

## Optimisation of miRNA transfection conditions and studying the effect of miRNA down-regulation on CK $\alpha$ gene expression in HepG2 cell line

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**ABSTRACT:** MicroRNA (miRNA) is a small RNA molecule of 22 nucleotides long that regulates gene expression by binding to the 3'-untranslated region of a specific mRNA and subsequently leads to the degradation of the targeted mRNA. MiRNA plays vital role in human physiology and developmental processes. Thus, analysis of miRNA regulation of gene expression provides valuable information in the study of miRNA related diseases such as cancer and certain metabolic disorder. In order to study the effect of miRNA on the expression of a specific gene in mammalian cell culture, the transfection efficiency of miRNA mimics must be optimised for the specific cell line. This study aims to optimise the conditions for GAPDH-targeting miRNA transfection of HepG2 cell line and to investigate the effect of GAPDH-miRNA on the expression of choline kinase alpha (*ck $\alpha$* ) gene expression. The GAPDH-miRNA concentration and transfection duration were optimised for the strongest down-regulation of GAPDH mRNA and protein levels. MiRNA with the concentrations of 25, 50 and 100 nM and transfection durations of 24, 36, 48 and 72 hours were tested in triplicate experiments with non-targeting miRNA as negative control. After the transfection, GAPDH and *ck $\alpha$*  relative mRNA levels were quantified by real-time PCR and GAPDH protein was detected by Western blot detection. The results showed that 25 nM and 48 hour transfection duration resulted in the lowest GAPDH mRNA and protein levels without apparent cytotoxic effect to HepG2 cells. The optimised transfection parameters determined in this study can be used as the general miRNA transfection protocol for HepG2 cell line. The GAPDH-miRNA is also suitable for experiments to validate potential miRNAs targeting *ck $\alpha$*  as

it did not affect the expression level of *cka* for all the concentrations and transfection durations tested.

**Keywords:** miRNA, transfection, HepG2 cell line, choline kinase alpha.

## Introduction

MicroRNA is a short, single-stranded non-coding RNA molecule of typically about 20-23 nucleotides long. It negatively regulates gene expression at post-transcriptional level by either mRNA degradation or translational repression (Rotllan and Fernandez-Hernando, 2012). MiRNAs are synthesized from either canonical (primary miRNAs from intergenic, intronic or polycistronic genomic loci) or non-canonical (primary miRNAs transcribed directly as endogenous short hairpin RNAs or spliced from introns) pathways (Rottiers and Naar, 2012). After some processing by Dicer and RNAase III enzyme complex in the cytosol to form 22-nucleotide double stranded miRNAs, Argonaute proteins unwind the miRNA duplex to produce AGO-containing RNA-induced silencing complex (RISC)-miRNA assembly that targets specific mRNA for down-regulation (Rottiers and Naar, 2012). It has been predicted that the expressions of more than 60% of human protein-coding genes were regulated by miRNAs. Higher repression efficiency of miRNAs is achieved by a single miRNA having multiple target sites in the 3' UTR of a particular mRNA (Mack, 2007) or a single mRNA is targeted by many miRNAs (Lim *et al.*, 2005, Saito and Saetrom, 2012). The involvement of miRNA in gene regulation that relates to vital biological processes such as cell growth, cell differentiation, apoptosis, lipid metabolism and various human disorders including cancers and viral infection has made miRNA becomes one of the widely studied biological molecules (Ambros, 2004, Huntzinger and Izaurralde, 2011, Hwang and Mendell, 2006, Rottiers and Naar, 2012, Santhakumar *et al.*, 2010). MiRNA has caught the attention of cancer researchers and developmental biologists because of its involvement in colon, breast and brain cancers as well as animal development (Hu *et al.*, 2011, Iorio *et al.*, 2005, Rosenbluth *et al.*, 2013, Takagi *et al.*, 2009, Wienholds and Plasterk, 2005). More recently, miRNAs have been tested as new therapeutic targets for many diseases by utilising either miRNA antagonists or miRNA replacements (Broderick and Zamore, 2011, van Rooij *et al.*, 2012).

Transfection of miRNA mimic into eukaryotic cells is used to study the function of a particular miRNA or to regulate the expression of a gene targeted by a specific miRNA. MiRNA mimics are chemically synthesized double-stranded RNA molecules designed to mimic endogenous mature miRNA molecules when introduced into cells. The availability of commercial miRNA mimics covering wide range of miRNA species from various suppliers have made experiments involving miRNA mimics become more convenient and relatively less costly. Many commonly used positive and negative control miRNA mimics are also verified by the suppliers in terms of their efficiency and target specificity. The users only need to perform basic optimisation for the transfection protocol and cell line in use. Synthetic miRNA mimics can also be modified to maximize the formation of mature miRNA-RISC complex and minimize the loading of passenger strand into the complex.

MiRNAs can be delivered into cells either by electroporation or more commonly by lipid mediated transfection. Selection of miRNA transfection reagent depends mainly on cell line. In a typical experiment involving gene down-regulation by miRNAs in mammalian cell lines, non-targeting miRNA with sequence usually derived from organism that has no homology to any known mammalian gene like *Caenorhabditis elegans* will be used as negative control. A positive control miRNA targeting constitutively expressed gene must also be included as a validation that the experimental system is working as expected (Cheng *et al.*, 2005). Optimisation of miRNA transfection efficiency is usually done by using positive control miRNAs targeting housekeeping genes such as glyceraldehyde-3-phosphate dehydrogenase (GAPDH) that are constitutively expressed in most cell types and their mRNA and protein levels can be measured easily (Borawski *et al.*, 2007). Optimisation of parameters like miRNA concentration and transfection duration is also performed to rule out any possibility of cell toxicity caused by the transfection protocol and to determine the best conditions for miRNA down-regulation of the target gene.

The optimisation performed in this study was intended to be subsequently applied in experiments to investigate the effect of miRNA down-regulation of human choline kinase gene expression. Choline kinase is the first enzyme in Kennedy pathway for the synthesis of phosphatidylcholine, an essential lipid building block of membranes. This enzyme phosphorylates choline by using ATP and  $Mg^{2+}$  as cofactor to form

phosphocholine (Mori *et al.*, 2007). Human choline kinase is encoded by two independent genes known as *ck $\alpha$*  and *ck $\beta$* . The *ck $\alpha$*  gene produces CK $\alpha$ 1 (NP\_997634) and CK $\alpha$ 2 (NP\_001268.2) isoforms while the *ck $\beta$*  gene gives rise to CK $\beta$  (AAH82263) isoform. The CK $\alpha$ 2 is longer than CK $\alpha$ 1 by having an additional stretch of 18 amino acids starting at the amino acid position 155. CK $\alpha$  has been implicated in cellular carcinogenesis and its expression level was much higher in breast, lung, prostate, liver and colorectal cancers, thereby making it a promising target for potential antitumor therapy (Gruber *et al.*, 2012). CK $\beta$  on the other hand plays important role in the developmental process of mouse embryo. Defect of *ck $\beta$*  gene causes muscular dystrophy in mice (Wu *et al.*, 2009). Our previous siRNA knockdown of CK $\alpha$  and CK $\beta$  showed that the balance between the two isoforms determine the fate of cell cycle progression (Gruber *et al.*, 2012). Thus, it would be interesting to search for miRNAs targeting choline kinase isoforms and investigate the regulation of cell cycle or cellular lipid metabolism by miRNAs.

In this study, optimisation of the miRNA transfection specifically for HepG2 cell line was carried out by using DharmaFECT 2 transfection reagent. MiRNA concentration and transfection duration were optimised for maximum down-regulation of GAPDH gene expression by miRNA targeting this housekeeping gene. GAPDH gene expression was measured by real-time quantitative PCR and Western blot. The effect of GAPDH-miRNA on the expression of *ck $\alpha$*  gene was also investigated to ensure that the positive control miRNA does not affect the expression of *ck $\alpha$* . Results obtained in this study are useful for validating potential *ck $\alpha$* -targeting miRNAs in HepG2 cell line.

## **Materials and methods**

### *Cell culture*

The HepG2 (hepatocellular carcinoma) cell line obtained from the American Type Culture Collection (ATCC no. HB-8065) was maintained at 37°C, 95% humidity and 5% CO<sub>2</sub> in Dulbecco's Modified Eagle's Medium (DMEM) with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. The HepG2 cells were seeded in 24-

well plate at a concentration of  $1.5 \times 10^5$  cells/well (diluted with antibiotic-free complete medium) one day before transfection with miRNAs.

#### *Transfection of HepG2 cells with non-targeting and GAPDH miRNAs*

Non-targeting (miRIDIAN microRNA Mimic Negative Control #1; Dharmacon) and GAPDH-targeting (Mimic Housekeeping Positive Control #2; Dharmacon) miRNAs were re-suspended in 1 x miRNA buffer (Dharmacon) to prepare a stock solution of 20  $\mu$ M. The non-targeting (negative control) miRNA sequence was based on the *C. elegans* cel-miR-67 that was confirmed to have minimal sequence identity with miRNAs from human, mouse and rat. The GAPDH targeting miRNA (referred to as GAPDH-miRNA) targets the 3' UTR of GAPDH mRNA. The miRNA stock solution was further diluted with RNase-free water to obtain a working solution with a concentration of 2  $\mu$ M miRNA. Prior to the transfection, the microRNAs were diluted with serum free Opti-MEM<sup>®</sup> I (Life Technologies) medium such that the final concentrations in the treatment plate were 25, 50 and 100 nM. In a separate tube, DharmaFECT 2 transfection reagent (GE Lifesciences) was also diluted with the serum free Opti-MEM<sup>®</sup> I medium according to the manufacturer's protocol. Then, the diluted miRNAs and transfection reagent were mixed for 20 minutes at room temperature to form the transfection complexes. The culture medium from the 24-well plate was removed and 400  $\mu$ l of complete medium plus 100  $\mu$ l of the transfection complexes were added into each well and cultured at 37°C with 5% CO<sub>2</sub>, for duration of 24 to 48 hours for mRNA level analysis and 48 to 96 hours for protein level analysis. For optimisation of miRNA concentration, the cells were transfected for 48 hours.

#### *Extraction of total cellular RNA*

After the transfection, the medium was removed and the cells were trypsinized, harvested and washed with PBS before RNA extraction using the RNeasy Mini Kit (Qiagen) according to the manufacturer's protocol. Any residual DNA was eliminated by adding the RNase-Free DNase I (Qiagen) onto the spin column and incubated for 20 minutes at room temperature. The concentration of the purified total RNA was measured at 260 nm and the OD<sub>260/280</sub> was also determined by using BioPhotometer

Plus (Eppendorf). RNA integrity and size distribution were also checked by running the purified RNA samples on 1% agarose gel.

### *Synthesis of cDNA*

The first strand of cDNA was synthesized from the extracted total RNA using the RevertAid™ H Minus First Strand cDNA Synthesis kit (Fermentas) with MyCycler Thermal Cycler (Bio-Rad). One microgram of total RNA was mixed with 0.5 µL each of oligo(dT)<sub>18</sub> (50 µM) and random hexamers (50 µM) each and the volume was topped up to 12 µL with distilled water. The mixture was incubated at 65°C for 5 minutes. Next, 4 µL of 5 x reaction buffer, 1 µL of RiboLock™ RNase Inhibitor (20 U/µL), 2 µL of dNTP mix (10 mM) and 1 µL of RevertAid™ H Minus M-MuLV Reverse Transcriptase (200 U/µL) were added into the mixture and incubated at 25°C for 5 min, 42°C for 1 hour and the reaction was terminated by final incubation at 70°C for 5 min. The synthesized cDNA was kept at -20°C until used.

### *Quantitative real-time PCR*

The quantitative real-time PCR reactions were performed using an ABI 7500 Fast Real-Time PCR System (SDS, Applied Biosystems). The reactions were carried out in 96-well plates (Axygen Scientific). Relative quantification method with UBC and YWHAZ as reference genes was used. Negative control which was not added with template DNA was run together with other samples to detect for possible contamination or non-specific amplification in the reaction. The PCR reactions with a final volume of 25 µL were consisted of 12.5 µL Power SYBR Green I Master Mix (Applied Biosystems), 1 µL of gene specific primers, 1 µL of 1:2 diluted cDNA and water. The cycling parameters were 2 min at 50°C, 10 min at 95°C, followed by 40 cycles of 10 sec at 95°C and 1 min at 60°C. After the amplification, PCR specificity was verified by melting curve analysis with temperature ranging from 60 to 95°C and 0.1°C increments. All reactions were run in triplicate. The reference genes (UBC and YWHAZ) were selected based on our previous study (Chua *et al.*, 2011). The forward primer sequence for UBC is 5'-CTGATCAGCAGAGGTTGATCTT-3' and its reverse primer sequence is 5'-GTCTTGCCAGTGAGTGTCTT-3'. For YWHAZ, the forward

primer sequence is 5'-TTCTTGATCCCCAATGCTTC-3' and the reverse primer sequence is 5'-AGTTAAGGGCCAGACCCAGT-3'. The forward and reverse primer sequences for GAPDH are 5'-CAAGGTCATCCATGACAACCTTTG-3' and 5'-GTCCACCACCCTGTTGCTGTAG-3', respectively. The forward primer sequence for *cka* is 5'-TCAGAGCAAACATCCGGAAGT-3' and the reverse primer sequence for *cka* is 5'-GGCGTAGTCCATGTACCCAAAT-3'. The *cka* primers were designed to amplify both *cka1* and *cka2* (total *cka*) mRNA transcripts. The PCR efficiencies of the primers were determined by plotting standard curves of  $C_T$  versus log copy number by using cDNA as template and the PCR efficiency was calculated by qPCR efficiency calculator available at Life Technologies website. Relative gene expression level normalised to the geometric mean of UBC and YWHAZ  $C_T$  values was determined by  $2^{-\Delta\Delta C_T}$  method (Livak and Schmittgen, 2001).

#### *Preparation of cell lysate*

MiRNA transfected HepG2 cells were trypsinised, washed with PBS and harvested by centrifugation at 250 x g for 5 minutes. Twenty volumes of ProteoJET™ Mammalian Cell Lysis Reagent were added into the cell pellet and incubated for 10 min at room temperature with 1200 rpm mixing on a thermomixer (Eppendorf). The mixture was then centrifuged at 16,000 x g for 15 minutes at 4°C and the supernatant was used as cell lysate. Protein concentration was determined by Bradford reagent. The cell lysate was kept at -80°C until used.

#### *Western blot detection of GAPDH*

A total of 30 µg cell lysate were separated on 12% SDS-PAGE and transferred onto nitrocellulose membrane using semi-dry method at 13 V for 2 hours. The membrane was then immersed in blocking buffer [TBS-T plus 5% skim milk: 10 mM Tris-HCl, pH 7.5 containing 150 mM NaCl, 0.1% (v/v) Tween 20 and 1% (w/v) skim milk] with gentle shaking at 25°C for 1 hour before being incubated with rabbit anti-GAPDH primary antibody (Abcam) (diluted 1:5,000 with blocking buffer) at 4°C for overnight. The membrane was subsequently washed three times with TBS-T for 10 min. After the washing step, the membrane was incubated with horseradish

peroxidase-conjugated goat anti-rabbit IgG secondary antibody (Abcam) (diluted 1:5,000 with the blocking buffer) for 1 hour at room temperature. The membrane was washed again three times with TBS-T for 10 min each. The signal was developed by incubating the membrane with SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific) for 5 min and detected by using Fusion FX Chemiluminescence system (Vilber Lourmat).  $\beta$ -actin was used as loading control in this experiment and it was detected with anti- $\beta$ -actin monoclonal antibody (Abcam) at a dilution of 1: 2,500 and anti-mouse IgG secondary antibody (Abcam) (1:5,000). ImageJ 1.49 software available at [www.imagej.nih.gov](http://www.imagej.nih.gov) was used to quantitate the signal intensity from Western blot.

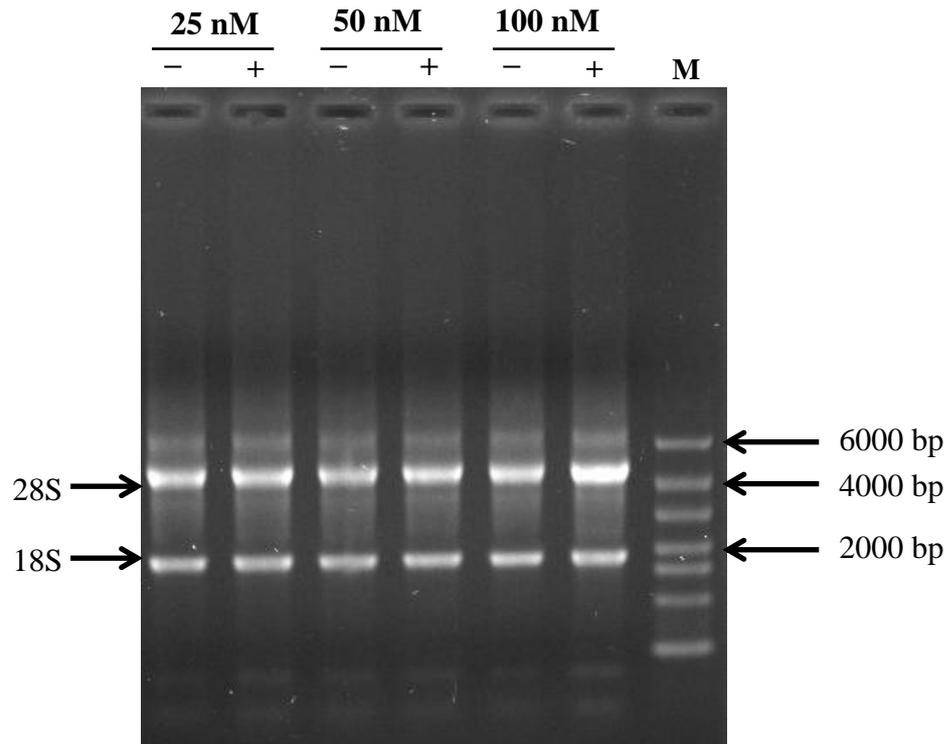
### *Statistical analysis*

All data were analyzed by either t-test or one-way ANOVA with Bonferroni *post-hoc* test using SPSS 22.0 software. A *p*-value of <0.05 was considered to be statistically significant.

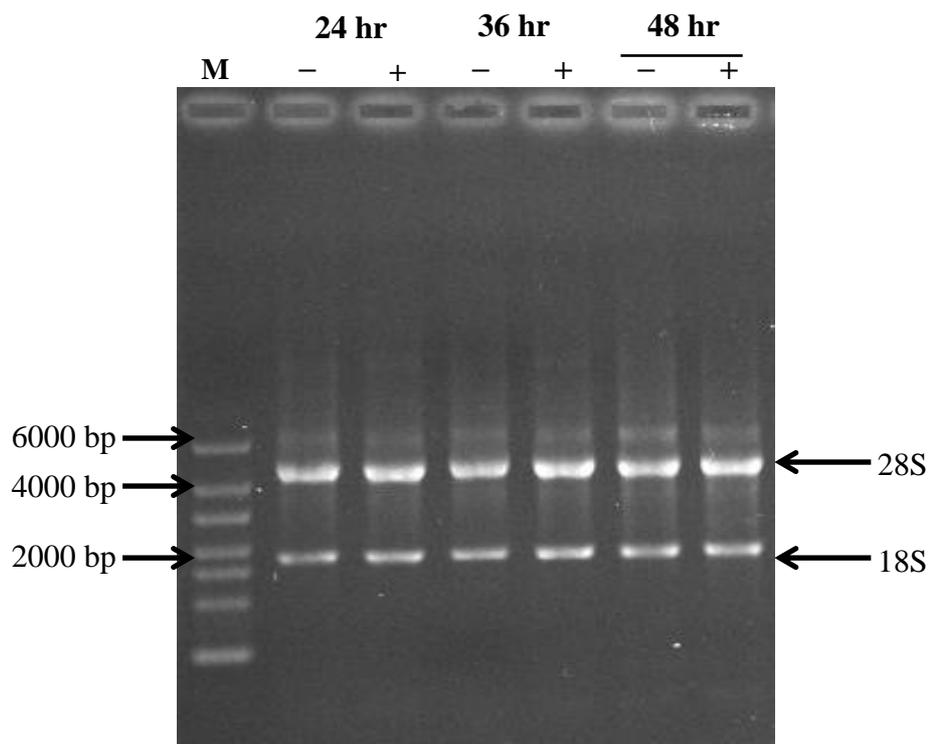
## **Results**

### *Integrity and purity of extracted total RNA*

The integrity of purified RNA samples is important for successful and accurate real-time PCR quantification of a target gene expression. Therefore, all purified RNA samples were run on agarose gel and stained with ethidium bromide to inspect its purity and sign of degradation. **Figure 1** shows the agarose gel electrophoresis of total RNA purified from the HepG2 cells transfected with different concentrations (25 nM, 50 nM and 100 nM) of GAPDH and non-targeting microRNAs. **Figure 2** shows the agarose gel electrophoresis of total RNA purified from the HepG2 cells transfected with both microRNAs for different time durations (24 hr, 36 hr and 48 hr).



**Figure 1:** Agarose gel electrophoresis of RNA samples from experiments to optimize miRNA concentration. Purified total RNA from HepG2 cells transfected with either non-targeting miRNA (-) or GAPDH-targeting miRNA (+) at different concentrations was loaded (1  $\mu$ g per lane) onto 1% agarose, run and stained with ethidium bromide. Lane M: High range RiboRuler™ RNA ladder (Life Technologies)

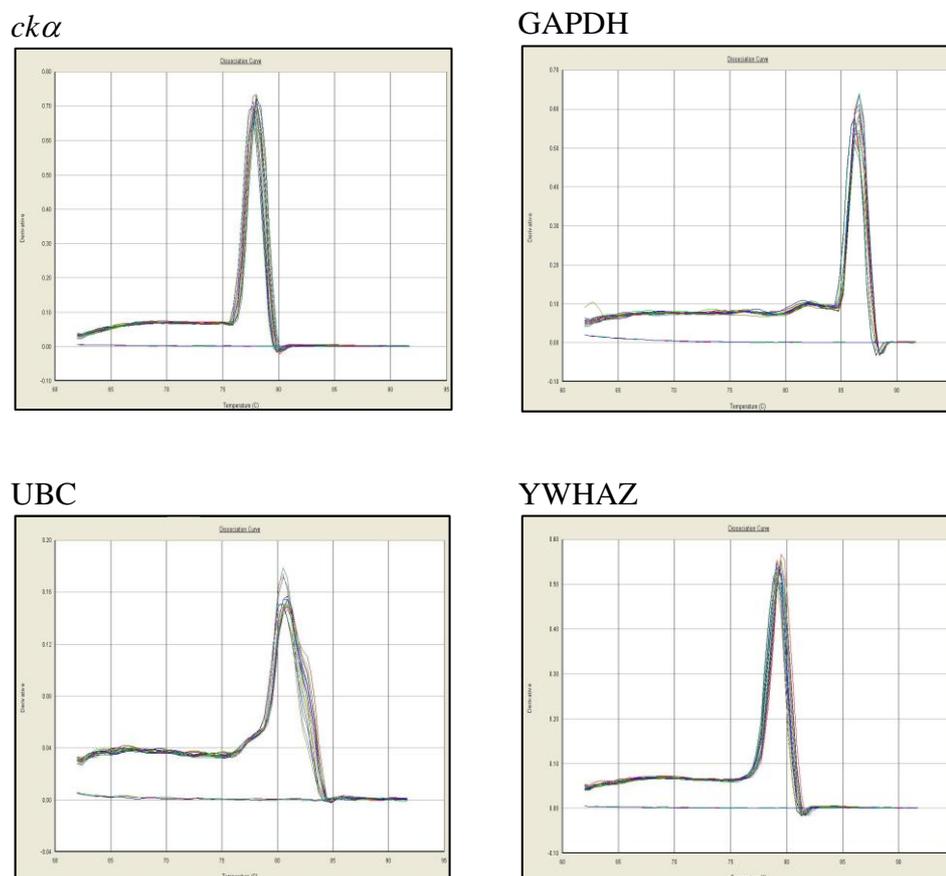


**Figure 2:** Agarose gel electrophoresis of RNA samples from experiments to optimize miRNA transfection duration. Purified total RNA from HepG2 cells transfected with either non-targeting miRNA (-) or GAPDH-targeting miRNA (+) for different durations was loaded (1  $\mu$ g per lane) onto 1% agarose, run and stained with ethidium bromide. Lane M: High range RiboRuler™ RNA ladder (Life Technologies)

In both of the ethidium bromide-stained gels shown in **Figure 1** and **Figure 2**, the upper 28S ribosomal RNA band was about twice the intensity of the lower 18S ribosomal RNA band and no observable smearing below rRNA bands indicate the integrity of the purified RNA samples. The absence of high molecular weight band also excludes the possible contamination by genomic DNA. Furthermore, the  $OD_{260/280nm}$  ratio of all the RNA samples were between 1.9 and 2.1, supporting the purity observed in the gels. Overall, all the purified RNAs used in this study went through quality check and only good quality purified RNA samples were used for obtaining reliable results in subsequent experiments.

*Specificities and PCR efficiencies of GAPDH, cka, YWHAZ and UBC PCR primers*

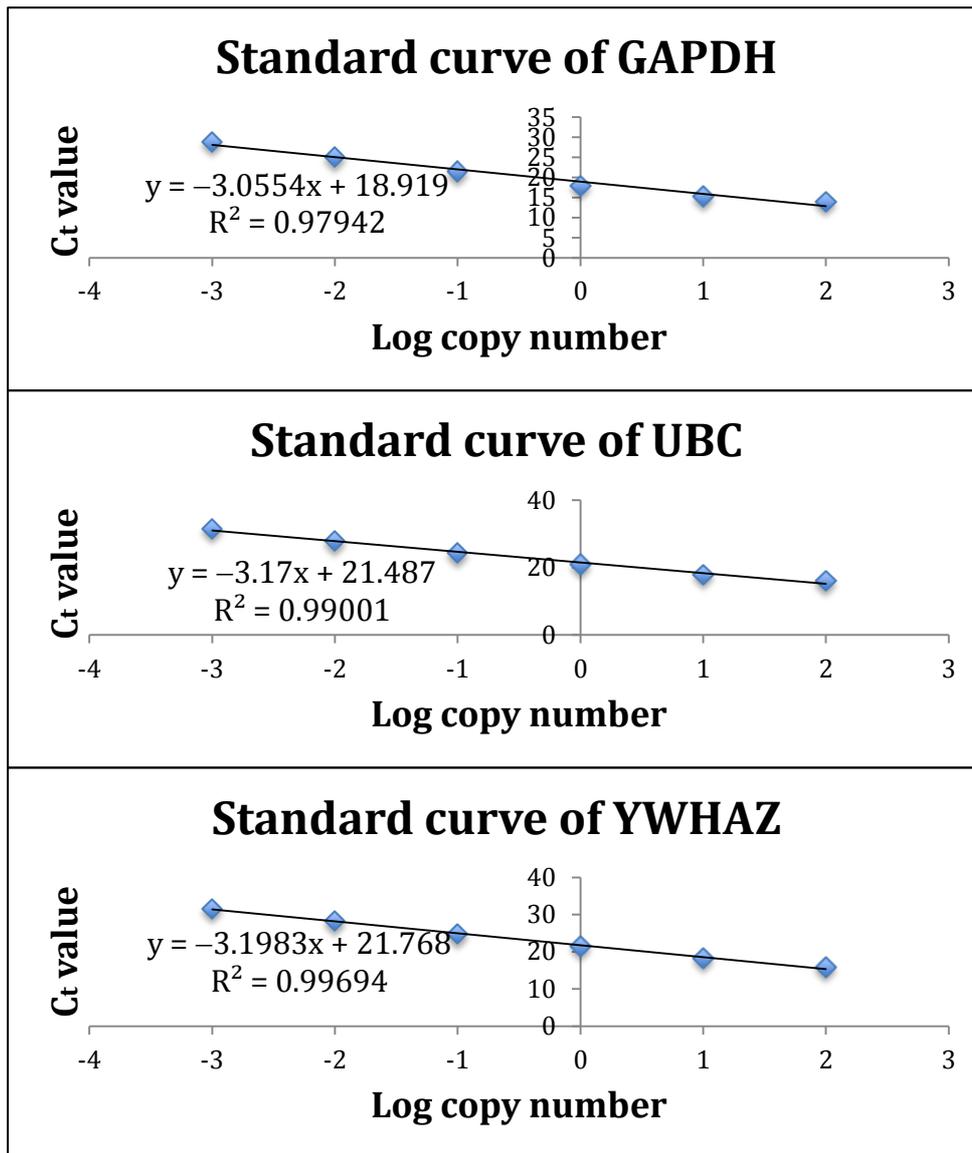
**Figure 3** shows representative real-time PCR melt-curve analysis from actual experiments to determine the levels of GAPDH and *cka* mRNAs. The analysis displayed a good specificity for all the primers used as indicated by a single peak without any unspecific amplification products or the presence of primer-dimers from the reactions. Melt curve analyses showed the melting temperatures ( $T_m$ ) for PCR products produced by GAPDH, *cka*, UBC and YWHAZ primers were 86.2°C, 77.8°C, 81.3°C and 79.2°C, respectively. Agarose gel electrophoresis also confirmed the specificity of the PCR products obtained from GAPDH, *cka*, UBC and YWHAZ primers to be 496 bp, 239 bp, 151 bp and 211 bp, respectively (data not shown).



**Figure 3:** Melt curve analyses of real-time PCR products for *cka*, GAPDH, UBC and YWHAZ

The PCR efficiencies of GAPDH, UBC and YWHAZ primers were determined in this study. Based on the standard curves shown in **Figure 4**, the slopes of all standard

curves were between -3.1 and -3.2, showing good amplification efficiencies of 112%, 107% and 105% for GAPDH, UBC and YWHAZ primers, respectively. All of the standard curves were linear in the range tested with the correlation coefficient ( $R^2$  values) greater than 0.98. The PCR efficiencies of *cka* primers have previously been determined to be 92% (Chua et al., 2015). The results show that all the primers used in this work were suitable for real-time PCR because of their high specificity and efficiency of between 90 to 110%.

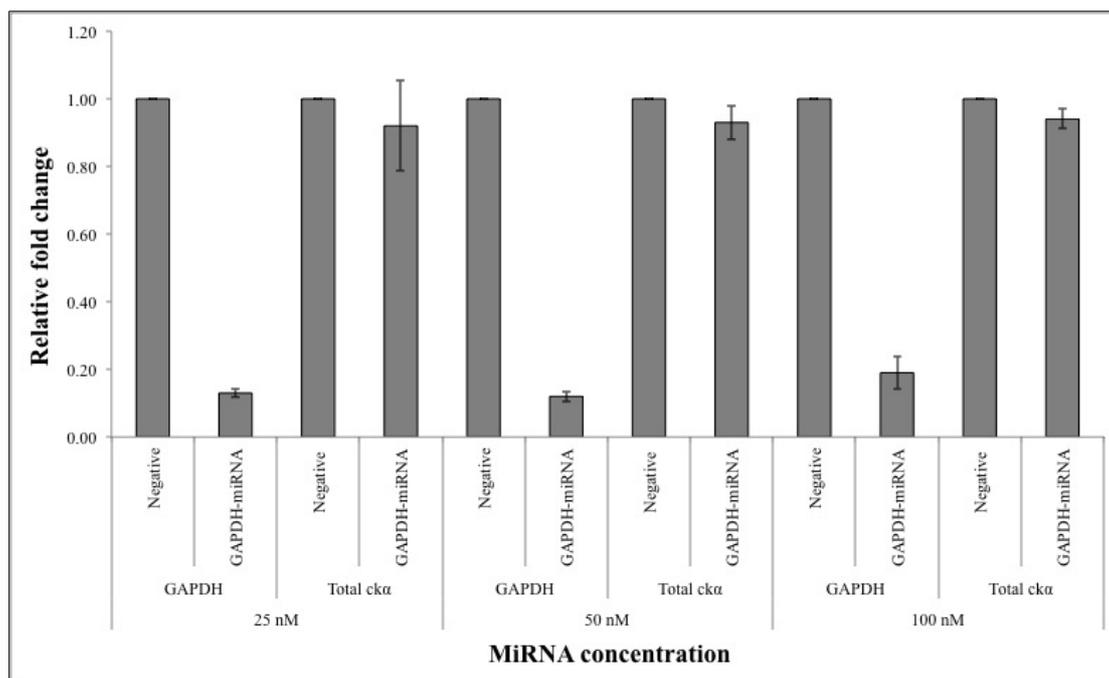


**Figure 4:** Standard curves plotted with GAPDH, UBC and YWHAZ primers used for the calculation of PCR efficiency. Each standard curve was generated from the mean of three independent serial dilutions of cDNA templates

### *Effect of microRNA concentration on GAPDH and cka gene expression*

The amount of miRNA mimic needed to efficiently down-regulate a target gene can vary greatly depending on the miRNA sequence, types of cell line used and experimental conditions such as transfected miRNA concentration and transfection duration (Jordan *et al.*, 1996). Therefore, it is necessary to perform optimisation experiments using various miRNA mimic concentrations to obtain optimal down-regulation of the target gene in a specific cell line. It is also important to show that the transfection protocol is not detrimental to cell growth or affect overall mRNA and protein expression of the cell line under investigation. This study looks at the effect of GAPDH- and non-targeting miRNAs transfection on the expression of human *ckα* because the optimised transfection parameters will be used for the identification of *ckα* targeting miRNAs.

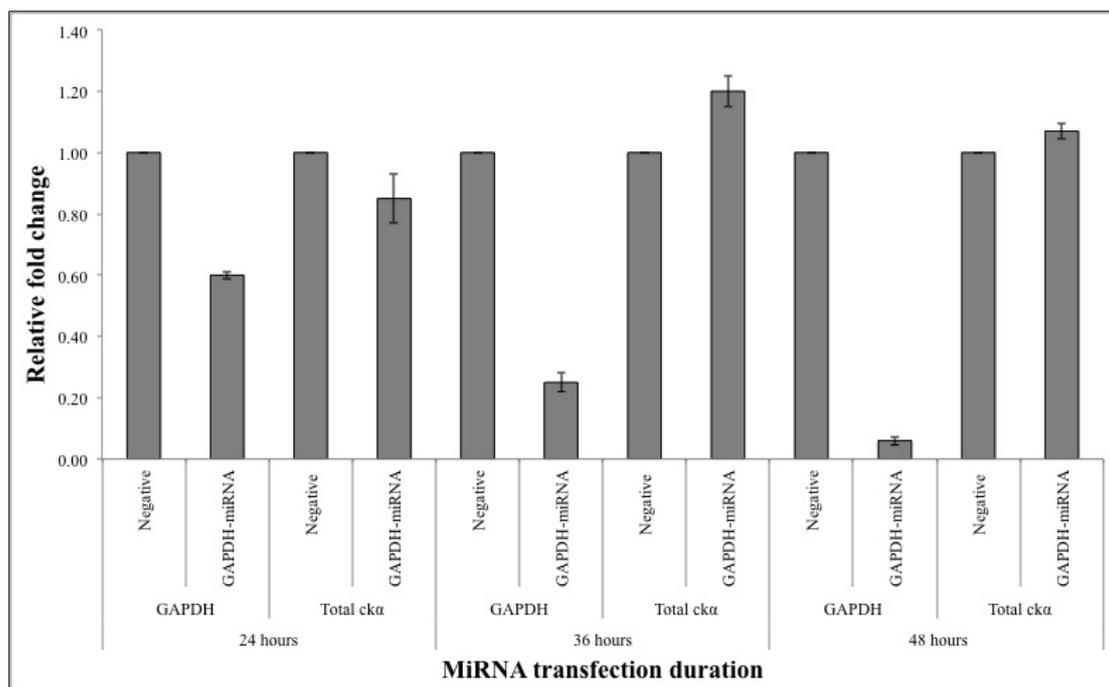
According to the results shown in **Figure 5**, the GAPDH mRNA levels were significantly ( $p<0.01$ ) down-regulated to 13%, 12% and 19% of the levels detected in cells transfected with non-targeting miRNA, respectively. Importantly, the expression levels of total *ckα* were not significantly ( $p>0.05$ ) affected by the transfection of GAPDH-miRNAs at all the tested concentrations. There was no significant difference between the down-regulation levels of GAPDH by transfection with 25 nM and 50 nM GAPDH-miRNA ( $p<0.01$ ), therefore 25 nM of miRNA was selected for subsequent experiments to optimise the transfection duration. The results also showed that the miRNA concentrations used did not affect the expression level of *ckα* expression.



**Figure 5:** Relative fold change of GAPDH and *cka* mRNA levels for 48 hr transfections with 25, 50 and 100 nM of non-targeting (negative) and GAPDH-targeting miRNAs. Error bars are standard error of mean (SEM) from three independent experiments

*Effect of microRNA transfection duration on GAPDH and cka gene expression*

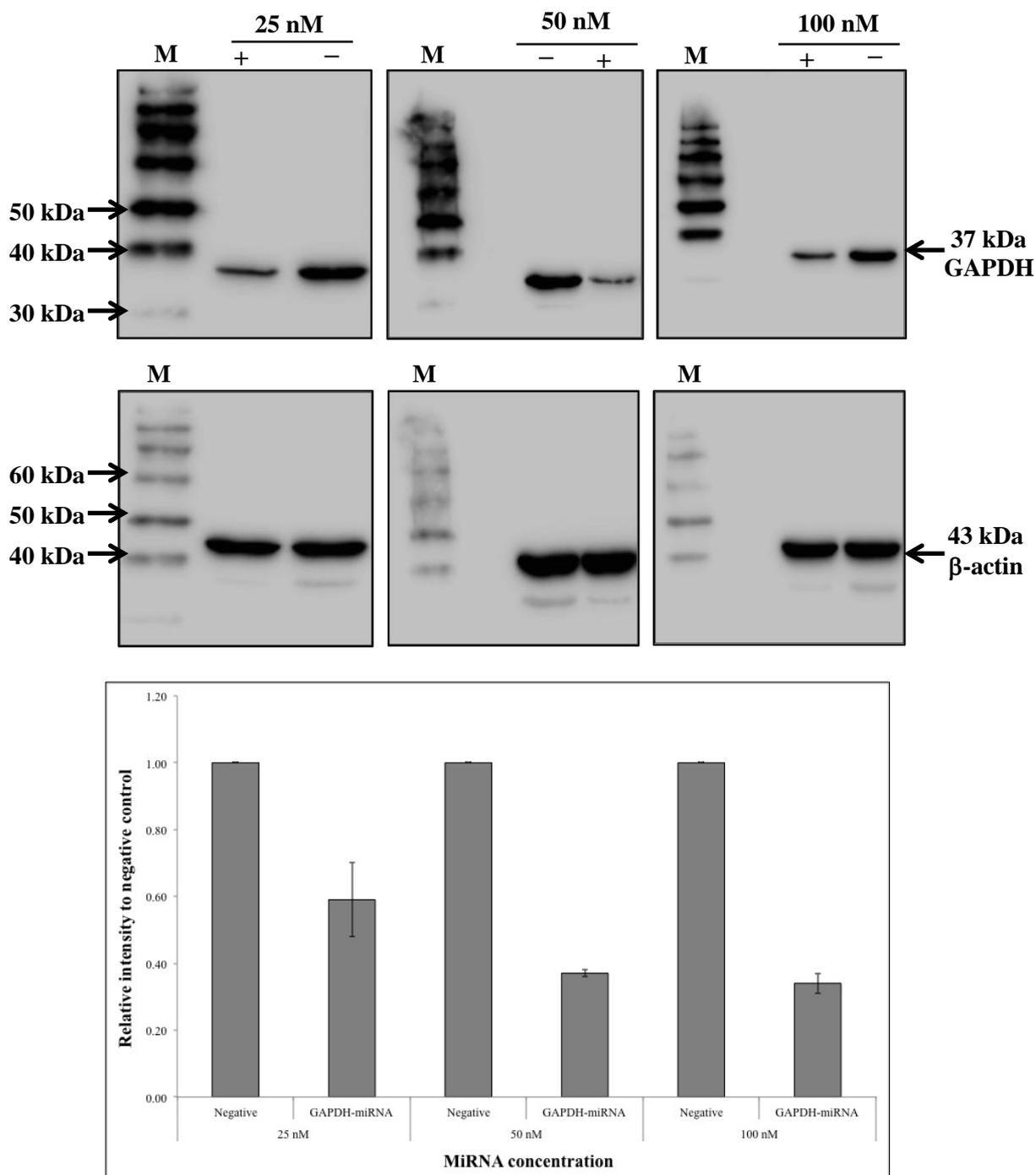
HepG2 cells were treated with 25 nM of GAPDH and non-targeting miRNAs for 24hr, 36 hr and 48 hr. **Figure 6** shows that the levels of GAPDH mRNA were significantly lower ( $p < 0.05$ ) after 24, 36 and 48 hours of transfection with GAPDH-miRNA. The strongest down-regulation was obtained after 48 hours transfection with the relative GAPDH mRNA level 17-fold lower than non-targeting miRNA treated cells. The level of *cka* mRNA was not significantly affected ( $p > 0.05$ ) by the transfection of GAPDH-miRNA compared with negative control miRNA for all three transfection durations tested. Hence, 48 hours was considered as the optimum miRNA transfection duration for future experiments with HepG2 cell lines.



**Figure 6:** Relative fold change of GAPDH and *ckα* mRNA levels for 24, 36 and 48 hours transfections with 25 nM of non-targeting (negative) and GAPDH-targeting miRNAs. Error bars are standard error of mean (SEM) from three independent experiments

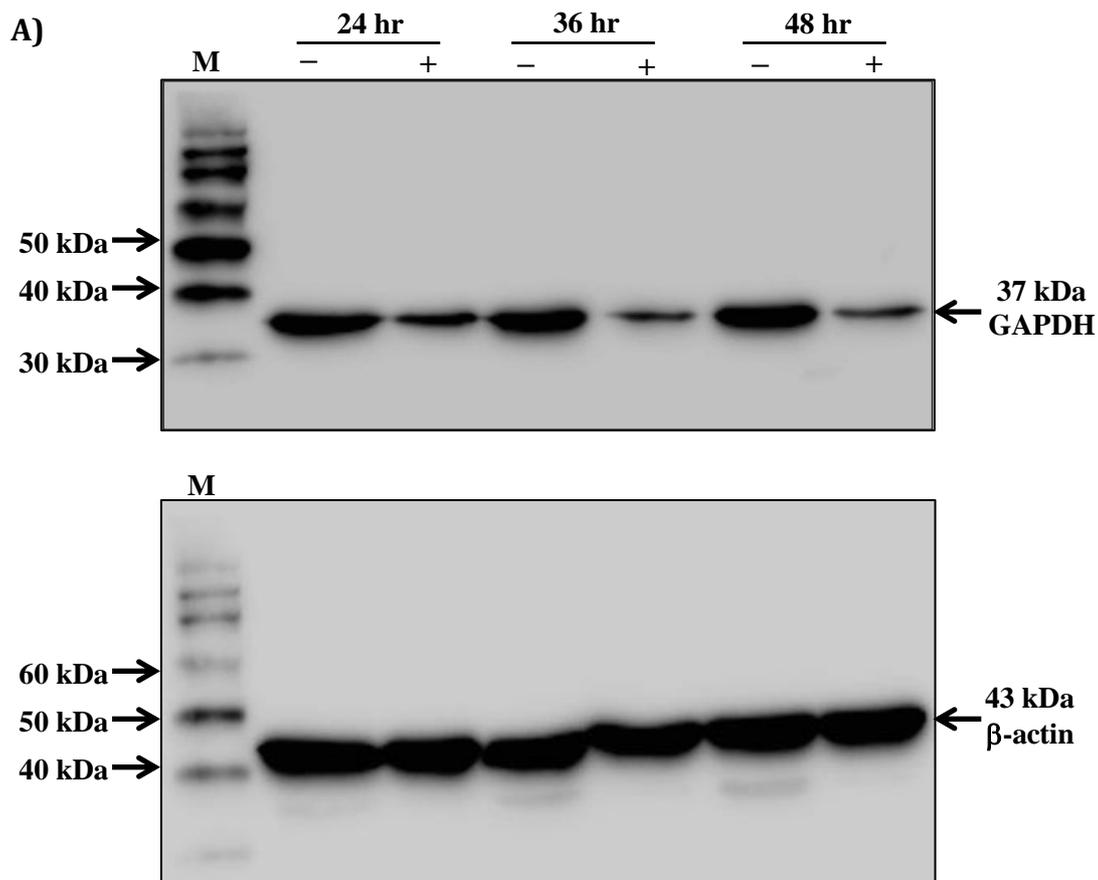
*Effect of GAPDH-miRNA concentration and transfection duration on the GAPDH protein level*

To further investigate whether miRNA down-regulation of GAPDH mRNA expression also led to lower level of GAPDH protein, Western blot detections of GAPDH level in HepG2 cells transfected with different concentrations and time durations of GAPDH- and non-targeting miRNAs were carried out. As shown by the mean signal intensities of GAPDH normalized to the signal intensity of  $\beta$ -actin from three independent experiments (**Figure 7**, lower panel), the levels of GAPDH protein were 59, 37 and 34% compared to negative control miRNA for transfection with 25, 50 and 100 nM miRNAs, respectively.

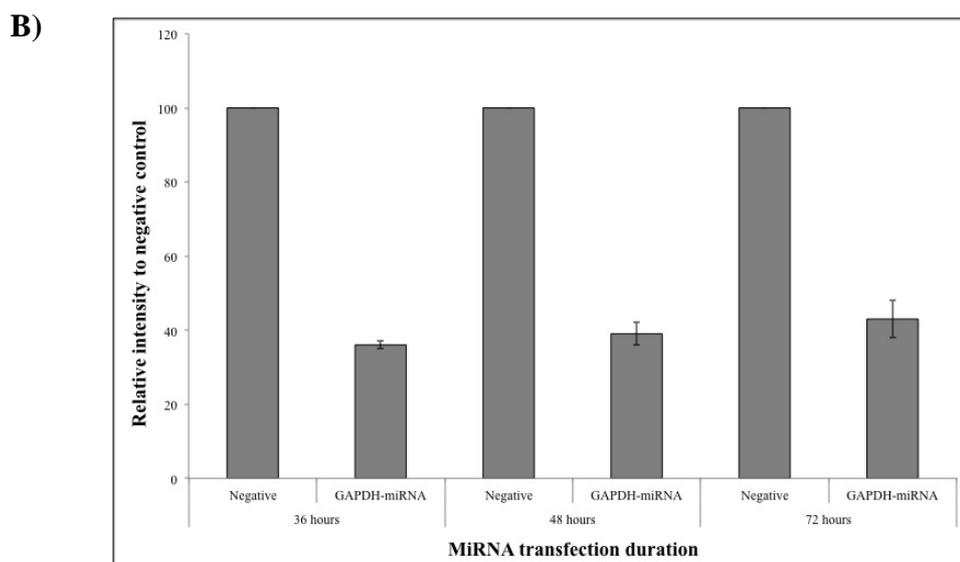


**Figure 7:** Western blot detection of GAPDH (upper panel) and  $\beta$ -actin (middle panel) in HepG2 cell lysate transfected with either non-targeting (-) or GAPDH-targeting (+) miRNA at 25, 50 or 100 nM for 48 hours. Lane M: Prestained molecular weight marker. The pictures are representatives of three independent experiments. The intensities of Western detection signals were quantitated by ImageJ 1.49 software and plotted as relative intensity in percentage (lower panel). Error bars are standard error of mean (SEM) from 3 independent experiments

**Figure 8** shows the levels of GAPDH in the cells transfected with 25 nM for 36, 48 and 72 hours were 36, 39 and 43% compared to negative control miRNA transfection, respectively. The transfection duration used for protein detection was longer than mRNA detection based on the assumption that miRNA down-regulates the mRNA level first and the effect on protein level would be observed later.



**Figure 8A:** Western blot detection of GAPDH (upper panel) and  $\beta$ -actin (lower panel) in HepG2 cell lysate transfected with 25 nM of either non-targeting (-) or GAPDH-targeting (+) miRNA for 24, 36 or 48 hours. Lane M: Prestained molecular weight marker. The pictures are representatives of three independent experiments.



**Figure 8B:** The intensities of Western detection signals were quantitated by ImageJ 1.49 software and plotted as relative intensity in percentage. Error bars are standard error of mean (SEM) from 3 independent experiments.

Generally, the effect of GAPDH-miRNA transfection of HepG2 cell line on the GAPDH mRNA and protein levels was very similar with all the miRNA concentrations and transfection durations tested resulted in significantly lower levels of GAPDH mRNA and protein. Yet, 25 nM miRNA concentration and 48 hours transfection time were considered the optimal miRNA transfection conditions for HepG2 based on the stronger down-regulation of mRNA level with these parameters. The selection of these optimal parameters was also due to the fact that mRNA is the actual target of miRNA and our subsequent experiments focus more on real-time measurement of mRNA levels rather than protein levels.

## Discussion

The effect of miRNA transfection on gene expression is usually investigated at the effect on mRNA and protein level. Quantitative real-time PCR is a method to accurately measure the level of mRNA in the cells treated with control and miRNAs of interest. For a successful real-time PCR experiment, the purity and quality of the starting material i.e the RNA extracted from the cells are particularly crucial. This is due to any available contaminants such as proteins, salts, phenol, DNA or EDTA can later on interfere with the reverse transcription, amplification and fluorescence

detection of the SYBR green. Additionally, complete removal of genomic DNA in RNA samples was important in order to avoid false positives or unspecific amplification of genomic sequences, which lead to incorrect quantification of mRNA level (Fleige and Pfaffl, 2006).

GAPDH is a well-known housekeeping gene used to assess the transfection efficiency of RNA mimic molecules like miRNAs. GAPDH is also a common reference gene used in real-time PCR quantification of gene expression (Barber *et al.*, 2005). Our results confirmed that the transfection protocol produced the expected down-regulation of GAPDH gene expression when the HepG2 cells were transfected with GAPDH-targeting miRNA at all the tested concentrations and transfection durations. The down-regulation of both GAPDH mRNA and protein levels confirmed that GAPDH-miRNA induced the degradation of mRNA, which subsequently led to lower level of protein and the mechanism of action was not by blocking the mRNA translation. The mechanism of action was expected as the GAPDH-miRNA was designed to target the 3'-UTR of GAPDH mRNA.

The main aim of this study was to determine the optimum miRNA transfection protocol for HepG2 which can be used as the basis for the transfection of other miRNAs potentially targeting *ckα* transcripts. We showed that the use of as low as 25 nM GAPDH-miRNA was already sufficient to down-regulate the target gene. It has been reported that miRNA concentration higher than 100 nM could decrease the cell viability due to toxicity (Bollin *et al.*, 2011). Higher concentration of miRNA might also cause off-target effect like in the case of siRNA transfection (Borawski *et al.*, 2007). We have used miRNA concentrations between 25 and 100 nM although 200 nM has been used for the transfection of miR-375 of MIN6 cells (Poy *et al.*, 2004) and miR-1 and miR-133 of C2C12 cells (Chen *et al.*, 2006). Up to micromolar concentration of miR-134 has been used to transfect primary neurons (Schratt *et al.*, 2006). In this work, significant down-regulation of GAPDH was already observed at 24 hour transfection and the effect became stronger with longer transfection duration. The optimum transfection duration of 48 hours (maximum duration tested) GAPDH-miRNA had also been used in other studies (Kuhn *et al.*, 2008, Sikand *et al.*, 2012).

## Conclusion

In conclusion, this work has clearly showed that the optimised transfection protocol for GAPDH-targeting miRNA can be reliably used for transfection of HepG2 cell line with other miRNAs, including those that potentially target *ck $\alpha$*  gene expression. The use of GAPDH targeting miRNA as positive control when validating *ck $\alpha$* -targeting miRNAs becomes more feasible as the results reported here showed that *ck $\alpha$*  mRNA level was not affected by all the tested GAPDH-miRNA concentration and transfection duration. It must be noted that after the miRNAs targeting *ck $\alpha$*  has been identified with the current transfection parameters, optimisation of the *ck $\alpha$* -targeting miRNA concentration and transfection duration must be repeated because the optimum transfection parameters are highly dependent on the miRNA and its target.

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