CHAPTER 3

METHODOLOGY

3.1 Introduction

This research was conducted at the Faculty of Medicine and Health Sciences Universiti Sains Islam Malaysia (USIM) over the two years from Dec 2020 until Dec 2022. The experimental model involved two human chronic myeloid leukaemia (CML) cell lines: the K562-s (imatinib-sensitive) and the K562-r (imatinib-resistant) cells. Both cell lines were subjected to various treatment groups including: the hsa-miR-3131, hsa-miR-891a-3p, Osa-miR-1858a/b, imatinib (10 μM) and untreated control group. The microRNA (miRNA) mimics used in this study were selected based on the in-silico target prediction analyses and were sourced from Qiagen and ThermoFisher Scientific.

3.2 Materials

3.2.1 Cell lines and culture media

Imatinib resistance chronic myeloid leukaemia cells, K562-r (ATCC®-3344™) and imatinib sensitive chronic myeloid leukaemia cells, K562-s (ATCC®-3343™) were obtained from American Type Culture Collection (ATCC) (Manassas, VA, USA). Materials used were RPMI-1640 medium (ATCC® 30-2001™), L-glutamine solution, 200 mM (ATCC® 30-2214™), Fetal Bovine Serum (FBS) (ATCC®30-2021™), Penicillin-Streptomycin-Amphotericin B solution (ATCC®PCS-999-002™),

Penicillin-Streptomycin solution (ATCC® 30-2300™), Hank's Balanced Salt Solution (HBSS), 1X (ATCC® 30-2213™) and imatinib (Sigma catalogue # CDS022173-25MG), miRCURY LNA™ miRNA mimic (5) No modification: hsa-miR-3131 and hsa-mir-891a-3p (Qiagen, USA), mirVana™ miRNA mimic MC14222 (osa-mir1858a/b) (ThermoFisher Scientific, USA). miRCURY LNA™ Premium miRNA mimic (5) – 3 Biotin: hsa-miR-3131 and hsa-mir-891a-3p (Qiagen, USA).

3.2.2 Cell Count

Materials used were Trypan Blue Solution, 0.4% (Thermo Scientific, USA). Equipment used was Neubauer Counting Chamber (Hawksley Cristalite, UK) dan coverslips (Menzel-Glaser, German), centrifuge machine serial (Eppendorf, German) and Olympus CKX41 phase contrast microscope (Olympus, USA). Other apparatus used were micropipette (1-1000 µl) (Eppendorf, German).

3.2.3 Cell proliferation/viability assay (MTS)

The material used was CellTiter96® Aqueous One Solution Cell Proliferation Assay kit (Promega, USA), 96-well plates suitable for tissue culture, multichannel pipettes and a 96-well microplate reader (Epoch™ Microplate Spectrophotometer).

3.2.4 Cell cycle assay

The material used was the DNA Cycle TEST™ Plus kit (Becton Dickinson, USA). The apparatus used were 12 X 75 mm tubes with a 35µm filter (Becton

Dickinson, USA) and flow cytometry machine BD FACSAria™ II with FACScan and Modfit software (Becton Dickinson Biosciences, USA).

3.2.5 RNA extraction, cDNA synthesis and qPCR

Materials used were GeneAll® Hybrid-R™ RNA purification kit (GeneAll Biotechnology, South Korea), AffinityScript QPCR cDNA Synthesis Kit (Agilent Technologies, Inc.), Brilliant III Ultra-Fast SYBR® Green QPCR Master Mix (Agilent Technologies, Inc.), absolute ethanol, chloroform (Merck, German), RNase-free pipette tips, sterile 0.2 to 1.5 ml microcentrifuge tubes and disposable gloves. Equipment used are microcentrifuge capable of attaining 12,000x g at 4°C and room temperature, Implen NanoPhotometer® P-300 (GmbH, Germany) and CFX96 Touch™ C1000 Touch Thermal cycler (Biorad, USA). Another apparatus used was a micropipette (1-1000 µl) (Eppendorf, German).

3.2.6 MiRNA Taqman Assay

The primers and probes used in the TaqMan assay were based on mature miRNA sequences obtained from the miRBase database. Pre-validated TaqMan miRNA assays specific to each miRNA, including plant miRNAs, were purchased from Thermo Fisher Scientific. For plant miRNAs, design criteria generally include the small size (21–24 nucleotides), high sequence specificity, minimal secondary structures, appropriate GC content (40–60%), and avoidance of dimers or hairpins. The stem-loop RT primer design used in TaqMan assays enhances specificity and sensitivity in detecting mature miRNAs (Chen et al., 2005). The assays utilized were: hsa-miR-3131 (Assay ID: 242305_mat, Size: XS), hsa-miR-891a (Assay ID: 466879_mat, Size: XS), osa-miR1858a (Assay ID: 007250_mat, Size: XS), RNU6B (Assay ID: 001093, Size: S),

and RNU48 (Assay ID: 001006, Size: S). These assays are optimized with proprietary primer and probe sequences for enhanced target specificity as provided by Thermo Fisher Scientific.

3.2.7 ABL1 protein ELISA assay

The material used was ABL1 (Human) Cell-Based ELISA Kit (Abnova, USA), Poly-L-Lysine (Sigma Cat#P4832 for suspension cells), 37% formaldehyde, deionised or sterile water. A microplate reader was used to measure absorbance at 490 nm and micropipettes (1-1000 µl) (Eppendorf, German). The apparatuses used include squirt bottle, manifold dispenser, multichannel pipette reservoir and 15 ml centrifuge tubes.

3.2.8 DNA microarray gene profiling

Human Gene Expression Microarray Service using Agilent One-colour SurePrint G3 Human Gene Expression V3 Microarray, 8 x 60K Format for 8 samples in 1 slide and Bioinformatics Analysis Service using GeneSpring Software.

3.2.9 1.5% electrophoresis agarose gel

Materials used were GeneRuler 100 bp DNA Ladder, ready-to-use (Thermo Scientific, USA), TopVision Agarose Tablets (Thermo Scientific, USA), TBE Buffer (Tris-borate-EDTA) (10X) (Thermo Scientific, USA), FloroSafe DNA stain (1st Base, Malaysia) and DNA Gel Loading Dye (6X) (Thermo Scientific, USA). The equipment used was an electrophoresis set (MS Major Science, Taiwan), power supply (MS Major

Science, Taiwan) and gel documentation system (Analytik Jena UVP ChemStudio PLUS, Fisher scientific, USA).

3.3 Experimental Design

During Phase I of the study, an in-silico analysis was conducted to identify microRNAs interacting with the 3' UTR of ABL1. In Phase II, miRNA transfection efficiency was evaluated using the miRNA TaqMan assay. Afterwards, the miRNA-mRNA binding interaction was validated using the biotin pull-down assay. The effects of miRNA transfection were assessed by examining changes in *ABL1* and *BCR-ABL1* gene expression, the cell's functional assay through the cell cycle and cell viability, and the expression of ABL1 protein. Then, microarray gene profiling was used to determine differently expressed genes (DEGs), and qPCR was used to confirm the up- and downregulation of the genes. This study's workflow is displayed in Figure 3.1.

3.4 Methods

3.4.1 In-silico analysis

3.4.1.1 Sequence of BCR-ABL1 and miRNAs

The sequence of 3'UTR of ABL1 was obtained from Ensembl Genome Browser (Cunningham et al., 2018). Meanwhile, a list of plant miRNA sequences was retrieved from Pirro, Zanella, et al. (2016) and analysed using psRNATarget (Version 2, 2017 release, The Zhao Lab, China), a plant's small RNA (sRNA) Target analysis server to identify plant sRNA targets. The software functions by analysing complementary matching between the sRNA sequence and target mRNA sequence using a predefined

scoring schema and by evaluating target site accessibility (Dai et al., 2018). Meanwhile, the human miRNA sequences were obtained from miRbase (Kozomara & Griffiths-Jones, 2011).

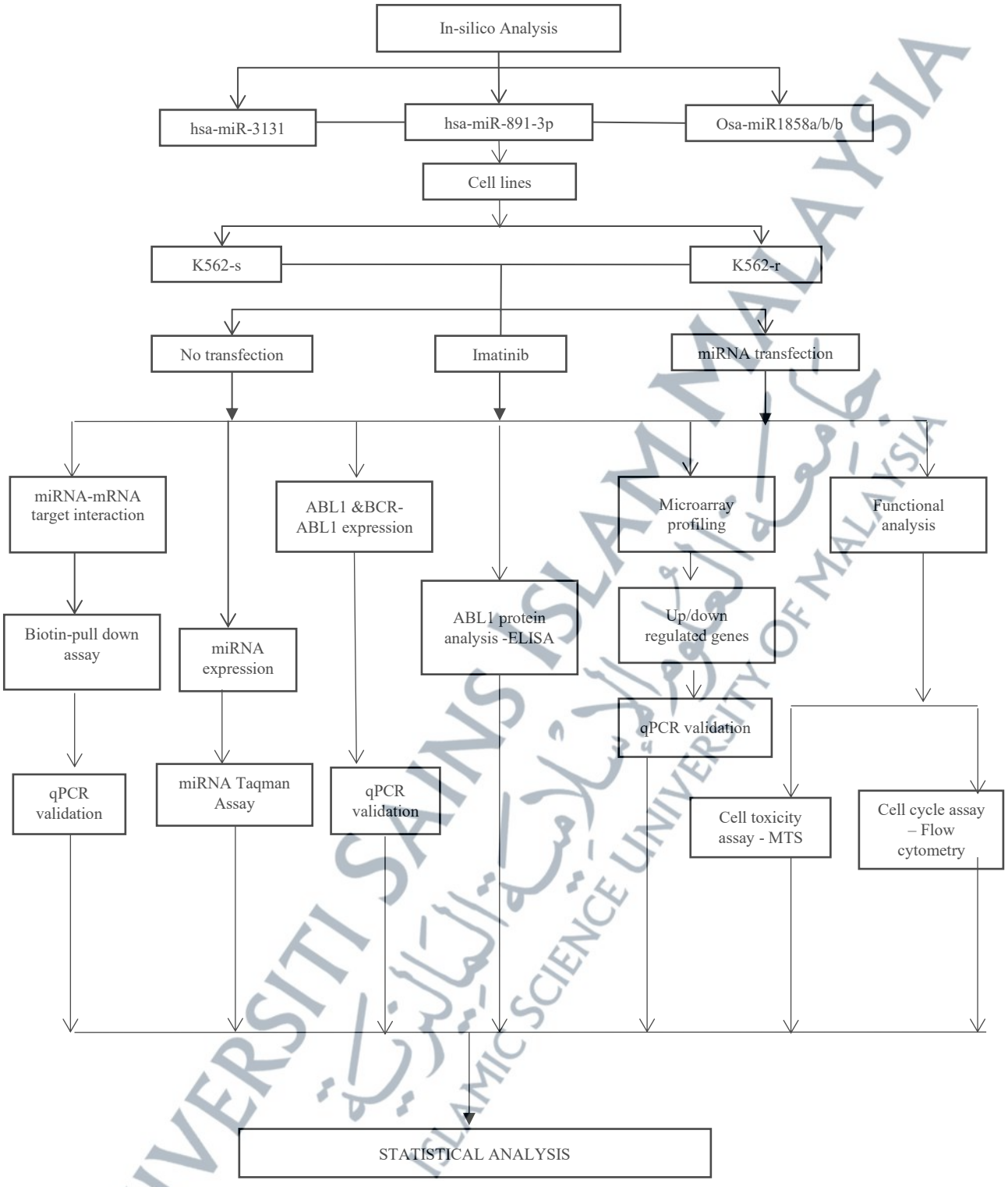


Figure 3.1: Work flowchart.

3.4.1.2 Predicting plant miRNA-mRNA target interaction

The psRNATArget and Schema V2 criteria were used in predicting the interaction. The 3'UTR of the ABL1 sequence was imported into the software, the miRNAs were selected based on expectations equal to or less than 5 which indicates high confidence binding. MiRNAs with scores >5 was excluded due to lower predicted binding affinity or specificity. Subsequently, RNA22 v2 microRNA target detection software (Jefferson™ Computational Medicine Center, Philadelphia, USA) was used to determine miRNA folding energy, p-value, and heteroduplex structure by inserting miRNA sequences and single target sequence (Loher & Rigoutsos, 2012; Miranda et al., 2006). Next, miRNA sequences were inserted into TargetRank to obtain the list of target genes (Nielsen et al., 2007).

3.4.1.3 Predicting human miRNA-mRNA target interaction

Screening of human miRNAs targeting 3'UTR of ABL1 was performed using DIANA TOOLS (DIANA web server, v5.0, University of Thessaly, Greece) with the micro-T CDS and miTG scores being set at more than 0.9 (Paraskevopoulou et al., 2013). Target genes of each selected miRNA were also identified using microT-CDS with the minimum threshold set at 0.7. This threshold is commonly used in miRNA target prediction studies as it balances sensitivity and specificity, allowing inclusion of biologically relevant targets while minimizing false positives (Maragkakis et al., 2009). Predicted miRNAs were later assessed based on their folding energy, p-value, and predicted target site using RNA22 software.

3.4.1.4 Protein-protein interaction (PPI) network

The interactions between genes could be analysed quickly by constructing the protein interaction network. STRING (version 11.5, STRING consortium 2022, Swiss Institute of Bioinformatics), an online website (http://string-db.org), was used to build interactive networks of target genes. By restricting the condition to "human species", networks were constructed, and the "TSV" format file of the gene interaction relationship was downloaded, and then the files were imported into the Cytoscape software (v3.9.1, Cytoscape Consortium, San Diego, California) (http://www.cytoscape.org/) for further analysis and clustering (Shannon et al., 2003). Cytoscape was employed with the MCODE plug-in to perform module analysis of the target network and protein clustering. The module selection criteria were as follows: degree cutoff ≥ 2, node score cutoff ≥ 0.2, K-score ≥ 2, and max depth = 100. The first neighbours of selected nodes used the "directed: ongoing" feature to analysed the neighbouring protein of ABL1 further.

3.4.1.5 Gene ontology and pathway enrichment analysis

Selected genes from Cytoscape networks were further analysed in STRING using the Cytoscape plug-in for their functional enrichment analysis. The roles of these genes in the metabolic pathways were elucidated based on the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway (Kenehisa Laboratories, Japan).

3.4.2 MiRNA mimics synthesis

MiRNA mimics identified from *in silico* analysis are hsa-miR3131, hsa-mir891a-3p were custom-designed in a locked nucleic acid (LNA) technology supplied by Qiagen. Meanwhile, the Osa-miR-1858a/b was purchased from Ambion Technologies (Thermofisher Scientific, USA). The sequences are provided in Table 3.1. For use in the biotin pull-down assay, the miRNA mimics were modified with a biotin molecule attachment at the 3' end. This modification allows the mimics to be captured using streptavidin-coated beads, enabling the isolation of miRNA–mRNA complexes for downstream identification of direct miRNA targets (Ørom & Lund, 2007). Meanwhile, the osa-mir1858a/b, a plant-based miRNA, is unavailable for biotin modification. Hence, the biotin-pulldown assay was only performed using the human-based miRNA, including the hsa-miR-3131 and hsa-miR-891a-3p.

Table 3.1: Mature miRNA mimic sequence.

miRbase ID	miRbase Accession	Mature miRNA sequence
hsa-miR-3131	MIMAT0014996	UCGAGGACUGGUGGAAGGGCCUU
hsa-miR-891a-3p	MIMAT0026717	AGUGGCACAUGUUUGUUGUGAG
osa-miR1858a/b	MI0008240	GAGAGGAGGACGGAGUGGGGC

3.4.3 Cell culture

3.4.3.1 Apparatus and Materials Sterilisation

All apparatus or consumables involved in cell culture must be sterile to maintain the aseptic techniques. The apparatus was sterilised using an autoclave machine set at a high temperature of 121°C for 20 minutes and dried in the oven at 70°C before being used. Meanwhile, all media used was filtered using a 0.2 μm syringe filter.

3.4.3.2 Culture Media Preparation

Complete culture medium (CCM) was prepared by adding RPMI-1640 medium with 10% fetal bovine serum (FBS), 1% L-glutamine and 1% penicillin-streptomycin to a volume of 500 ml. Media was filtered and aliquoted in 50 ml tubes for storage. The media was stored at 4°C until used.

3.4.3.3 Cell Culture Harvesting

Cells were cultured using aseptic techniques. CML cell line K562-r (ATCC® CRL-3344) and K562-s (ATCC® CRL-3343) were cultured in RPMI-1640 medium supplemented with 10% FBS, 1% L-glutamine with 1% penicillin-streptomycin. Cells were passaged three times per week to maintain exponential growth and seeded in a 25 cm^2 or 75 cm^2 culture flask at a seeding density of 2 X 10^5 cells. The K562-r cell was treated with 1 uM imatinib to maintain its resistance at each seeding. Cells were incubated at 37°C and 5% CO_2 and harvested when they achieved 70-80% confluency. Media was changed every two days by collecting cells into 50 ml tubes and centrifuging at 125xg for 10 minutes. The supernatant was decanted, and the pellet was resuspended in a new 1 ml CCM. The cell was subcultured in a 1:2 ratio and put into a new culture flask. Otherwise, if media changing is not done, half of the cell's volume in the old flask can be transferred into a new flask with new CCM to a total volume of 15 ml in T75 or 5 ml in T25.

3.4.3.4 Cell count

Ten microliters of the cell suspension were mixed with 10 µl of 0.4% trypan blue 0.4% (Gibco™). Next, 10 µl of the mixture was loaded into onto the haemocytometer slide (Figure 3.2). The cells were observed under a phase-contrast microscope. Live cells appeared colourless and shiny, while dead cells stained blue due to uptake of the dye. Only live cells were counted from four squares and averaged. Cell concentration (cell/ml) was calculated using the formula:

$$\text{Cells/ml} = n \times 10^4 \times \text{dilution factor}$$

n = average of cell counts from four squares.

The number of cells per ml was then multiplied by the total volume of the cell suspension to obtain the final cell count.

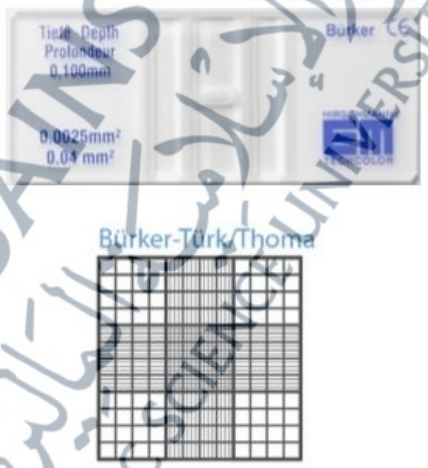


Figure 3.2: haemocytometer slide and the grid.

3.4.3.5 Cell stock storage

Storage of cell culture stock is essential to ensure continuous and long-term usage during experiments. Cells which achieved 70-80% confluency in each flask were collected for harvesting. CCM was mixed with 10% DMSO to resuspend the cells pellet

for freezing medium. The cell mixture was later transferred to cryovials and stored in the freezing container, Nalgene® Mr. Frosty, at -80°C for 24 hours for slow cooling. For long-term storage, cells were moved into a liquid nitrogen tank.

3.4.3.6 Cell thawing and revive

Cryovials were thawed in a 37°C water bath for approximately two minutes. Cells were then transferred to 9 ml media and centrifuged at 125xg for 10 minutes. The cell pellet was resuspended in complete culture media and was transferred to a 75 cm^2 culture flask and incubated at 37°C and 5% CO_2 for 24 hours. The next day, viable cells were counted using a trypan blue and haemocytometer, and cells were mixed in a 1:1 ratio (Bhutra et al., 2014).

3.4.4 Imatinib preparation

Imatinib (Sigma catalogue # CDS022173- 25MG) was dissolved in nuclease-free water to 20 mM master stock, aliquoted, and stored at -20°C. Imatinib working solution was prepared in 1 mM, aliquoted and stored at -20°C. The study used 10 µM of imatinib as a positive control. This concentration was chosen based on previous research by (Arunasree et al., 2008) which found that the imatinib IC_{50} was at 10 µM. While this may be slightly higher than necessary for the sensitive K562-s cells, short-term exposure at this dose did not result in excessive cytotoxicity, as verified by cell viability assays conducted in this study. Using a uniform concentration across both cell lines allowed for clearer comparative assessment of resistance. In addition, according to another study, 10 µM is comparable to 5897 ng/ml of imatinib in plasma, which can only be attained at extremely high dosages in patients, making it a useful benchmark for testing imatinib resistance *in vitro*. Importantly, this standardized dose ensured that

any residual sensitivity in K562-r cells could be clearly distinguished from the fully sensitive phenotype of K562-s (Schaefer & Behrends, 2017).

3.4.5 MiRNA Transfection

The biological activity of the miRNA mimics was studied by transfecting it into the cell lines using the Lipofectamine RNAimax method (Invitrogen, Thermo Fisher Scientific, USA) following the manufacturer's instruction. Briefly, in the 6-well plate, K562-r and K562-s cells were seeded at 5 X 10^5 cells per well in 1.75 ml media until 60-80% confluence. Lipofectamine RNAiMAX was diluted in Opti-MEM medium and mixed with diluted miRNA in a 1:1 ratio. Details of dilutions are stated below:

(a) 9 µl of lipofectamine RNAiMAX was mixed with 141 µl Opti-Mem,
(b) 3 µl miRNA (10µM) was mixed with 147 µl Opti-Mem making up a 30 pmol miRNA.

The miRNA-lipid complex was incubated for 5 minutes at room temperature, and 250 µl of the complex was added to cells. The total volume per well was 2 ml. Cells were incubated for 48 hours at 37°C and 5% CO_2 before visualisation or analysis (Deng et al., 2018; Hershkovitz-Rokah et al., 2015; Li et al., 2016).

3.5 Taqman™ MicroRNA Expression Assay

TaqMan microRNA assay was used to validate transfection efficiency by quantitating the expression level of miRNA after transfection into the cells. The quantification of miRNAs was performed using TaqMan™ MicroRNA Assays (Thermo Fisher Scientific) as listed in Section 3.2.6. Each assay employs proprietary primer and probe sequences, which are not disclosed in the product documentation.

These assays have been validated by the manufacturer to ensure accuracy and specificity in the detecting the targeted miRNAs, thereby providing reliable results for the quantification process. Following the manufacturer's protocol, such as RNA extraction, reverse transcription, or qPCR steps, the samples were processed, and miRNA expression levels were quantified accordingly.

3.5.1 MiRNA extraction

MiRNA was extracted using the miRNA-hybrid extraction kit (GeneAll, Korea) following the manufacturer's protocols. Briefly, cells were collected and subjected to centrifugation. The pellet was lysed in 500 µl of RiboEx™ per ~1 x10^7 cells by repetitive pipetting or vortexing. Homogenate was incubated for 5 minutes at room temperature to dissociate nucleoprotein complexes completely. 100 µl of chloroform was added and vigorously vortexed for 15 seconds. The mixture was incubated for 2 minutes at room temperature and centrifuged at 12,000 x g for 15 minutes at 4°C. The mixture was separated into a lower phase, an interphase, and a colourless upper aqueous phase. The upper layer of the aqueous phase was transferred into a fresh 1.5 ml centrifuge tube. One volume of 50% ethanol was added to the transferred aqueous phase and mixed thoroughly by inverting. All mixture was transferred into a Column Type B. The tube was centrifuged at room temperature ≥10,000 x g for 30 seconds. The mini column was then transferred to a new 2 ml collection tube and stored at room temperature. The pass-through now contained the small (micro) RNA. 500 µl absolute ethanol was added to the collection tube containing the pass-through and mixed by pipetting. 650 µl of the mixture was transferred to a Column Type W (mini). The tube was centrifuged at room temperature at ≥10,000 x g for 30 seconds. 500 µl of Buffer RBW was added to the mini-column and centrifuged again at ≥10,000 x g for 30 seconds

at room temperature. 500 µl of Buffer RNW was added to the mini column, centrifuged at ≥10,000 x g for 30 seconds at room temperature, and repeated twice. The tube was centrifuged for an additional 1 minute at ≥10,000 x g at room temperature to remove residual wash buffer. The mini column was transferred to a new 1.5 ml microcentrifuge tube. 50 µl of nuclease-free water was added into the centre of the membrane in the mini-column and incubated at room temperature for 1 minute. Lastly, RNA was eluted by centrifugation at ≥10,000 x g for 1 minute at room temperature. Purified miRNA was stored at -80°C before use. The assay workflow is mentioned in Figure 3.3.

3.5.2 MiRNA reverse transcription

The reverse transcription reaction mix was prepared as suggested by the manufacturer. One reaction consists of 0.15 µl of 100 mM dNTPs (with dTTP), 1 µl of MultiScribe™ Reverse Transcriptase, 50 U/µl, 1.5 µl of 10X Reverse Transcriptase Buffer, 0.19 µl of RNase Inhibitor, 20 U/µl, 4.16 µl of nuclease-free water for a total volume of 7 µl. The tube was inverted to mix, centrifuged briefly to collect content, and placed on ice. A reaction tube combined 7 µl of RT reaction mix with 5 µl of total RNA (1-10 ng). The solution was mixed thoroughly and briefly centrifuged. 3 µl of 5X RT primer was added to each tube and sealed. The tube was briefly centrifuged and stored on ice. Reverse transcription was performed following the manufacturer's instructions of 16 °C for 30 minutes, 42°C for 30 minutes, 85 °C for 5 minutes and 4°C (hold). The RT reaction product can be stored at -25°C to -15°C for up to one week.

3.5.3 Amplification of RT reaction products

The reactions were prepared in an area free from template contamination (e.g., plasmid DNA, cDNA, or PCR products) and siRNA transfections residues to ensure accurate qPCR amplification. The reactions were done in duplicates for each reaction. The following reactions were included: a small RNA assay for each cDNA sample, endogenous control assays for each cDNA sample and no-template controls (NTCs) for each assay on the plate. Before beginning, 60X assays were diluted to 20X working solution. Assays and cDNA templates were thawed on ice, gently vortexed and centrifuged. The PCR reaction mix was prepared by adding 1.00 µl TaqMan Small RNA Assay (20X), 10.00 µl PCR master mix, and 7.67 µl nuclease-free water to make a total reaction of 18.67 µl for one reaction. The mixture was vortexed and centrifuged briefly. The reaction was transferred into a 96-well plate, and a 1.33 µl cDNA template was added to 20 µl per well. The cycling condition was as follows: enzyme activation at 95°C for 10 minutes, denaturation at 95°C for 15 seconds and annealing/extension at 60°C for 60 seconds. The cycle was repeated 40 times.

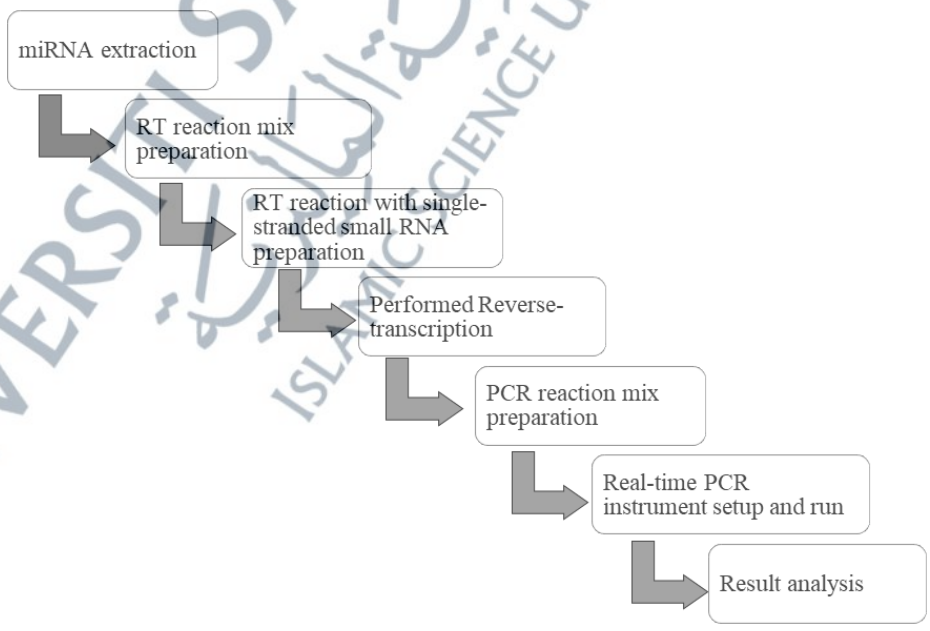


Figure 3.3: TaqMan miRNA assay workflow.

3.6 Biotinylated miRNA Pull-down Assay

This procedure measures the direct binding of miRNA to its mRNA target to establish functional interaction. It involves a one-step approach designed to detect and confirm the physical interaction between the miRNA and its corresponding target mRNA. First, a 3' biotinylated locked nucleic acid (LNA) miRNA mimic was transfected into the cells. Then, the miRNA: mRNA complex in the cellular lysate is captured using streptavidin-coated magnetic beads. The streptavidin-biotin interaction allowed the miRNA-mRNA complex to be pulled and captured with a magnetic stand. The mRNA target bound to its complementary miRNA is later purified and quantified via qPCR.

3.6.1 Production and transfection of 3'-biotinylated miRNA

Biotin-tagged miRNA duplexes were purchased from Qiagen. These Biotin-tagged miRNA duplexes contained the Locked Nucleic Acid (LNA) technology. The manufacturer's instructions were followed for further processing. K562-r and K562-s were seeded at 5 x 10^5 per well in 1.75 mL of complete RPMI-1641 medium supplemented with 10% Fetal Bovine serum (FBS). The cells were cultured in an incubator at 37 °C, 5% CO2 overnight. After 24 hours, the health and conditions of the plated cells were observed under a light microscope. Cells showing normal morphology, such as round shape, smooth membrane, and suspension growth without aggregation or blebbing were considered healthy and suitable for miRNA transfection. Cells with normal morphology are needed before proceeding to the next step. miRNAs transfection was done according to section 3.4.5. The miRNA lipid complexes were transferred to the cell and incubated for 48 hours.

3.6.2 Washing step I: Preparation of streptavidin-coated magnetic beads

Thoroughly, the streptavidin magnetic beads were resuspended by vortexing, and 30 µL of bead suspension (per sample) was transferred to a new microcentrifuge tube. The tube containing the bead suspension was placed on the magnetic bead separator stand ("magnet") for 2 min, and the supernatant was removed carefully. Next, the beads were added to 100 µL of bead wash buffer (10 mM Tris-Cl pH 7.5, 0.5 mM EDTA, 1 M NaCl). The tube was removed from the magnet and subjected to vortex for 15 seconds at room temperature. Once again, the tube containing beads was placed on the magnet for 2 min, and the supernatant was carefully discarded. Washing was repeated three times. After the final wash step, the tube was removed from the magnet. 100 µL of RNase freeing solution (0.1 M NaOH, 0.05 M NaCl) was added to the beads, mixed well by vortex for 15 s, and incubated at room temperature for 5 min. The tube containing beads was placed on the magnet for 2 min, and the supernatant was discarded. The process was repeated three times. Later, bead resuspension solution (0.5 M NaCl) was added to the beads, vortexed for 15 s, and incubated at room temperature for 5 min. Resuspended beads were placed on the magnet for 2 min, and the supernatant was removed carefully. Lastly, 200 µL of bead-blocking solution (1 µg/µL BSA, 2 µg/µL Yeast tRNA) was added to the beads. The mixture undergoes gentle vortexing for 15 seconds at room temperature. The mixture was incubated at 4°C for 16 hours (overnight) on a multi-tube rotator.

3.6.3 Preparation of cell lysates

K562-r and K562-s transfected cells were harvested and transferred into a sterile 2 mL microfuge tube. The cell was subjected to centrifugation at 300x g for 5 minutes. The supernatant was removed, and the pellet was resuspended in 1x sterile PBS (Phosphate Buffer Saline), pH 7.2 and centrifuged again to remove residual media. The tube containing the pellet was immediately plunged into ice. Next, 260 µL of ice-cold, freshly prepared complete cell lysis buffer was added to each sample in the microcentrifuge tube, and the pellet was resuspended into a homogenous suspension by pipetting. The cells were then lysed using the freeze-thaw method by incubating the tubes at -80 °C for 15 min. Then, cells were allowed to thaw out on the ice. The cell lysate was centrifuged at 16,000 x g for 5 min in a refrigerated benchtop centrifuge (4°C). Cleared cell lysate was transferred to a sterile microcentrifuge tube on ice. Ten microliters of cell lysate were put aside into another tube as the control lysate (before pull-down). Lastly, 5M NaCl was mixed with the cleared lysate to a final concentration of 1M, and the samples were maintained on ice. For example, 60 µL of 5M NaCl to 240 µL of lysate, giving a final concentration of 1M NaCl in 300 µL.

3.6.4 Washing step II: Preparation of streptavidin magnetic beads

The tube containing the prepared beads from section 3.6.2 was placed on the magnet for 2 min. The supernatant was carefully removed. Next, 150 µL of ice-cold, freshly prepared, complete pull-down wash buffer was added to the beads. The mixture was vortexed for 15 seconds and incubated at room temperature for 60 seconds. The tube was placed on the magnet for 2 min, and the supernatant was carefully removed

and discarded. This washing step was repeated three times. Lastly, the beads were resuspended in 300 µL of complete pull-down wash buffer.

3.6.5 Pull-down of target mRNA-miRNA complexes

300 µL of the cell lysate from section 3.6.3 was transferred to the microfuge tube containing 300 µL beads. The mixture was incubated on a rotating mixer for one hour at room temperature to allow miRNA to bind to their target mRNA. Next, the tube was placed on the magnet for 5 min. The supernatant was carefully removed and discarded. 300 µL of ice-cold complete pull-down wash buffer was added to the beads. The tube was vortexed for 15 seconds at room temperature and placed on the magnet for 5 min. The supernatant was carefully removed, and this washing step was repeated three times. The beads were suspended in 100 µL of nuclease-free water and incubated on ice. Beads now contained the miRNA and target mRNA and were immediately subjected to RNA extraction.

3.6.6 RNA extraction from biotin-pull down assay

A modified RNA cleanup protocols based on the RNeasy Mini Kit (Qiagen, Germany) was employed to dissociate the biotinylated miRNA-mRNA complexes from the magnetic beads in the pull-down assay. Briefly, after the pull down, the sample comprising biotin-tagged miRNA bound to its target mRNA and attached to streptavidin magnetic beads was adjusted to 100 µL with RNAse-free water. Next, 350 µL of Buffer RLT was added, and the mixture was thoroughly homogenized by repetitive pipetting and vortexing. The tube was then incubated at 37°C with shaking at 300 rpm for 10 minutes to promote the release of the RNA complexes from the beads. Following

incubation, magnetic separation was performed to retain the beads while collecting supernatant containing the release RNA. The collected supernatant was then subjected to centrifugation at 6000 xg for 1 minute before proceeding with RNA purification. The supernatant was transferred into a new tube. A 250 µL of 100% ethanol was added to the supernatant and mixed well by pipetting. 700 µL of the mixture was then transferred to the column and placed in a 2 ml collection tube. The tube was centrifuged for 15 seconds at 10,000 xg. The flow-through was discarded, and the column was put back into the collection tube. Next, 500 µL of buffer RPE was added to the spin column and centrifuged for another 15 seconds at 10,000 xg to wash the spin column. Flow-through was discarded. The step was repeated but with 2 minutes of centrifugation. Next, the spin column was inserted in a new 1.5 ml collection tube, and 30 µL of RNAse-free water was added directly to the centre of the spin column membrane. The tube was centrifuged at 10,000xg for 1 minute for RNA elution. RNA was stored at -80°C until further use. RNA concentration was later quantified using the method as described in sub-section 3.7.1. For quantification and amplification of the miRNAs' target, the ABL1 gene, qPCR protocols and primer sequence were used from subsections 3.8 and 3.9.

3.7 RNA Extraction

All consumables and apparatus such as Eppendorf tubes, tips, Schott bottles and water used for RNA extraction are DNase/RNase free. Total RNA was extracted from cells using the GeneAll Hybrid-R following manufacturer protocols (GeneAll, Korea). Basically, for cells grown in suspension. Approximately 1 X 10^7 cells were pelleted by centrifugation. Cells were then lysed in 1ml of RiboEx™ (GeneAll, Korea) by repetitive pipetting or vortexing. The homogenate was incubated for 5 minutes at room

temperature to allow nucleoprotein complexes to dissociate completely. A 0.2 ml chloroform was added per 1 ml of RiboEx™ and was shaken vigorously for 15 seconds and incubated for 2 minutes at room temperature. Homogenate was centrifugated at 12,000 x g for 15 minutes at 4°C, and the colourless upper aqueous phase was transferred to a fresh tube. One volume of buffer RB1 was added to the sample and mixed thoroughly by inverting it. A maximum of 700 µl mixture was transferred to a mini spin column and centrifuged at ≥ 10,000 x g for 30 seconds at room temperature. 500 ul of buffer SW1 was added to the mini spin column and centrifuged at ≥ 10,000 x g for 30 seconds at room temperature. 500 ul of Buffer RNW was added to the mini spin column and centrifuged at ≥ 10,000 x g for 30 seconds at room temperature. Flow-through was discarded, and the mini spin column was reinserted into the same tube. The column was centrifuged at ≥ 10,000 x g for an additional 1 minute at room temperature to remove residual wash buffer. The mini spin column was transferred to a new 1.5 ml tube. 50 µl of Rnase-free water was added to the centre of the membrane in the mini spin column and let stand for 1 minute. Lastly, RNA was eluted after being centrifuged at ≥ 10,000 x g for 1 minute at room temperature. Purified RNA can be stored at 4°C for immediate analysis and -70°C for long-term storage. Purified RNA is free of DNA and protein, and A260/280 is between 1.8 and 2.1.

3.7.1 RNA quantification and quality determination

The quantity and quality of extracted RNAs were determined using Implen NanoPhotometer® P-300 (GmbH, Germany). RNA purity was determined by the absorbance ratio at 260 nm to 280 nm (A260/280), where values between 1.8 – 2.1 are generally indicative of high purity. Ratio lower than this range suggest the presence of

protein contamination or other contaminants absorbing at 280 nm, whereas ratio exceeding 2.1 may reflect contamination with phenol, guanidine, or the presence of single-stranded nucleic acid (Manchester, 1995). The absorbance ratio of 260/230 was also evaluated, with an acceptable range of 1.8-2.2. Lower value may indicate contamination with EDTA, carbohydrate or phenol, which absorb at 230 nm, while unusually high values can arise from residual extraction reagents, background noise, or instrument artefacts (Wilfinger et al., 1997).

3.8 cDNA Synthesis

Isolated RNAs were reverse transcribed into complementary DNAs (cDNAs) using the AffinityScript QPCR cDNA Synthesis kit (Agilent, USA). cDNA synthesis was performed using 100 ng of total RNA. cDNA master mix was prepared in a 0.2 ml microcentrifuge tube by adding RNAse-free water to a total volume of 20 µl, 10 µl of cDNA synthesis master mix (2X), 3 µl of random primers, 1 µl of AffinityScript RT and 100 ng of total RNA (x µl). The mixture was put in a thermal cycler with cycling conditions of 25 °C for 5 minutes, 42 °C for 15 minutes, 95 °C for 5 minutes and 4°C as the holding stage. For long-term storage, the reaction can be stored at -20 °C.

3.9 Annealing Temperature and Primer Concentration Optimisation

The primer annealing temperature and concentrations were optimised using gradient PCR. Gradient temperatures were set at 52°C, 55°C, 58°C, 61°C and 64°C while primer concentrations used were 200 nM, 300 nM, 400 nM and 500 nM. The primers used are listed in Table 3.2. ABL1 sequences were retrieved from (Qin &

Huang, 2016), and the BCR-ABL1 primer was from (Jennings et al., 2014). GAPDH, PGK1, ACTB and PP1B primers were taken from (Cao et al., 2012).

Table 3.2: Primer sequences for ABL1, BCR-ABL1 and housekeeping genes amplifications.

Gene	Primer sequence (5' to 3')
ABL1 forward	TGGAGATAACACTCTAAGCATAACTAAAGGT
ABL1 reverse	GATGTAGTTGCTTGGGACCCA
BCR-ABL1 forward	CATTCCGCTGACCATCAATA
BCR-ABL1 reverse	ACACCATTCCCCATTGTGAT
	Housekeeping genes (HKG)
GAPDH forward	CAACAGCGACACCCACTCCT
GAPDH reverse	CACCCTGTTGCTGTAGCCAAA
PGK1 forward	AAGAACAACCAGATAACAAACAAC
PGK1 reverse	GTGGCTCATAAGGACTACCG
ACTB forward	TTGGCAATGAGCGGTTCC
ACTB reverse	GTTGAAGGTAGTTTCGTGGATG
PP1B forward	GTCCGTCTTCTTCCTGCTG
PP1B reverse	CATCTTCATCTCCAATTCGTAGG

3.9.1 Determination of qPCR efficiency

QPCR efficiency for each primer was determined by a standard curve of different cDNA concentrations in 10-fold dilutions. The standard curve was constructed, and PCR efficiency was estimated in a range between 90% and 110% with a coefficient of correlation, R^2 equals or more than 0.9.

3.9.2 Selection of housekeeping genes

Two housekeeping genes (HKGs) were selected for the qPCR reaction from the list of HKG available: the GAPDH, ACTB, PGK1 and PP1B. The selection of HKG was based on the stability of the genes in all treatment groups. Ct values from the reaction were exported into an online software https://www.heartcure.com.au/reffinder/ a web-based comprehensive tool developed for evaluating and screening reference genes from extensive experimental datasets. It integrates major computational programs such as geNorm, Normfinder, BestKeeper, and the comparative Delta-Ct method to compare and rank the tested candidate reference genes. Based on the rankings from each program, it assigned an appropriate weight to an individual gene and calculated the geometric mean of their weights for the final ranking (Xie et al., 2012).

3.10 Amplification of ABL1 and BCR-ABL1 Gene Using qPCR

ABL1 gene expressions were determined with ABL1 and BCR-ABL1 specific primers, as stated in Table 3.2. Quantitative polymerase chain reaction (qPCR) was performed using Brilliant III Ultra-Fast SYBR® Green QPCR Master Mix (Agilent, USA). The reactions were carried out in a thermocycler with a cycling program of activation at 95°C for 3 minutes followed by 40 cycles of denaturation at 95°C for 5 seconds, annealing and extension together at 60°C for 10 seconds. Finally, melting analyses were performed to confirm the absence of false-positive peaks. All reactions were performed in duplicate for each sample. A no template control (NTC), in which nuclease free water was added instead of cDNA template, was included as negative control in all reactions. Relative expression levels (Cq values) of the ABL1 gene were

quantified using the $2^{-\Delta\Delta Cq}$ method with the housekeeping gene as internal control and data normalisation.

3.11 Colorimetric Cell-Based Enzyme Link Immunostaining Assay (ELISA) of ABL1 Protein Quantification and Determination.

The protein activity of the proto-oncogene tyrosine-protein kinase ABL1 was determined with the ABL1 (Human) Cell-Based ELISA kit (Abnova, USA). Mouse Anti-GAPDH Antibody was used as the internal positive control, while HRP-Conjugated Anti-Rabbit IgG Antibody and HRP-Conjugated Anti-Mouse IgG Antibody were used as the negative controls. 200 µl of 30,000 cells were seeded in a culture medium in each well of a 96-well plate. The plate was coated with 100 µl of 10 µg/mL of Poly-L-Lysine solution (Sigma Cat# P4832) in each well for at least 1 hour at 37°C with gentle shaking on the orbital shaker before adding cells. Plates were washed twice in sterile water and left to dry for at least 2 hours before using them. Cells were then incubated overnight at 37°C, 5% CO_2. After 24 hours, cells were transfected with miRNA for 48 hours (Deng et al., 2018; Hershkovitz-Rokah et al., 2015; Li et al., 2016). Later, the culture medium was removed, and the cells were washed twice with 200 µl of 1x TBS solution. The cells were then fixed in 100 µl fixing solution (8% formaldehyde) for 20 minutes at room temperature. Afterwards, the fixing solution was removed, and the plate was washed three times with 200 µl 1x wash Buffer for five minutes each time with gentle shaking on the orbital shaker. 100 µl quenching buffer was added and incubated for 20 minutes at room temperature. The plate was washed thrice with 1x wash Buffer for 5 minutes, with gentle shaking on the shaker. Next, 200 µl of blocking Buffer was added and incubated for 1 hour at room temperature. Once

again, the plate was washed thrice with gentle shaking. A 50 µl of 1x primary antibodies (Anti-ABL1 Antibody or Anti-GAPDH Antibody) was added to the corresponding wells, covered with parafilm and incubated for 16 hours (overnight) at 4°C. The plate was washed thrice with 1x Wash Buffer for 5 minutes, with gentle shaking on the shaker. Then, 50 µl of 1x secondary antibodies (HRP-Conjugated Anti-Rabbit IgG or HRP-Conjugated Anti-Mouse IgG) was added to corresponding wells and incubated for 1.5 hours at room temperature with gentle shaking on the shaker.

The washing step was repeated three times. Afterwards, 50 µl of the ready-to-use substrate was added to each well and incubated for 30 minutes at room temperature in the dark with gentle shaking on the shaker. Lastly, 50 µl of Stop Solution was added to each well, and plate Optical density (OD) reading was read at 450 nm immediately using the microplate reader. Following the colourimetric measurement of HRP activity via substrate addition, the crystal violet whole-cell staining method was used to determine cell density. After staining, results were analysed by normalising the absorbance values at 590 nm for cell count, by which the plating difference can be adjusted. Cell count normalisation was performed for data analysis and calculation of results. The OD 450 values obtained for the target protein can be normalised using the OD 590 values obtained for crystal violet staining. Figure 3.4 describes the protocols in a simplified manner.

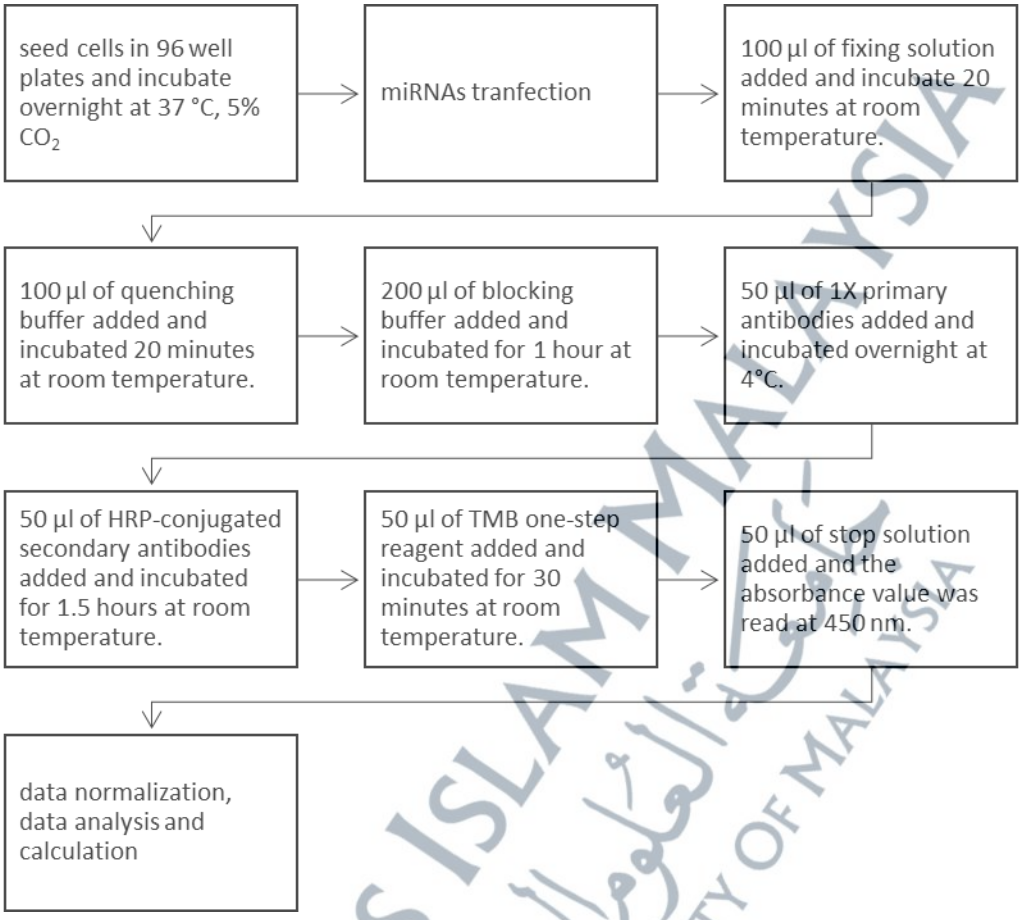
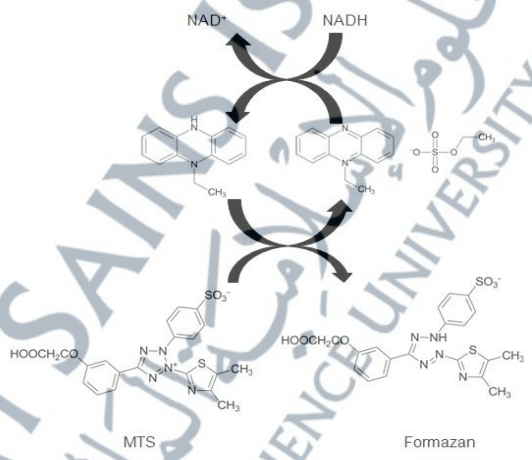


Figure 3.4: Flowchart of ABL1 cell-based ELISA assay.

3.12 Cell Viability/Proliferation Determination

Cell viability and proliferation of transfected cells were determined using CellTiter 96® AQueous One Solution Cell Proliferation Assay (Promega, USA, cat #G3580) following manufacturer instructions. This assay is based on a colourimetric method for determining the number of viable cells in proliferation or cytotoxicity assays. It contains a novel tetrazolium compound [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H- tetrazolium, inner salt] (MTS) and an electron coupling reagent (phenazine ethosulfate; PES) use as a substrate for MTS assay. Dehydrogenase enzymes of metabolically active cells reduce the substrates into a coloured formazan product soluble in a tissue culture medium (Figure 3.5) (Riss et al.,

2016). Absorbance is measured based on the amount of formazan produced at 490 nm. Cells were seeded at 5,000 cells per well in a 96-well plate one day before transfection until 60-80% confluent in a 90 µl medium. The next day, miRNA was transfected into the cell culture. Briefly, 1.5 µl lipofectamine reagent was diluted in 25 µl Opti-mem, and 0.5 µl miRNA was diluted in 25 µl Opti-mem. Diluted miRNA was added to diluted lipofectamine in a 1:1 ratio. The mixture was incubated for five minutes, and later, 10 µl of the miRNA-lipid complex was added into the cell culture, making up to a miRNA with a final concentration of 1 pmol per well. Each miRNA was transfected into three different wells for technical replicates. For blank, a medium with no cells was used to obtain a value for background absorbance. Cells were incubated for another 48 hours before adding MTS solution.



Source: (Riss et al., 2016)

Figure 3.5: Intermediate electron acceptor phenazine ethosulfate (PES) transfers electrons from NADH in the cytoplasm to reduce MTS in the culture medium into an aqueous soluble formazan.

Later, 20 µl of CellTiter 96® AQueous One Solution Reagent was added per well, and the plate was incubated for 4 hours in a humidified, 5% CO2 atmosphere. Absorbance was measured at 490 nm using a 96-well plate reader. Cell viability for

each miRNA transfection was calculated by dividing the absorbance (A490) treated cells with cells without treatment. A summary of cell viability calculation is as follows:

$$\text{cell viability (\%)} = \frac{\text{Abs transfected cellls} - \text{Abs blank}}{\text{Abs control cells} - \text{Abs blank}} \text{x } 100$$

Where, Abs = absorbance at 490 nm.

3.13 Cell Cycle Assay

Flow cytometric analysis of cellular DNA content is used to measure and identify abnormal cell populations. Cellular DNA content provides information for cell-cycle analysis; thus, the DNA index of diploid and aneuploid cells is established. This assay identified CML's cell cycle stages that might be affected by miRNA transfection. First, a DNA quality test was done to get an optimum result using the BD™ DNA QC Particles kit (Biosciences, USA) using the same FACS machine. The cell cycle analysis was done using BD™ Cycletest Plus DNA kit reagent (Biosciences, USA). For cell suspension preparation, cells were placed into a labelled 17 x 100 mm tube. Tubes were centrifuged at 300 xg at room temperature for 5 minutes.

The supernatant was aspirated, leaving 50 µl of residual fluid. The pellet was resuspended in one millilitre of buffer and gently vortexed. These steps were repeated twice. The cells were counted using a haemocytometer and adjusted to $1x10^6$ cells/mL with buffer solution. A total number of 5.0×10^5 cells was needed for cell staining. The cell suspension was centrifuged at room temperature at 400 xg for 5 minutes. The supernatant was decanted, and the pellet was resuspended in 250 µL of Solution A (trypsin buffer). The tube was gently mixed by tapping it by hand. The mixture was incubated for 10 minutes at room temperature. Next, 200 µL of Solution B (trypsin

inhibitor and RNase buffer) was added and gently mixed by tapping the tube with a hand. The tube was incubated for 10 minutes at room temperature. Then, 200 µL of ice-cold Solution C (PI stain) was added and gently mixed by tapping with a hand. The tube was incubated again for 10 minutes in the dark on ice. After incubation, samples were filtered through a 35-µm cell strainer cap into a 12 x 75 mm tube. The prepared tubes were stored at 2°C– 8°C in the dark before analysis using the flow cytometer and FACScan software. For optimal results, samples must be acquired from the flow cytometer within 3 hours after adding Solution C. The DNA Index can be calculated from the ratio of the mode (or mean) of the G0/G1 peaks of the test and control cell populations for a particular specimen.

3.14 Microarray Gene Expression

The RNA samples' concentrations were determined using a bioanalyser (Agilent, USA) to determine their RNA quality and integrity before the microarray. Due to the limited number of available microarray slides, only two miRNA-treated groups were selected, alongside imatinib and untreated controls. One representative was chosen from human miRNAs and one from plant miRNAs. For the human group, hsa-miR-3131 was selected over hsa-miR-891a-3p based on its higher efficacy in downregulating both ABL1 and BCR-ABL1 gene expression, as determined by qPCR analysis. For the plant group, osa-miR1858a/b was selected as it was the only plant-derived miRNA tested. Imatinib served as a positive control, while untreated cells acted as a negative control. Samples were sent in duplicate, making each slide of eight and 16 RNA samples for both cells. Next, RNA concentration samples were normalised to 50 ng before preparing Cyanine-3 (Cy3) labelled cRNA using the One-Color Low Input Quick Amp

Labelling Kit (Agilent, Valencia, Ca) following the manufacturer's instructions. Cy3 labelled RNA was then purified by Rneasy Mini Kit (Qiagen, Valencia, CA) to purify the amplified cRNA samples. Dye incorporation and cRNA yield were checked with the NanoVue Plus Spectrophotometer (GE Healthcare, UK). A 0.825 mg of Cy3-labeled cRNA with specific activity ≥ 6 pmol Cy3/mg cRNA was fragmented at 60°C for 30 min in a reaction volume of 25 ml containing 25X Agilent fragmentation buffer and 10X Agilent blocking agent following the manufacturer's instructions.

After the fragmentation step, 25 ml of the mixture was combined with 25 ml of 2x Hi-RPM Hybridization Buffer and immediately hybridised to Agilent SurePrint G3 Human Gene Expression v3 8x60K (Design ID:072363) for 17 hours at 65 °C in a rotating microarray hybridisation oven. After hybridisation, microarrays were washed for 1 min at room temperature with GE wash buffer 1 and 1 min with 37 °C GE Wash buffer 2. Slides were immediately scanned using Agilent SureScan Microarray Scanner (G4900DA) with 3 mm resolution at wavelengths of 532 nm (Cy3) using the extended dynamic range (10-100%) setting. Normalised intensities were extracted using the Agilent Feature Extraction Software with protocol GE1_1200_Jun14, and all eight samples' data (duplicate) in a txt format were obtained. The files were analysed using Agilent GeneSpring Software version 14.9.1. Figure 3.6 shows a simplified workflow for sample preparation and array hybridisation design, while Figure 3.7 shows the flow of analysis of microarrays' results. The workflow outlines the downstream analysis of the microarray dataset, beginning with quality control by filtering genes based on expression levels to remove low-quality data. The filtered genes were visualised in a volcano plot to identify differentially expressed genes (DEGs), which were then subjected to clustering, gene ontology analysis, and pathway analysis to classify

expression patterns, assign functional annotations, and determine involvement in biological pathways.

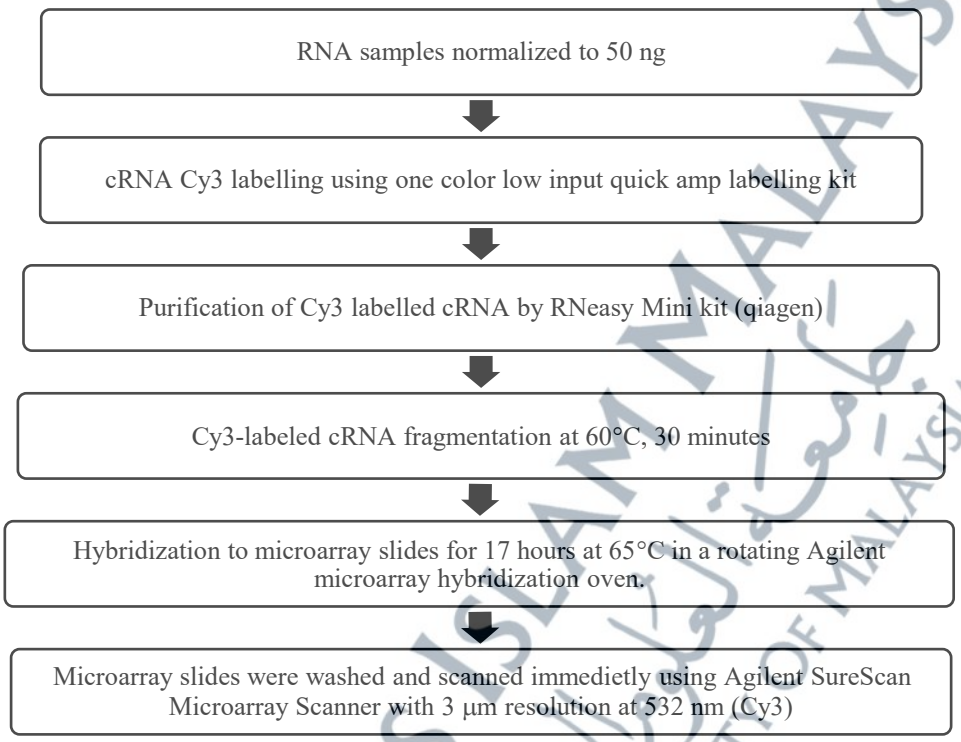


Figure 3.6: Standard workflow for sample preparation and array hybridization design.

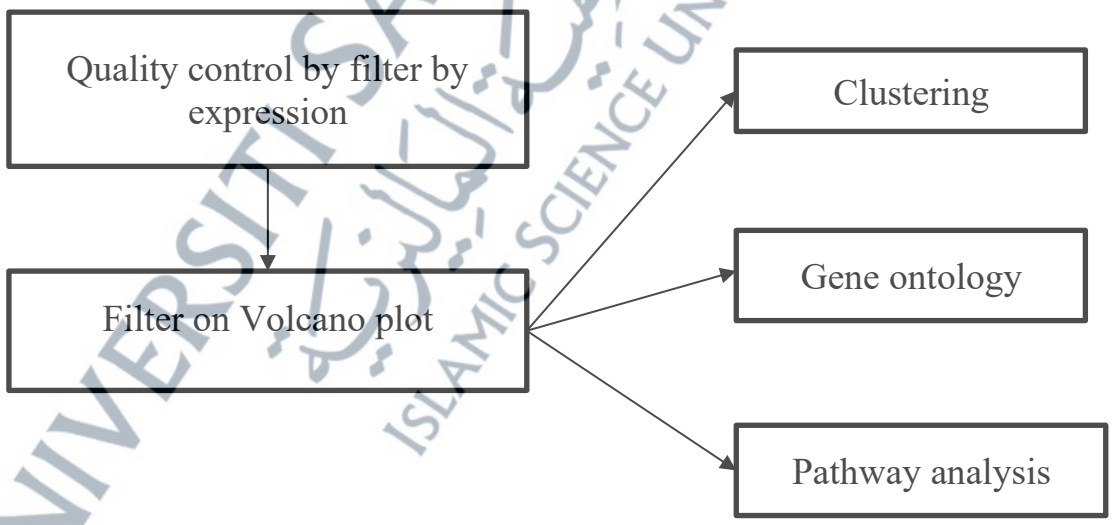


Figure 3.7: Flow of the analysis from microarray report.

3.14.1 Real-time polymerase chain reaction (qPCR) for microarray genes expression

From the microarray results, genes related to the study were selected for further validation by qPCR. Selection criteria included a fold change greater than 2, a statistically significant P-value, and their involvement in key pathways associated with chronic myeloid leukaemia (CML). Based on this criteria, five downregulated and three upregulated genes were chosen. All primers were designed in-house using Primer3, except for TERT primer, which was obtained from Y. Zhang et al. (2012) (Table 3.3).

Table 3.3: List of primers for gene validation.

Gene	Primer sequence (5' to 3')
IL6 Forward	CACAGACAGCCACTCACCTC
IL6 Reverse	GCCTCTTTGCTGCTTTCACA
CSF1 Forward	CTGCCCTCCCACGACATG
CSF1 Reverse	TCCTCGGTGATACTCCTGCT
TNF Forward	AGGCAGTCAGATCATCTTCTCG
TNF Reverse	GCTTGAGGGTTTGCTACAACA
MAP4K1 Forward	TACCAAGTGACAGGCTCCCT
MAP4K1 Reverse	TGACCTCCCCAGCATCATTG
IL1B Forward	CCTGTCCTGCGTGTTGAAAG
IL1B Reverse	GGGAACTGGGCAGACTCAAA
MYC Forward	TAGTGGAAAACCAGCCTCCC
MYC Reverse	TCCTCCTCGTCGCAGTAGAA
PAK1 Forward	CACCACTCCACCAGATGCTT
PAK1 Reverse	CCACACTCACTATGCTTCGTAA
TERT Forward	ATGCGACAGTTCGTGGCTCA
TERT Reverse	ATCCCCTGGCACTGGACGTA

Primarily, RNA was converted into cDNA using LunaScript® RT SuperMix Kit (NEB, England) with 4 µl LunaScript RT SuperMix, 1µg RNA and nuclease-free water until 20 µl. The reaction was put in a thermocycler at 25°C for 2 minutes, 55°C for 10

minutes and 95°C for 1 minute. Next, the amplification reaction was done using the Luna Universal qPCR Master Mix (NEB, England) in a 20 µl reaction. The qPCR components were 1X Luna Universal qPCR Master Mix, 250 nM primers, 100 ng cDNA and nuclease-free water. Then, thermocycling protocols start with an initial denaturation at 95°C for 1 minute, denaturation at 95°C for 15 seconds, annealing and extension at 60°C for 30 seconds, and then melt curve analysis. A gene's relative expression levels (Cq values) were quantified using the $2^{-\Delta\Delta Cq}$ method with GAPDH and Beta-actin as the internal control and data normalisation.

3.15 Statistical Analysis

All data analysis was performed using SPSS version 26. Data distribution was assessed using the Shapiro–Wilk test to evaluate normality. As the data did not follow a normal distribution, the non-parametric Mann-Whitney U test was used to compare the categorical independent variable to the numerical dependent variable in a two-group comparison. P-values of 0.05 or less were defined as statistically significant. The graphs were generated with Graph Pad PRISM 8.4.0.