Plasmid DNA Miniprep Kit Protocol

Plasmid purification was performed by Miniprep kit (Biolab, New England)

Protocol:

1. Pellet 1–5 ml bacterial culture by centrifugation for 30 seconds. Discard supernatant.
2. Resuspend pellet in 200 µl Plasmid Suspension Buffer (B1) (pink). Vortex or pipet to ensure cells are completely resuspended.
There should be no visible clumps.
3. Lyse cells by adding 200 µl Plasmid Lysis Buffer (B2) (blue/green). Invert tube immediately and gently 5–6 times until color changes to
dark pink and the solution is clear and viscous. Do not vortex! Incubate for one minute.
4. Neutralize the lysate by adding 400 µl of Plasmid Neutralization Buffer (B3) (yellow). Gently invert tube until color is uniformly yellow
and a precipitate forms. Do not vortex! Incubate for 2 minutes.
5. Clarify the lysate by spinning for 2–5 minutes at 16,000 x g.
6. Carefully transfer supernatant to the spin column and centrifuge for 1 minute. Discard flow-through.
7. Re-insert column in the collection tube and add 200 µl of Plasmid Wash Buffer 1. Plasmid Wash Buffer 1 removes RNA, protein and
endotoxin. (Add a 5 minute incubation step before centrifugation if the DNA will be used in transfection.) Centrifuge for 1 minute.
Discarding the flow-through is optional.
8. Add 400 µl of Plasmid Wash Buffer 2 and centrifuge for 1 minute.
9. Transfer column to a clean 1.5 ml microfuge tube. Use care to ensure that the tip of the column has not come into contact with the flowthrough.
If there is any doubt, re-spin the column for 1 minute before inserting it into the clean microfuge tube.
10. Add ≥ 30 µl DNA Elution Buffer to the center of the matrix. Wait for 1 minute, then spin for 1 minute to elute DNA.

Gel electrophoresis

The detection was done by agarose gel electrophoresis, using 2% agarose gel, the TBE buffer (Trizma, Boric and EDTA) and loading buffer and visualized by ethidium bromide staining **(Sambrook and Russell, 2001)**.

I. Equipments

a) Horizontal electrophoresis unit (Ultraviolet product, UVP-Delta, pharmacia, USA) and standard power pack (P.25 Biometra, Germany).
b) InGenius gel documentation (gel doc) system (Syngene Bio Imaging, Synoptics Ltd, England).
c) pH meter electrode (Dakton, USA).

III. Procedure

a) 11 µl of DNA product was added to 2 µl of the loading buffer.
b) 11 µl of that mixture were applied to each well.
c) 11 µl of –ve control product was added to 2 µl of the loading buffer then it was applied to a well in the gel of each run.
d) 10µl of the molecular weight marker was added to 2 µl of the dye + 4-6 µl distilled water then it was applied to a well in the gel of each run.
e) The samples were run at 80 volts for 30 minutes or till the tailing of the gel.
f) The gel was taken to be visualized by ultraviolet transillumination and photographed in InGenius gel documentation system, Gene Snap automatic image capture software version [7.4.5.0] from Syngene was used.

Transfection protocol

Cell lines

The human hepatocellular carcinoma cell lines, HepG2 and SNU449, cells used in this study were obtained from ATCC. HepG-2 cells were maintained in Dulbecco's modified Eagle's medium (DMEM, Gibco-BRL, Gaithersburg, MD, USA) while SNU449 cells were maintained in RPM1640 media (Sigma, USA). The media of both cells contained 10% heat-inactivated fetal bovine serum (FBS, Gibco-BRL) and cultured at 37 C in 5% CO2/ atmospher. Cultures were observed using an inverted

microscope to assess the degree of cell density and confirm the absence of bacterial and fungal contamination.

First genetic construct was introduced into cells using Lipofectamine™ 2000 reagent (Invitrogen, Carlsbad, CA, USA) according to manufacturer's instructions. Second crispr-based genetic construct was introduced into cells simultaneously with pFETCh_vector expressing hsa_circ_0000064 & HDR using Lipofectamine™ 2000 reagent**.**

One or two days after transfection, at least two million HCC cells were used for isolation of total RNA including miRNA followed by qRT-PCR to detect the expression of cirRNA associated ceRNA network. For cotransfection the same plasmid was used together with GFP expressing vector at ration of 3:1 (cirRNA: GFP)

Extraction of total RNA from HepG2 cell line(including circular RNA and miRNA):

- **Kits used:**

Total RNA was extracted from HepG2 cell line using miRNeasy® HepG2 cell line/Plasma Kit (QIAGEN®, USA).

- **Principle:**

The miRNeasy Kit combines Acid Guanidinium- Thiocyanate-Phenol-Chloroform extraction-based lysis of samples and silica-membrane–based purification of total RNA.

- **Procedure**

1. Preparation of samples.
2. Lysis (5 volumes of Qiazol lysis reagent 500 µl).
3. Phase separation (100 µl chloroform).
4. RNA precipitation (450 µl 100% ethanol).

5. RNA wash (700 µl RWT, 500 µl RPE ,500 µl 80% ethanol).
6. RNA solubilization (RNase free water).
7. Measurement of RNA concentration.

1. Measurement of RNA concentration:

- 72µl of DEPC-water were added to 3µl of RNA solution (dilution 1:25). The sample was pipetted up and down several times to ensure adequate mixing.
- The sample was read at 260 nm for RNA detection and 280 nm for protein detection using the spectrophotometer.
- The samples were considered with high RNA quality if RNA: Protein ratio (260:280 ratio) is more than 1.8.
- 40µg RNA/ml is equivalent to 1 absorbance, based on an extinction coefficient calculated for RNA at neutral pH.
- Thus, the concentration of RNA in a sample (µg/ml) = sample absorbance at 260nm × 40/1 × dilution factor (25).

- Consequently, the concentration of RNA in a sample
- $(\mu g/\mu l) = \frac{\text{O.D (at 260 nm)} \times 40 \times \text{ dilution factor (25)}}{1000}$

2- Reverse transcription Polymerase Chain Reaction (RT-PCR):

- **Kits used:**

Real-Time two-step RT-PCR was performed on the extracted RNA prepared in the previous step using Qiagen miScript II RT kit (Qiagen, USA).

- **Principle:**

Non coding RNAs are polyadenylated by poly(A) polymerase and reverse transcribed into cDNA using oligo-dT primers **.** Polyadenylation and reverse transcription are performed in parallel in the same tube. The oligo-dT primers have a 3' degenerate anchor and a universal tag sequence on the 5' end, allowing amplification of mature miRNA and circular RNA as well in the real-time PCR step.

Figure ( ): Poly A tailing based RT-PCR

- **Equipment:**
  - Thermo Hybaid PCR express (Thermo Scientific, USA)

- **Procedure:**

1. The template RNA was thawed on ice, while the RNase-free water, 10x miScript Nucleics Mix, 5x miScript HiSpec Buffer and 5x miScript HiFlex Buffer were thawed at room temperature (15–25ºC).
2. The reverse transcription reaction master mix required for first-strand cDNA synthesis was Prepared (Table 2) then mixed with RNA template and stored on ice.

Table 2: Reverse Transcription Reaction components:

Component	Volume/reaction
10x Nucleics mix	2µl
5x miScript HiSpec Buffer	2µl
5x miScript HiFlex Buffer	2µl
miScript Reverse Transcriptase Mix	2µl
RNase-free water	Variable(7µl)
RNA template	Variable(5 µl)
Total reaction volume	**20µl**

3. Incubation for 60 min at 37ºC was done.

4. To inactivate miScript Reverse Transcriptase, tubes were incubated at 95ºC for 5 min, mixed and then placed on ice.

3-Syber Green based Quantitative Real Time PCR (Polymerase Chain Reaction):

- **Principle:**

Detection of RNA levels by real time RT-PCR to ensure sensitive and specific RNA detection and quantification with high amplification efficacy.

- **Kits used:**

Quantitative Real Time PCR was performed by using QuantiTect® primer Assay & miScript primer Assays.

- **Equipment:**

- Real-Time PCR instrument (7500 Fast Real-Time PCR system, Applied Biosystems, Foster City, California, United States).

- **Procedure for detection OF MiRNA expression:**

1. miScript SYBRGreen PCR Master Mix (2x), template cDNA , primers, and RNase-free water were thawed.
2. A reaction mix was prepared according to the following tables (Table 3).

Table 3: Using miScript SYBR Green kit to prepar Reaction Mastermix for two- step RT-PCR:

Component	Volume/reaction
2x miScript SYBRGreen PCR Master Mix	12.5µl
10x miScript universal primer	2µl
10x miScript primer assay	2µl

Template cDNA (added at step 4)	Variable (1 µl)
RNase-free water	Variable (7.5 µl)
Total reaction volume	**25µl**

3. The reaction mix was mixed thoroughly, and appropriate volumes were dispensed into PCR tubes.
4. Template cDNA was added to the individual PCR tubes containing the reaction mix.
5. Real-time cycler was programmed and data acquisition was performed during the extension step (Table 4).

Table 4: Real time PCR program.

Step	**Time**	**Temperature**
PCR initial activation step	5 min	95°C
2-step cycling:		
Denaturation,	15 s	94°C
Annealing	30 s	55°C
Extension	30 s	70°C
Number of cycles	40-45 cycles	

6. The PCR tubes were placed in the real-time cycler, and the cycling program was started.

- **Procedure for TRIM mRNA and circular RNA expression :**

7. Quantitect SYBRGreen PCR Master Mix (2x), template cDNA , primers, and RNase-free water were thawed.

8. A reaction mix was prepared according to the following tables (Table 5).

Table 5: Using Quantitect SYBRGreen kit to prepar Reaction Mastermix for two- step RT-PCR :

Component	Volume/reaction
2x Quantitect SYBRGreen PCR Master Mix	10µl
Primer	1µl
Template cDNA (added at step 4)	1µl
RNase-free water	8µl
Total reaction volume	**20µl**

9. The reaction mix was mixed thoroughly, and appropriate volumes were dispensed into PCR tubes.
10. Template cDNA was added to the individual PCR tubes or wells containing the reaction mix.
11. Real-time cycler was programmed and data acquisition was performed during the extension step (Table6).

Table 6: Real time PCR program.

Step	Time	Temperature
PCR initial activation step	5 min	95°C
2-step cycling:		

Denaturation,	15 s	94°C
Annealing	30 s	55°C
Extension	30 s	72°C
Number of cycles	40-45 cycles	

12. The PCR tubes were placed in the real-time cycler, and the cycling program was started.

5- Data analysis:

- Quantitation Strategies:

- The relative expression of total TRIM gene and circular RNA was analysed using the ΔΔCT method through the following steps:

  $RQ = 2^{-\Delta\Delta CT}$

- Normalization Strategies:

In this study, appropriate normalization strategies were carried out to control experimental error introduced during the multistage process required to extract and process the RNA: Ensuring similar sample size for RNA extraction, Equalizing the amount taken from the extracted RNA for reverse transcription (5µl) and Selecting a reference gene from the validated reference gene panels. They are the gold standard for normalization because they are internal controls that are affected by all sources of variation during the experimental workflow in the same way as genes of interest (ACTB is the housekeeping internal control in this study for TRIM and circular RNA and RNU-6 as a housekeeping for miRNA)

6- Immunohistochemistry

Cross-Adsorbed Secondary Antibody Alexa Fluor® 488 conjugate (A-11034) was used at a concentration of 4 µg/ml in phosphate buffered saline containing 0.2% BSA for 45 minutes at room temperature, for detection of TRIM2 (**TRIM2 Antibody (PA5-57431) in IF** in the nucleus (Panel a:green). Goat anti- Rabbit IgG (H+L) Highly Cross-Adsorbed Secondary Antibody Alexa Fluor® 488 conjugate (A-11034) was used at a concentration of 4 µg/ml in phosphate buffered saline containing 0.2% BSA for 45 minutes at room temperature, for detection of TRIM2 in nuclei (green).No nonspecific staining was observed with the secondary antibody alone, or with an isotype control. The images were captured at 60X magnification.

7- Flowcytometry

Single cell suspension was prepared from harvested cultured cells fixed with100ul immunofixation buffer, incubated at room temperature for 20-30minutes protected from light, Permeabilized by permibilization buffer, washed with PBS and labelled with **TRIM2 Antibody (PA5-57431) in IF** in the nucleus (Panel a:green). Goat anti- Rabbit IgG (H+L) Highly Cross-Adsorbed Secondary Antibody Alexa Fluor® 488 conjugate (A-11034) was used at a concentration of 4 μg/ml and analysed by FlowCytometery.