

Y chromosome genes may play roles in directed differentiation of human embryonic stem cells.

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Supplementary Methods

1. MOI determination

Before cell transduction, we needed to know about MOI (Multiplicity of infection) of the target cell. The transduction efficiency of lentiviral vectors on hESC was determined with the pLIX-403-GFP lentiviral vector. Cells were transduced with varied MOI between 5 and 50. Three days after transduction, the percentage of GFP positive hESCs was analyzed using flow cytometry. We found out, at MOI of 40, about 30% of cells were transduced. The increase in the MOI to 50, did not increase in the transduction rate.

For transduction first, hESC-RH6 were seeded as single cells onto the matrigel-coated plates. After 24 hours, when the cell density reached appropriate confluence (around 70 %), target cells were transduced with a suitable amount of virus to achieve the desired MOI. After a day, we selected the transduced cells by puromycin treatment. We found that 2 μ g/ml was the optimal concentration after around 6 days (Supplementary Fig 2. A-C).

2. Polybrene toxicity assay.

We performed a polybrene toxicity titration before transduction. At first, the cells were grown in a complete culture medium with a range of polybrene concentrations from 0 to 10 μ g/ml in a 12-well plate for 24 hours. Two wells were used as negative controls. The next day, the old medium was replaced with a polybrene-free complete culture medium, and the cells were incubated at 37°C and 5% CO₂ for an additional 72 hours. The cell viability was checked for polybrene toxicity; the highest concentration of polybrene leads to less than 10% cell death compared to no polybrene (control wells) (Supplementary Fig 2. E, F)

3. Puromycin toxicity assay.

The cells were grown in complete culture medium with a range of puromycin concentrations from 0, 0.5, 1, 1.5, 2, 2.5, and 3 μ g/ml in a 6-well plate for 24 hours. The medium was refreshed with a puromycin-free complete culture medium, and the cells were grown for an additional six days (Supplementary Fig 3. B). Cell viability was evaluated for puromycin toxicity; minimum concentration should be used to kill all of the negative control cells (validated as non-transduced cells).

4. DNA isolation.

Genomic DNA extraction from resistant colonies was performed manually, where approximately 1×10^5 cells were digested with 300 μ l lysis buffer (50 mM Tris HCL, 10 mM SDS, 100 mM EDTA) and 1 μ g/ml proteinase k (Sinaclon, Iran), at 55°C for overnight and salting out with 5M NaCl, centrifuged at 13000 rpm for 5 minutes at 4°C. Then, the supernatant was transferred to a new microtube and mixed with an equal volume of ethanol 96% and 0.1 Sodium acetate (3M C₂H₃NaO₂), centrifuged at 13000 rpm for 5 minutes at 4°C. The pellet was washed in 70% ethanol, then centrifuged at 13000 rpm for 5 minutes at 4°C. Consequently, the supernatant was removed and the pellet left to dry before being resuspended in 20 μ l diluted water and incubated overnight. The supernatant was then pooled and stored at 2-8°C until the concentration and purity were determined. The quantity and quality of extracted DNA were determined spectrophotometrically using a Biowave DNA Life Science Spectrophotometer (Terra Universal, Inc, USA). The integrity of DNA was evaluated by separation in a 1% agarose gel electrophoresis, thus for Size Determination of DNA fragments, 50 ng of DNA was loaded to each well with an O'Gene Ruler 1kb DNA Ladder (Fermentas).

5. PCR cycling program.

The cycling program used consisted of 35 repetitive cycles including initiation denaturation step at 95 °C for 2 min, second denaturation at 95 °C for 30 s, annealing step at 58 °C for 30 s and extension step at 72 °C for 90 s. The Final extension was at 72 °C for 10 min. Triplicate measurements were performed to assign the reproducibility of the PCR assay. Agarose (1%) gel electrophoresis for 60 min with 110 V was performed for separation and analysis of PCR products. Gel images were taken with a gel documentation imaging system (Vilber Lourmat Quantum, Germany).

6. Western Blot Analysis

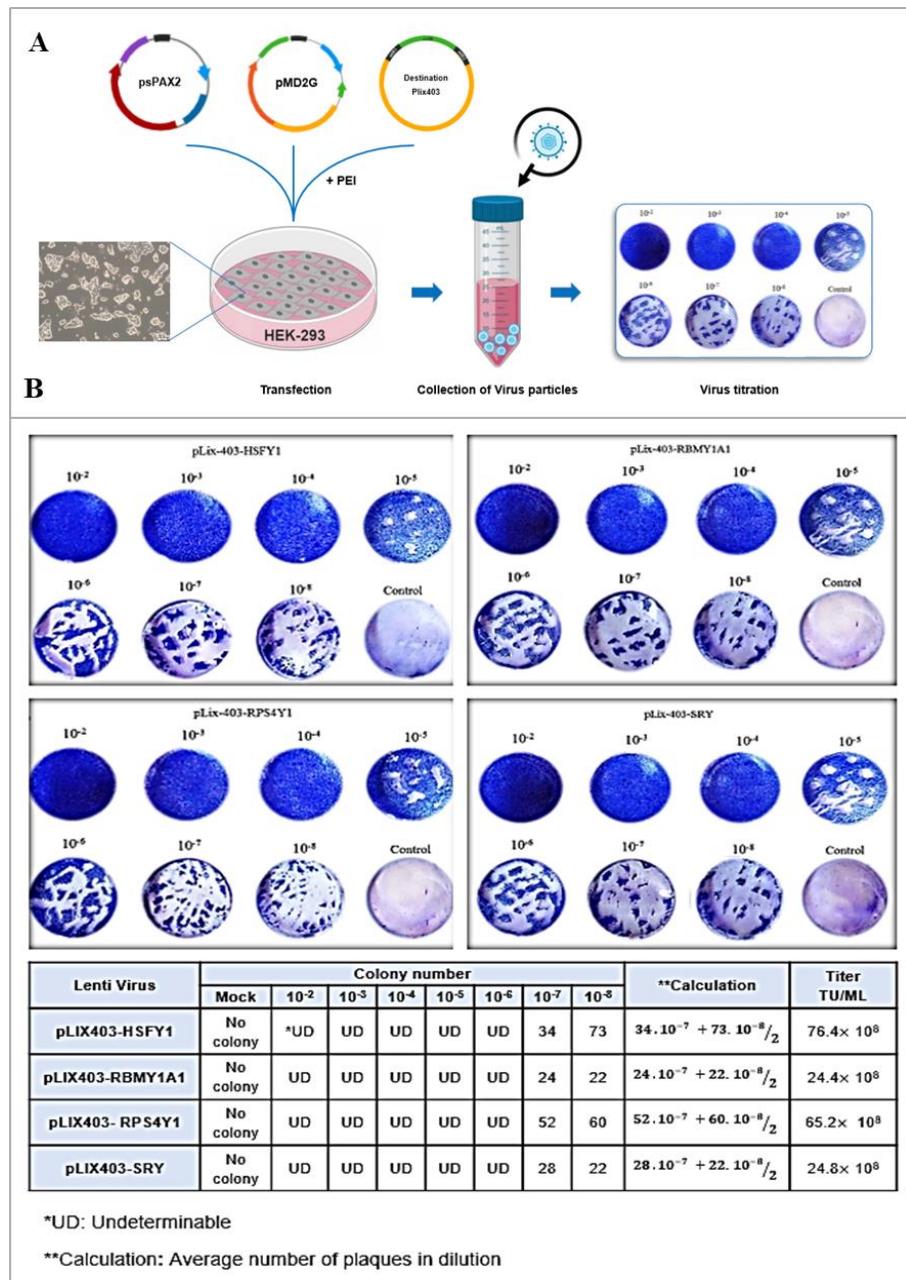
The protein concentrations were performed by the BCA protein assay kit; BSA was used as a standard. Proteins were separated according to their molecular weight on 12% SDS polyacrylamide gel (SDS-PAGE). The isolated proteins were transferred to a polyvinylidene difluoride (PVDF) membrane through wet blotting (BioRad, USA). The membranes were blocked with 2% skim milk for 1 hour, after that, they were incubated (at room temperature for 1.5 hours) with the respective primary antibodies (Supplementary Table 2). At the end of the incubation period, membranes were rinsed 3 times for 15 min with a solution of Tris-buffered saline with Tween 20 (0.1% v/v; TBST) and incubated with the specific secondary antibodies (Supplementary Table 2) for 1 hour. Subsequently, the membranes were washed with TBST, and the target protein bands were visualized by using Amersham ECL Advance Western Blotting Detection Kit (GE Healthcare, USA) on a UVITEC Chemiluminescence and Documentation System (UVITEC, Cambridge, UK).

Supplementary Table 1. List of primer sequences designed for the Y chromosome genes, pluripotency markers, three germ layers' markers, and cloning genes.

Gene	Sequence	Target genes
<i>RBMY</i>	attB1: 5'-GCCACCATGGTAGAAGCAGATCATC-3' attB2: 5'-CTATATCTGCTCGAGTCTCCT-3'	<i>RBMY1A1, RBMY1B, RBMY1D, RBMY1J, RBMY1F, RBMY1E</i>
<i>HSFY1</i>	attB1: 5'-GCCACCATGGCACATGTTCTCAG-3' attB2: 5'-CTAGTTGTAATTAGGGTATTTG-3'	<i>HSFY1</i>
<i>RPS4Y1</i>	attB1: 5'-CCCAAGAACCTTAAAGCGT-3' attB2: R: 5'-TAGCCACTGCTCTGTTGG-3'	<i>RPS4Y1</i>
<i>SRY</i>	attB1: 5'-CACC ATGCAATCATATGCTTCTGC-3' attB2: 5'-CTACAGCTTGTCCAGTGGC-3'	<i>SRY</i>
<i>GAPDH</i>	F: 5'-GAAATCCCACCATCTTCC-3' R: 5'-GGCTGTTGTCATACTTCTCAT-3'	<i>GAPDH</i> (Transcript variant 1-4)
<i>OCT4</i>	5'-CTGGGTGATCCTCGGACCT-3' R: 5'-CACAGAACATACGGCGGG-3'	<i>OCT-4</i>
<i>SOX1</i>	5'-GGTACTTGTAAATCCGGGTGC-3' 5'-CACAACTCGGAGATCAGCAA-3'	<i>SOX1</i>
<i>SOX2</i>	5'-GGGAAATGGAAGGGGTGCAAAAGAGG-3' 5'-TTGCGTGAGTGTGGATGGGATTGGT-3'	<i>SOX2</i>
Notch1	5'-CAGACCCACACCCAGTA-3' 5'-GGCAACGTCAACACCTT-3'	Notch1
<i>TP63</i>	5'-TTTCAGAGGAATCCACACA-3' 5'-ATGCATGCAAATGAGCTCTG-3'	<i>TP63</i>
<i>Nestin</i>	5'-TCCAGGAACGGAAAATCAAG-3' 5'-GCCTCCTCATCCCCTACTTC-3'	<i>Nestin</i>
<i>MIXL1</i>	5'-TCTTGAGGTAGATGTGAAGT-3' 5'-CTTTGAACCAATGTCCTCAGAG-3	<i>MIXL1</i>
<i>Nodal</i>	5'-GCGTACATGCTGAGCCTCTA-3' 5'-CTTGAAACCAATGTCCTCAGAG-3'	<i>Nodal</i>
<i>AFP</i>	5'-AAATCGTTCTCGTTGCTT-3' 5'-GCCACAGGCCAATAGTTGT-3'	<i>AFP</i>
<i>GATA4</i>	5'-CCTGTCATCTCACTACGG-3' 5'-GCTGTTCCAAGAGTCCTG-3'	<i>GATA4</i>
<i>FOXA2</i>	5'-GGAGCGGTGAAGATGGAA-3' 5'-TACGTGTTCATGCCGTTCAT-3'	<i>FOXA2</i>
<i>Brachyury</i>	5'-AATTGGTCCAGCCTTGGAAAT-3' 5'-CGTTGCTCACAGACCACA-3'	<i>Brachyury</i>
<i>NANOG</i>	F: 5'-AAAGTCTTAAAGCTGCCTAAC-3' R: 5'-CAGTCGGATGCTCAAAG-3'	<i>NANOG</i>
<i>HSFY1</i>	F: 5'- TTCATGGGATGAGAATGGAAC-3' R: 5'- GAAAGGTGGCTAGAAAGGCAG-3'	<i>HSFY1</i> (Transcript variant 1,2,3)
<i>RPS4Y1</i>	F: 5'- AGGAAGATTACTGTGGGAGTG-3' R: 5'- GTATCGTTCACCTTGATGAC-3'	<i>RPS4Y1</i>
<i>RBMY1</i>	F: 5'-TGCCACATAACTTGAGCTACC-3' R: 5'-ACTCGAGCTGTGGTATTTG-3'	<i>RBMY1A1, RBMY1B, RBMY1D, RBMY1J, RBMY1F, RBMY1E</i>
<i>SRY</i>	F: 5'-CGAAACTCAGAGATCAGCAAGCAGC-3' R: 5'-CCTGTAATTCTGTGCTCCTGG-3'	<i>SRY</i>

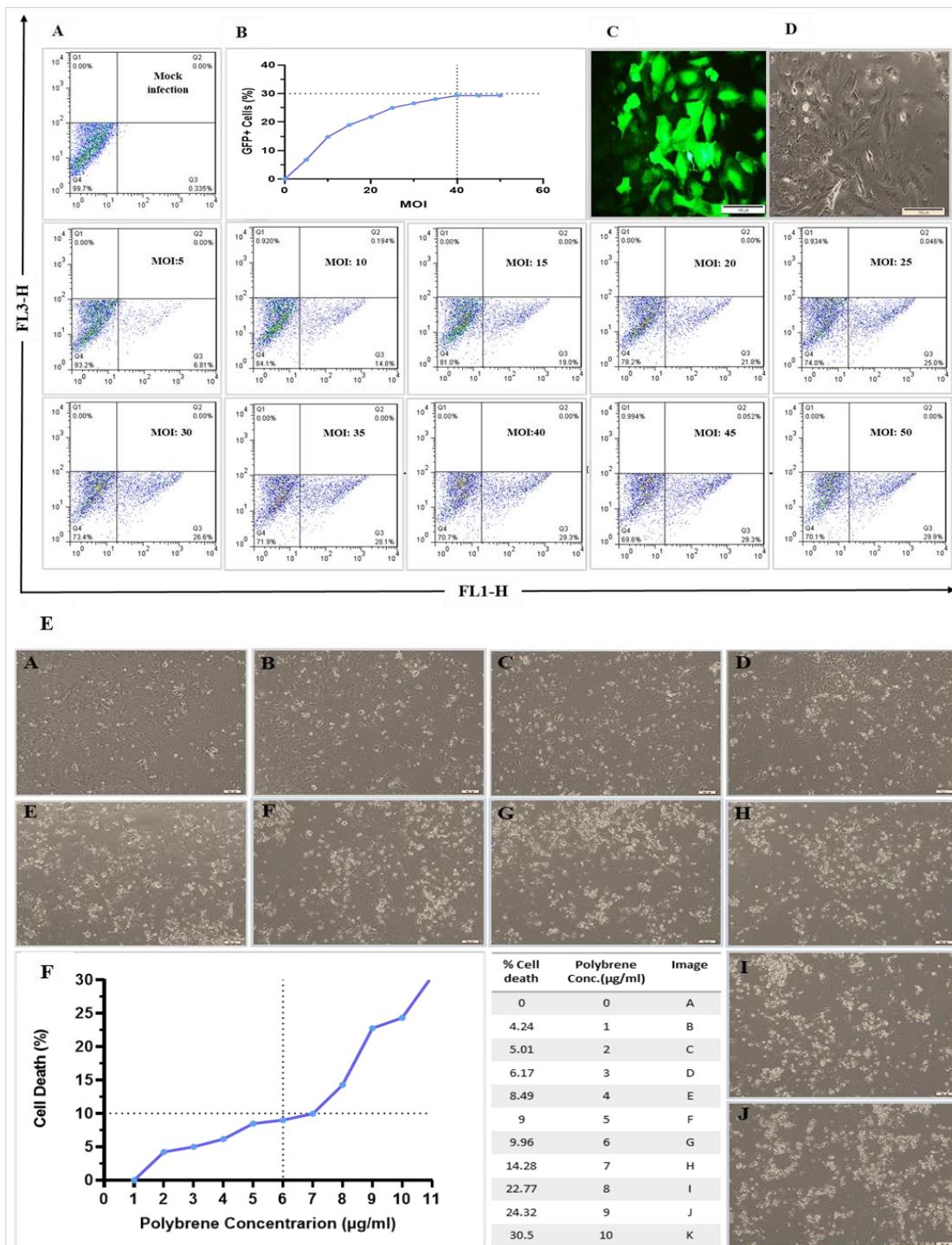
Supplementary Table 2. Details of sources and concentrations of antibodies used for western blot and immunocytochemistry (ICC).

Antibody	Species/Company	Dilution
Western blot		
Beta Actin	Rabbit IgG/Abcam/ ab8227	1:5000
HSFY1	Rabbit IgG/Homemade	1:1000
RBMY1	Mouse IgG/Sigma	1:200
RPS4Y1	Rabbit IgG/Homemade	1:1000
SRY	Human IgG/Sigma	1:200
Anti-Rabbit	Goat Anti-rabbit IgG/Abcam	1:20000
Anti-Mouse	Goat Anti-mouse IgG/Sigma	1:2000
Immunocytochemistry (ICC)		
RPS4Y/RPS4X	Rabbit IgG/Homemade	1 : 200
Alexa Fluor® 546	Donkey anti-rabbit IgG/Invitrogen	1 : 500
NANOG	Rabbit IgG/Abcam	1 : 200
OCT4	Mouse IgG/Abcam	1 : 200
SOX2	Rabbit IgG/Abcam	1 : 200
SOX1	Goat IgG/R&D System	1 : 200
NESTIN	Mouse IgG/Santa Cruz	1 : 200
Alexa Fluor® 546	Donkey anti-goat IgG/ Invitrogen	1 : 500
Alexa Fluor® 488	Goat anti-rabbit IgG/Abcam	1 : 500
Alexa Fluor® 488	Rabbit anti-mouse IgG/Abcam	1 : 500
Alexa Fluor® 594	Donkey anti- Rabbit IgG/Invitrogen	1 : 500



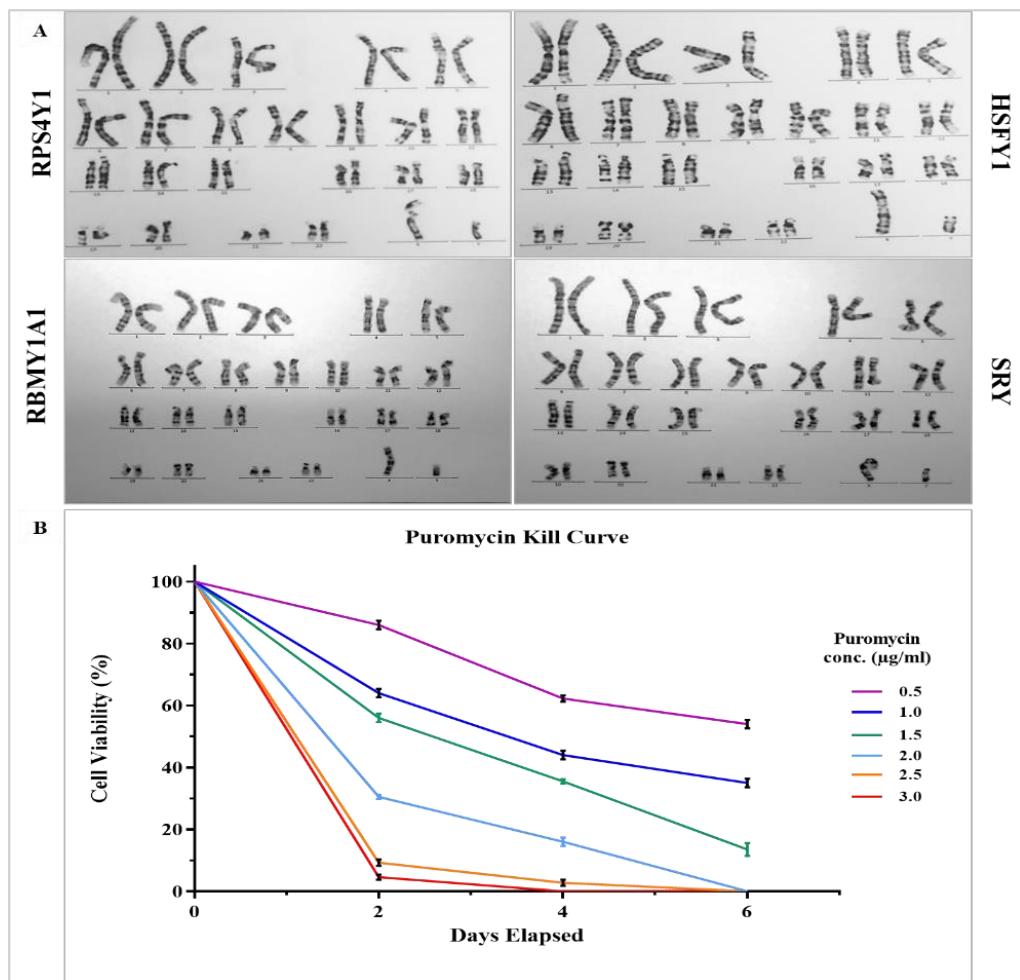
Supplementary Figure 1. A. Production and concentration of lentivirus. HEK293T cells were seeded in adherent culture. When the cell confluence was achieved to an appropriate number, the cells were transfected with the cocktail of lentiviral vectors using polyethyleneimine. The cell culture medium was collected after 24 to 72 hours and filtered. Lentiviral particles were concentrated by centrifugation. The puromycin (2 μ g/mL) selection on HT1080 cells and crystal violet staining was performed for viral particle titration. **B.** Plaque assay for lentiviruses. The HT1080 cells were seeded in an 8-well plate. Seven-fold serial dilutions of the lentiviral particles in a cocktail of transduction medium and polybrene (6 μ g/ μ l) were made, and after 24 hours, the 7-well plate was transduced with lentiviral particles. Untransduced control well, as mock was included by transduction medium, without any lentiviral particles. The medium containing puromycin was changed every 2-3 days until all untransduced cells have died. The cells were

fixed and stained with crystal violet. The countable plaques were in the 10^{-7} and 10^{-8} dilution wells. Infectious titers were estimated according to the formula in the above table.



Supplementary Figure 2. **A.** MOI calculation of hESC (RH6) line. The transduction efficiency of lentiviral vectors on hESCs was determined with the pLIX-403-GFP lentiviral vector. Cells were transduced with varied MOI between 5 and 50. Three days after transduction, the percentage of GFP positive ESCs was analyzed using flow cytometry. **B.** At MOI of 40, about 30% of cells were transduced. The increase in the MOI to 50, did not improve in the transduction rate. **C, D.** The transduced cell morphology is shown by the light and fluorescence microscopy. Scale bar: 100 μ m.

Polybrene toxicity assay. A polybrene toxicity test is performed by using a range of concentrations from 0 to 10 μ g/ml on the cells. **E.** (A-J) Images of microscopy of cell death after 72 hrs. are shown. Scale bar: 100 μ m. **F.** The highest concentration of polybrene that results in less than 10% cell toxicity compared to negative control is 6 μ g/m.



Supplementary Figure 3. **A.** Karyotype analysis for stable transgenic cell lines. **B.** Puromycin toxicity assay. Puromycin toxicity assay was done with a range of concentrations between 0 to 3 μ g/ml. The curve shows cell viability percent after 6 days. The lowest concentration of puromycin that kills 100% of the negative control was 2 μ g/m.