multiwell plates prior to standard qPCR amplification and analysis.

p0075 In order to control for variations in RNA input or RT efficiency when performing qPCR using either individual miRNA assays or PCR-based arrays, normalization to endogenous control genes is recommended. Inclusion of controls enables normalization of qPCR results for relative quantitation analysis. An ideal control demonstrates gene expression that is highly abundant and constant across cell types and tissues. Endogenous control assays may be custom designed or are available predesigned commercially and include members of the snRNA (e.g., U6 snRNA *(26,69)*) and snoRNA (e.g., snoRNA202 *(70)*) families. In addition to low-medium throughput miRNA profiling, qPCR is usually the method of choice for validating results from microarray analysis (Section 6) or RNA-seq profiling (Section 7) or miRNAs.

s0030 6 MICROARRAY PROFILING OF miRNAs

p0080 miRNA microarray technology is based on complementary hybridization of labeled miRNAs to an array of immobilized synthetic oligonucleotide probes on a gene chip. This technology allows for high-throughput analysis of miRNA expression and is cheaper to perform than RNA-seq. However, it is not as sensitive as qPCR or RNA-seq approaches. As it is based on current knowledge on genome sequence, miRNA microarray profiling does not permit the identification of novel previously uncharacterized miRNAs. Various commercial platforms are available for miRNA microarray profiling (Table 1). These platforms employ different approaches to label miRNAs for subsequent hybridization to the DNA probes on the microarray chip. One approach involves the enzymatically catalyzed ligation of a fluorophore-conjugated oligonucleotide to the 3′ end of the miRNA molecule using T4 RNA ligase prior to hybridization (SurePrint microRNA microarray; Agilent). Because Dicer processing during miRNA biogenesis results in the exposure of a 5′ phosphate on the mature miRNA that could lead to circularization during the ligation reaction, procedures involving T4-mediated ligation are preceded with a dephosphorylation step to remove the 5′ phosphate.

p0085 Alternative microarray approaches are available that involve the 3′ tailing of the miRNA molecule with poly(A), followed by labeling and hybridization. The NCode miRNA microarray platform (Life Technologies) involves the addition of the poly(A) tail to the miRNA prior to ligation of fluorescent dye molecules and subsequent hybridization to the antisense miRNA probes on the microarray chip. In the case of Affymetrix GeneChip microRNA arrays, the poly(A)-tailed miRNAs are labeled with a biotinylated signal molecule. A limitation of hybridization-based microarrays is that because of the small size of miRNAs, the Tm of the probes has a wide range that may diminish specificity and/or sensitivity for miRNAs with a low GC content *(10,56)*.

Comp. by: DElayaraja Stage: Proof Chapter No.: 2 Title Name: COACChennai
Date:4/3/14 Time:15:11:37 Page Number: 30

B978-0-444-62650-9.00002-6, 00002

As a result, it may be difficult to use a temperature that suits the annealing of all the probes to their target miRNAs during hybridization. To overcome the issue of diminished specificity, LNA probes may be used to increase the Tm value (miRCURY LNA microRNA arrays, Exiqon).

7 SMALL RNA-SEQ

p0090 Next-generation RNA-seq provides a more precise measurement of levels of transcripts and their isoforms than other methods and is not limited by variability in Tm, small variations in miRNA sequences among families or post-transcriptional modifications. NGS technologies rely on a combination of procedures involving template preparation, sequencing, and imaging, followed by sequence alignment to a reference genome and data analysis *(71)*. In the case of miRNAs, adapter sequences are firstly ligated to the 5′ and 3′ ends of the mature miRNA molecule, taking advantage of the natural structure common to most mature miRNA molecules of a 3′-hydroxyl group and 5′-phosphate that result from cleavage by the enzyme Dicer *(56)*. Adapter ligation is followed by an RT reaction, a PCR amplification, and a purification step. Sample quality should be assessed on the Agilent Bioanalyzer 2100 at each step of the cDNA library preparation to ensure that the integrity of the samples is sufficient for sequencing. The high-quality cDNA template preparation is attached to a solid support surface and this immobilization of spatially separated template sites allows for thousands of sequencing reactions to be performed simultaneously in order to obtain millions of short-sequence reads *(60,71)*. Both Illumina and Roche platforms (Table 1) sequence by synthesis. The Illumina platform supports massively parallel sequencing using a reversible terminator-based cyclic method that comprises nucleotide incorporation, fluorescence imaging, and cleavage. A fluorescently labeled terminator is imaged as each dNTP is added and then removed to allow incorporation of the next base. Since all four reversible terminator-bound dNTPs are present during each sequencing cycle, natural competition minimizes incorporation bias. By contrast, Roche sequencing technology involves pyrosequencing, where each nucleotide addition results in the activation of luciferase activity that produces light that is detected during the imaging process. As this technique does not require terminator removal at each step, increased sequencing speed can be achieved *(56)*. The SoLID platform sequences by ligation. Primers hybridize to the adapter sequences on template DNA and a set of four fluorescently labeled probes compete for ligation to the sequencing primer.

p0095 NGS machines usually produce raw data files in FASTA or FASTQ format, which contain millions of short-sequence reads. A significant level of computational expertise is thus required for the analysis of NGS small RNA-seq datasets, with many command line and web-based analysis platforms available (Table 2). Following sequencing, quality is assessed in order to determine whether the sequencing run was successful. Resources for

Comp. by: DElayaraja Stage: Proof Chapter No.: 2 Title Name: COACChennai
Date:4/3/14 Time:15:11:39 Page Number: 31

B978-0-444-62650-9.00002-6, 00002

t0010 **TABLE 2 Analysis Software Available for Small RNA-seq Data Analysis**

Resource	URL	Tools
Galaxy	https://usegalaxy.org/	Sequencing data quality assessment
FASTX-Toolkit	http://hannonlab.cshl.edu/fastx_toolkit/index.html	Sequencing data quality assessment and preprocessing, for example, adapter sequence removal, sequence trimming, and conversion of sequence reads to reverse complement
FastQC	http://www.bioinformatics.babraham.ac.uk/projects/fastqc/	Sequence data quality assessment
BWA	http://bio-bwa.sourceforge.net/	Alignment of sequence reads to a reference genome
Bowtie	http://bowtie-bio.sourceforge.net/index.shtml	Alignment of sequence reads to a reference genome
TopHat	http://tophat.cbcb.umd.edu/	Alignment of sequence reads to a reference genome
UCSC genome browser	http://genome.ucsc.edu/cgi-bin/hgGateway	Visualization of aligned sequence reads in the genome of interest
Integrative Genomics Viewer	http://www.broadinstitute.org/igv/	Visualization of aligned sequence reads in the genome of interest Integration with clinical and phenotypic data
Gene Expression Omnibus	http://www.ncbi.nlm.nih.gov/geo/	Publicly available functional genomics repository
Cufflinks	http://cufflinks.cbcb.umd.edu/index.html	Differential expression analysis
Gobyweb	http://campagnelab.org/software/gobyweb/	Differential expression analysis

quality control assessment are available from web sites including Galaxy *(72)*, FASTX-Toolkit, and FastQC. These web sites provide useful tools that determine whether there are any problems with the datasets before commencing with further downstream analysis. In addition, FASTX-Toolkit enables file preprocessing, including shortening of reads to remove barcodes or noise (FASTQ/A Trimmer), conversion of sequence reads to the reverse complement (FASTQ/A Reverse-Complement), or removal of adapter sequences

Comp. by: DElayaraja Stage: Proof Chapter No.: 2 Title Name: COACChennai
Date:4/3/14 Time:15:11:41 Page Number: 32

B978-0-444-62650-9.00002-6, 00002

from the sequence reads (FASTQ/A Clipper). Following quality control, sequence reads are then aligned to a reference genome to produce a genome-wide transcription map using bioinformatics tools such as BWA *(73)*, Bowtie *(74)*, or TopHat *(75)*. Aligned files may be visualized using the UCSC genome browser *(76,77)* or the Integrative Genomics Viewer (IGV) *(78)*. The UCSC genome browser provides a web-based tool for the display of any requested portion of the genome at the desired scale. Multiple annotation tracks of sequence data are publicly available on the web site. Alternatively, users may add their own NGS files to the browser as custom tracks for visualization, or custom tracks of interest that have been published in the literature and made available through resources such as the NCBI Gene Expression Omnibus database *(79)*. The IGV is freely available for download to personal computers as a desktop application, and also enables visualization of genomic data from public sources as well as visualization of users own datasets. In addition, the IGV allows for integration of clinical and phenotypic data. Finally, relative quantitation of differential miRNA expression in terms of the number of sequence reads for a particular miRNA relative to the total reads in a sample can be determined using differential expression platforms such as Cufflinks *(80)* and Gobyweb *(81)*. Following differential expression analysis, differentially regulated miRNAs identified by RNA-seq analysis are usually validated by qPCR approaches.

p0100 Bioinformatics analysis of the sequence reads identifies both known and novel miRNAs and provides precise sequence information, which is important to distinguish between miRNA molecules that differ by as little as a single nucleotide. Not all novel miRNAs identified by small RNA-seq are genuine functional mature miRNAs. A number of criteria have been suggested before a novel miRNA may be considered genuine and entered into the miRBase miRNA repository, including a sequence length of approximately 22 nt, conservation across species, a genomic origin that predicts a hairpin pre-miRNA sequence and identification in the data reads that correspond to both the -5p -3p arms of the pre-miRNA hairpin *(82,83)*.

s0040 8 *IN SILICO* miRNA ANALYSIS RESOURCES

p0105 A complete understanding of the function of miRNAs and their role in biological processes requires elucidation of the mRNA targets that they regulate. Having identified differentially expressed miRNAs through qPCR, microarray or RNA-seq approaches, a wide variety of computational resources exist for *in silico* data mining in relation to miRNA expression patterns, miRNA target prediction, and the identification of miRNA–disease linkages *(59)* (Table 3). miRBase is the key repository of miRNA sequences and nomenclature. miRBase *(84)* provides information about predicted precursor hairpin sequences and experimentally identified mature miRNA sequences. It also provides links to miRNA target prediction databases and

Comp. by: DElayaraja Stage: Proof Chapter No.: 2 Title Name: COACChennai
Date:4/3/14 Time:15:11:44 Page Number: 33

B978-0-444-62650-9.00002-6, 00002

t0015 **TABLE 3** A Selection of *In Silico* miRNA Analysis Resources

Resource	URL	Tools
miRBase	http://www.mirbase.org	A comprehensive database of miRNA nomenclature and sequences Links to target prediction web sites and RNA-seq datasets.
TargetScan	http://www.targetscan.org/	A tool for prediction miRNA targets in multiple species
PicTar	http://pictar.mdc-berlin.de/	A tool for prediction of miRNA targets in multiple species
Tarbase	http://www.diana.pcbi.upenn.edu/tarbase.html	A database of experimentally verified miRNA targets
microRNA.org	http://www.microrna.org/microrna/home.do	A database of miRNA targets and experimentally validated tissue expression levels
miRNAMap	http://mirnamap.mbc.nctu.edu.tw/	A database of miRNA targets and experimentally validated tissue expression levels
mir2Disease	http://www.mir2disease.org/	Comprehensive resource of miRNA dysregulation in human disease
mirWalk	http://www.umm.uni-heidelberg.de/apps/zmf/mirwalk/	miRNA target prediction and associations with pathways and disease
Magia	http://gencomp.bio.unipd.it/magia/start/	Integrative analysis of miRNA and gene-expression networks
mirConnex	http://mirconnx.csb.pitt.edu/job_config	Integrative analysis of miRNA and gene-expression networks
Cytoscape	http://www.cytoscape.org/	Network data integration and analysis

RNA-seq datasets deposited in the NCBI Gene Expression Omnibus repository *(10,54,79)*. Target prediction tools involve mRNA target prediction based on an inputted miRNA sequence or accession number *(85)*. Numerous target prediction resources are available. TargetScan identifies mRNA targets in many species by detecting the presence of conserved 7mer and 8mer sites that match the seed region of each miRNA *(86)*. Similar toTargetScan, PicTar uses genomewide alignment to predict target mRNAs in different species *(87)*. The false-positive discovery rates for PicTar and TargetScan have been reported to be between 20% and 30% with between 80% and 90% overlap in target predictions between the two for human targets *(88)*.

Comp. by: DElayaraja Stage: Proof Chapter No.: 2 Title Name: COACChennai
Date:4/3/14 Time:15:11:45 Page Number: 34

B978-0-444-62650-9.00002-6, 00002

p0110 Many online resources collect experimentally confirmed miRNA–mRNA interactions. One of these is Tarbase *(89)*, which collects interactions reported in the literature from a variety of experimental techniques. Numerous other online resources exist that contain information on experimentally validated interactions and expression patterns including microRNA.org *(90)* and miRNAMap *(91)*. Online tools can be used to study a disease state of interest including miR2disease *(92)*, which is a curated database for microRNA deregulation in human disease. miR2disease is updated frequently and contains information on miRNA–disease relationships, including expression patterns of specific miRNAs in specific disease states as well as the method used for detection of miRNA expression. miR2disease also contains information on experimentally verified miRNA target genes as well as links to other miRNA databases. miRwalk *(93)* is another comprehensive database of experimentally verified miRNA interactions associated with pathways and diseases. Information is provided on the chromosomal location of the miRNA as well as links to the original published study. Integrative analysis of miRNA expression profiles with large-scale mRNA expression datasets may also be performed using *in silico* tools such as MAGIA *(94)* and mirConnX *(95)*, which enable target prediction, integrated analysis of expression profiles, posttranscriptional regulatory network browsing, functional annotation, and enrichment analysis in combination with the network visualization tool Cytoscape *(96)*.

s0045 9 miRNA FUNCTIONAL ANALYSIS

p0115 In order to determine the function of miRNAs identified by the miRNA profiling techniques outlined above, various analytical tools are available to perform loss- and gain-of-function studies. The anti-miR (Life Technologies) and the miScript miRNA inhibitor (Qiagen) ranges consist of a panel of single-stranded molecules for specific inhibition of all miRNAs currently listed in miRBase. These RNA inhibitors can be transfected into cells in order to assess the effect of decreased miRNA expression on cell phenotype and function. Furthermore, the effect of miRNA inhibition on global gene expression can be studied using microarray or NGS approaches in order to identify target mRNAs regulated by an miRNA of interest. Gain-of-function studies can also be performed by transfecting miRNA mimic molecules directly into cells. miRNA mimics in the form of pre-miR miRNA precursors (Life Technologies) or miScript miRNA mimics (Qiagen) are small, chemically modified double-stranded RNAs that mimic endogenous miRNAs and enable functional analysis by upregulation of miRNA activity. Loss- or gain-of-function studies may also be performed to investigate the effect of miRNA expression on putative mRNA targets identified by bioinformatics analysis. To directly assess miRNA binding to their target mRNAs, the ability of miRNA mimics or inhibitors to regulate expression from a luciferase reporter construct (e.g., pMIR-REPORT miRNA reporter system, Life Technologies),

Comp. by: DElayaraja Stage: Proof Chapter No.: 2 Title Name: COACChennai
Date:4/3/14 Time:15:11:48 Page Number: 35

B978-0-444-62650-9.00002-6, 00002

in which the 3′UTR of the target mRNA has been subcloned, can be tested. Decreased target protein expression may be validated by Western blot analysis. Such experiments provide insight into the functional consequences of altered miRNA expression.

s0050 10 RECENT DEVELOPMENTS IN THE APPLICATION OF miRNA PROFILING TO CANCER RESEARCH

p0120 The role of miRNAs in cancer development and progression has been widely reported *(11,97–99)*. Generally speaking, miRNA profiling and functional studies in cancer research can be divided into (i) those that focus on deciphering the mechanistic role of miRNA dysregulation in the tumor phenotype with the ultimate aim of developing therapeutic strategies to target these mechanisms and (ii) those that involve biomarker discovery to identify miRNA profiles associated with disease type or predicted response to therapy. Various forms of the miRNA profiling methods outlined above have been used to describe miRNAs whose expression is altered in cancer cell lines, tumor tissue, and plasma or serum samples from cancer patients. Using a combination of qPCR and miRNA microarrays, numerous studies have delineated miRNAs that are differentially expressed in cancer of the breast *(34,37)*, lung *(42,43,46,47)*, pancreas *(100)*, liver *(40,41)*, and in B-cell lymphoma *(101)*. Additionally, dysregulated miRNAs have been associated with treatment outcome. By combining miRNA microarray analysis with stem-loop qPCR validation, Yang *et al*. described significantly decreased let-7i expression in chemotherapy-resistant epithelial ovarian cancer *(102)*. Tomimura *et al*. showed that increased miR-21 expression in hepatocellular carcinoma leads to increased resistance to the antitumor effect of combination therapy involving interferon-α and 5-fluorouracil *(40)*. Increased miR-21 expression has also been reported in colon adenocarcinoma and is associated with poor survival and therapeutic outcome *(39)*.

p0125 The significant body of work involving miRNA profiling of numerous tumor types has led to the generation of a substantial body of evidence on dysregulated miRNAs in human cancers. Subsequently, there has been a dedicated effort focused on understanding the functional role of such altered miRNA expression in disease progression and outcome. miRNAs with a demonstrated functional importance in cancer can be subdivided into oncogenic miRNAs (oncomiRs) and tumor suppressor miRNA (TS-miR) *(48,49)*. Much effort has been made to elucidate the functional consequences of oncomiR and TS-miR expression in order to unveil potential therapeutic targets for disease management. Significant progress has been reported in our understanding of the functional role of specific miRNAs and regulation of their targets. By applying miRNA profiling, target identification, and functional analysis, Liu *et al*. have identified miR-31 as an oncomiR in lung cancer *(43)*. First, miRNA microarrays and qPCR were performed to identify

Comp. by: DElayaraja Stage: Proof Chapter No.: 2 Title Name: COACChennai
Date:4/3/14 Time:15:11:50 Page Number: 36

B978-0-444-62650-9.00002-6, 00002

differences in miRNA expression in lung cancer tissue compared with adjacent normal tissue in mouse models of lung carcinoma. miR-31 was shown to be significantly increased in the murine lung cancer tissue and the finding was confirmed using human tissue samples. Functional analysis indicated that miR-31 inhibition decreased lung cancer cell growth and tumorigenicity. Using the bioinformatics resources TargetScan, Pictar, and miRanda, Liu *et al*. also identified the tumor suppressor genes, large tumor suppressor 2 (LATS2) and the PP2A regulatory subunit B alpha isoform (PPP2R2A) as targets of miR-31, and validated these targets using 3′-UTR luciferase-binding assays and by demonstrating inverse expression of miR-31 and LATS2 or PPP2R2A in mouse and human lung cancers *(43)*. In another study, Volinia *et al*. used microarrays to identify dysregulated miRNAs in samples from six solid tumor types, namely lung, breast, stomach, prostate, colon, and pancreatic *(35)*. They defined a solid tumor signature of overexpression of miRNAs, including miR-17-5p, miR-20a, miR-21, miR-92, miR106a, and miR-155. Using TargetScan, the retinoblastoma 1 tumor suppressor gene and transforming growth factor, beta receptor II were identified as predicted targets. The mRNA targets were confirmed experimentally using 3′-UTR luciferase assays and by monitoring target protein expression to support a functional role for the role of miRNAs in solid cancer pathogenesis. Using a similar approach involving combined miRNA profiling, target identification, and functional analysis, decreased expression of the miRNA let-7a has been suggested to contribute to the development of prostate cancer *(103)*. More recently, RNA-seq analysis has shed further light on how miRNA expression contributes to cancer progression, in particular, in the lung. Using a mouse model of lung adenocarcinoma, Valdmanis *et al*. utilized small RNA-seq to show increased expression of a cluster of miRNAs at the Dlk1-Dio3 locus, some of which are known to be involved in key cancer-associated pathways *(46)*. Perdomo *et al*. identified an association of miR-4423 with lung cancer in primates by utilizing a small RNA-seq approach *(47)*.

p0130 Based on their disease association and functional contribution to carcinogenesis, miRNAs are attractive therapeutic targets for cancer treatment. The rationale for targeting oncomiRs that are upregulated in cancer involves the use of oligonucleotides that block their activity. Likewise, the principle behind targeting TS-miRs is to replenish their expression *(104)*. Encouraging results from preclinical models have resulted in miRNAs entering clinical trials for therapies in some disease settings. For example, an effective and safe approach for sequence-specific antagonism of miR-122, which is implicated in Hepatitis C virus (HCV) infection, in the livers of nonhuman primates has been demonstrated *in vivo (105,106)*. Based on these studies, an miR-122 inhibitor, Miravirsen, is currently in Phase 2A clinical trials in humans for chronic HCV infection *(107)*. Miravirsen treatment demonstrated good tolerability in 36 patients and a dose-dependent reduction in HCV RNA

Comp. by: DElayaraja Stage: Proof Chapter No.: 2 Title Name: COACChennai
Date:4/3/14 Time:15:11:51 Page Number: 37

B978-0-444-62650-9.00002-6, 00002

levels that endured beyond the end of active was reported *(107)*. Although not yet in clinical trials, select miRNAs have emerged as potential clinical targets for cancer treatment. miR-34 has a well-defined role as a tumor suppressor and miRNA replacement therapy by means of miRNA mimics has shown promise in animal studies *(108)*. Like all therapeutics, the major barriers to targeting miRNAs in humans are ensuring high bioavailability, achieving target specificity, and reducing toxicity. The use of nanoparticles to target miRNA is a potential option for the treatment of cancers. Recently, an miR-155-targeting nanoparticle containing miR-155 antisense nucleotides, was shown to reduce the growth of B-cell lymphoma in a murine model of disease *(109)*, suggesting a promising therapeutic option for lymphoma and leukemia.

p0135 In relation to biomarker discovery, many studies have focused on identifying cancer-associated biomarkers in plasma or serum as a noninvasive means for diagnosis or predictive of response to therapy. A good example of this is miR-22, the plasma levels of which were used to distinguish lung cancer patients from healthy individuals by qPCR, thereby supporting its potential role as a diagnostic biomarker that could be further evaluated in clinical trials *(44)*. In prostate cancer, circulating levels of miR-375 and miR-141 have been reported to correlate with disease progression and lymph node metastasis *(53)*. miR-1, mir-92a, miR-133a, and miR-133b were identified as the most important diagnostic markers for breast cancer detection in a recent study by Chan *et al*. *(51)*. In terms of utilizing miRNAs as predictive biomarkers for response to therapy, qPCR on circulating levels of miR-125b are predictive of the chemotherapy response in breast cancer *(36)*. The translation of these findings into either noninvasive rapid diagnostic tools or as viable therapeutic targets depends on additional carefully designed and executed profiling studies.

s0055 11 CONCLUSIONS

p0140 Herein, we have described numerous approaches that may be employed to detect miRNAs, investigate their differential expression, and assess their functional properties in a wide variety of sample types. These include molecular profiling approaches such as qPCR, microarray, and next-generation RNA-seq analysis, as well as computational approaches to predict miRNA targets and investigate miRNA association with disease states. We have also described loss- and gain-of-function methods used to elucidate the functional importance of a given miRNA, together with tools that may be used to confirm direct miRNA:mRNA targeting. The methods of choice for an investigator depend on multiple factors, including the specific aims of a given experiment, the availability of local expertise, budget considerations, and access to specific platforms. The recent advances in RNA-seq technology have provided researchers with an exciting opportunity for the specific and sensitive high-throughput detection and quantification of known and

Comp. by: DElayaraja Stage: Proof Chapter No.: 2 Title Name: COACChennai
Date:4/3/14 Time:15:11:54 Page Number: 38

B978-0-444-62650-9.00002-6, 00002

previously unidentified miRNAs. As the cost of these techniques is falling, RNA-seq will become a more accessible platform for rapid and large-scale miRNA profiling, offering the potential to provide invaluable insight into the role of these key regulatory molecules a variety of settings. It is clear that the elucidation of the biological, pathological, and clinical roles of miRNA regulation, expression, and functional properties has greatly contributed to our understanding of pathogenesis. Continued investigation involving both miRNA discovery as well as miRNA targeting will be of great importance to the translation of miRNA biology into clinical applications for diagnosis of disease and novel therapeutics.

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Comp. by: DElayaraja Stage: Proof Chapter No.: 2 Title Name: COACChennai
Date:4/3/14 Time:15:11:56 Page Number: 39

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B978-0-444-62650-9.00002-6, 00002

Non-Print Items

Abstract

microRNAs (miRNAs) are small, evolutionarily conserved, noncoding RNAs that posttranscriptionally regulate specific gene products resulting in altered protein expression. miRNAs target specific messenger RNA molecules either by guiding degradation through a mechanism similar to RNA interference or by inhibiting translation. As such, miRNAs control the expression of thousands of genes in a broad spectrum of normal physiological contexts and in disease settings. Recent advances in high-throughput methods for profiling microRNA expression and for the identification of microRNA targets have ushered in a new era in the research of transcriptional regulation. Understanding microRNA expression patterns and microRNA targets provides an insight into gene regulation, biomarker identification, and potential strategies for therapy. This chapter provides an overview of recently developed technologies for the investigation of microRNA expression and function, including profiling by quantitative PCR, microarray analysis, and next-generation RNA sequencing, as well as useful bioinformatics tools for differential expression analysis and target prediction. In addition, the contribution of these miRNA technologies to advances in cancer research is discussed.

Keywords: microRNA; Messenger RNA; Expression profiling; RNA sequencing; Microarray; Quantitative PCR

Comp. by: DElayaraja Stage: Proof Chapter No.: 2 Title Name: COACChennai
Date:4/3/14 Time:15:12:12 Page Number: 45