

## A novel medium-throughput biological assay system for HTLV-1 infectivity and drug discovery

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ARTICLE INFO	ABSTRACT
<p><b>Article type:</b> Original article</p> <hr/> <p><b>Article history:</b> Received: Nov 18, 2016 Accepted: Aug 10, 2017</p> <hr/> <p><b>Keywords:</b> Biological assay Drug screening HTLV-1 Hut102 Luciferase Reporter gene</p>	<p><b>Objective(s):</b> Here, a reporter cell line containing two reporter vectors were developed, in order to monitor the Human T-Lymphotropic Virus type1(HTLV-1) infectivity and the cell viability simultaneously.</p> <p><b>Materials and Methods:</b> The reporter cell line was constructed by stably transfected baby hamster's kidney cell line (BHK-21), with the genomes expressing two different reporters in separate plasmids. The first reporter gene is transactivated by the HTLV-1 tax protein, while the second reporter is continuously expressed when introduced into a mammalian cell. In order to show its functionality, the effect of the drug mix on HTLV-1 was assayed by this system and was compared to the results obtained by other methods.</p> <p><b>Results:</b> HTLV-1 reporter cell line was found to produce high level of luciferase when co-cultured with MT-2 and Hut-102 cells but not with Jurkat cell. Moreover, the combination therapy against HTLV-1 can reduce luciferase expression of the cell when co-cultured with MT-2 and Hut-102 comparable to the ELISA (R=0.932, P-value =0.002). In addition, the results revealed the superiority of the present system over the molecular methods.</p> <p><b>Conclusion:</b> The results demonstrated that the biological assay system is a beneficial tool for the medium-throughput anti-HTLV-1 drug screening and inhibitory effect.</p>

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### Introduction

The diseases associated with of HTLV-1 were known and introduced in Iran long time before the discovery of the virus itself by Bernard Poiesz in 1980 (1-3).

HTLV-1; Which stands for: Human T-cell Lymphotropic Virus type 1, is a public health threat in endemic areas, with more than 20 million infected people worldwide. In some foci like Sabzevar and Mashhad (2 major cities of Khorasan Province in Iran), the prevalence is up to 3% (4-8). Unfortunately, despite the considerable progress in HTLV-1 researches over the past three decades, still there isn't an available Food and Drug Administration (FDA), approved medicine or beneficial tool for anti HTLV-1 drug screening (8-11). The quantitative assessment of the virus and its infectivity is an important step for various basic and clinical studies as well as the analysis of drug susceptibility. HTLV-1 has the non-cytolytic and cell associated properties in cell culture (12). Thus, available classical virological methods such as: plaque assays are not usually used for the quanti-

tation of HTLV-1 infectivity. In other hand, the other techniques i.e. ELISA and PCR-based assays are either expensive or do not allow accurate quantitation of the number of the infectious viral titer over time (13-15). Hence, the unavailability of a rapid, economic, high-throughput assay for titrating HTLV-1, has hampered the efforts both to screen the new compounds and also the systematic analytical study on HTLV-1(16). The reporter cell systems are more commonly used in the drug susceptibility assays and the detection of virus infectivity (12, 17-20). Different reporter cells have been reported for HTLV-1 in the literature, such as: pA18G-BHK-21 cells (21), H1GFP cells (22) and H9/K30luc cells (16). All these cell lines carry a reporter gene regulated by the HTLV-1 long terminal repeat (LTR) promoter, inducing them to produce the reporter protein while they are infected with HTLV-1. Even though pA18G-BHK-21 cells with  $\beta$ -galactosidase reporter and H1GFP cells with green fluorescent protein can detect viral infection by some degrees. It seems that H9/K30luc cells with the third generation-

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luciferase reporter vector had a higher sensitivity as compared to them (16).

To facilitate the analysis and monitoring the HTLV-1 infection, a unique HTLV-1 reporter cell line with two reporter vectors was designed in this research. At first, these two reporter vectors were constructed and tested, and then BHK-21 cell was stably transfected by them. The suitability of the cell line in measuring the virus replication was tested by using the co-culture with 2 different HTLV-1 producing cell lines. Finally the system was used to evaluate the effect of the drugs combination on the HTLV-1 replication.

## Materials and Methods

### Plasmids and constructs

pGL4.17 (Promega, USA), pEGFP-N1 vector (Clontech CA, USA) and pCDNA3.1Hyg+ mammalian expression vectors (Invitrogen, USA) were purchased. pCDNA3.1TAX Hyg+ (23) was used for the induction of Luc expression. Two different reporter plasmids, pGL4LTRLuc and pCDNA3.1 SV40-EGFP Hyg+, were constructed (Figures 1b, 1c). The first vector has been previously described (24). To sum up, HTLV-1 LTR promoter containing three tax respond elements (TRE I, II and III), was cut out from pUCLTRLacZ (25) and then it was subcloned into the MCS site of plasmid pGL4.17 (Promega, USA), at the upstream of the Luciferase reporter gene. The second reporter vector, pCDNA3.1SV40-EGFP Hyg+, was constructed by cloning of the SOEing Polymerase Chain Reaction (PCR) product, SV40 promoter linked to EGFP (SV40-EGFP), into pCDNA3.1Hyg+, to express the EGFP continuously when it is introduced into a mammalian cell. Firstly, SV40 promoter from pCDNA3.1Hyg+ and fragment of pEGFP-N1 containing the EGFP were amplified by PCR using the following set of primers:

SV<sub>40</sub>FWD =5' CAAGATCTGGTTAAAAAATGAGCTGATTTA 3', SV<sub>40</sub>Rev =5'TTGCTCACCATCACGT GCTGATCAGAT 3'.

EGFP FWD=5' GTGATGGTGAGCAAGGGCGAG3', EGFP-Rev= 5' TGGACAAACCACAACCTAGAATGCAGTG 3'.

Then, the integration of SV40 promoter was done using the Touchdown PCR protocol by the SV<sub>40</sub>FWD and EGFP Rev as forward and reverse primers at the EGFP gene upstream (Figure 1a). After that, it (SV40-EGFP) was cloned, simultaneously when the CMV promoter was removed in pCDNA3.1+Hyg between the XbaI- BglII sites (Thermo Fisher Scientific, LithuaniaLtd). Once the selected colonies were grown in the Luria-Bertani broth (LB, Himedia) overnight, the recombinant vector was purified by the QIAGEN Plasmid Mini Kit (Qiagen, Germany).

### Cell lines and culture

Baby hamster kidney cells (BHK-21), which has demonstrated high susceptibility to HTLV-1 (Cat No: C107) was obtained from the National Cell Bank of Iran (Pasteur Institute of Iran, Tehran). BHK-21 cell line was grown in high-glucose Dulbecco's modified

Eagle's medium (Gibco) supplemented with 10% heat inactivated fetal bovine serum (FBS), 100 units-100 µg/ml/ml penicillin-streptomycin(PAA, austeria) and 2 mM L-glutamine at 37 °C in 5% CO<sub>2</sub>. T-cell lines, HTLV-1-producer cell lines, MT-2 and HUT 102 cells (HTLV-1 producing CD<sub>4</sub><sup>+</sup>human T-cell lines as effector cells) (26) and Jurkat (HTLV-1 infection free CD<sub>4</sub><sup>+</sup> T lymphocyte) were maintained in RPMI 1640 (PAA, Austeria) with the same supplements.

### Transient transfection and reporter expression evaluation

The BHK-21 cell was plated at a density of  $4 \times 10^4$  cells per well in 48-well plates and allowed to grow overnight. 0.2 µg of pCDNA3.1SV40-EGFP Hyg+ plasmid was transfected into the BHK-21 cell using Polyfect (Qiagen, Hilden), according to the manufacturer's instructions. 8 hrs after post-transfection, medium was replaced by fresh medium. Throughout 48 hr, EGFP expression was showed using fluorescent microscopy (Figure 2a).

### Establishment of the reporter cell line

#### First stably transfection and clonal selection

The BHK-21 cells were cultured at a density of  $5 \times 10^5$  cells per well in 6-well plates to reach a confluency of 70 percent and allowed to grow overnight. Six µg pCDNA3.1SV40-EGFP Hyg+ plasmid linearized with Bgl II digestion, was transfected into the BHK-21 cells using Polyfect (Qiagen, Hilden) according to the manufacturer's recommendation. After transfection the plate was centrifuged 5 min at 280 g. Four hr after post-transfection, the medium was replaced with fresh medium. Forty-eight hr after transfection, the cells were cultured in complete medium supplemented with 0.2 mg/ml Hygromycin (Hyg B) (Invivogen, USA). To obtain stable clones, during several weeks the Hyg-resistant colonies were isolated further by plating them at three rounds of limiting dilution (27) onto 96-well plates during several weeks. The single clones with no EGFP expression were removed. Several clones from remaining cells with high constitutive EGFP expression (Figure 2b) were stably transfected with pGL4LTRLuc. Before that correlation between EGFP activity and cell viability was evaluated by flow cytometry using propidium iodide (PI) staining and dye exclusion method as described elsewhere (28). Besides, different expression was shown by two distinct clones (Figure 3a). Also the validity of using EGFP activity, to monitor reporter cell numbers, was evaluated by measuring EGFP activity in various numbers of cells and determining the correlation between EGFP activity and cell numbers using flourimeter (Biotek, USA). Reporter cells were plated into 96-well plates triplicate (3 wells for each test) at different density of cells from  $10 \times 10^3$  to  $100 \times 10^3$  cells per well. And then they were assayed by flourimeter (Figure 3b).

### *Second stably transfection and clonal expansion (Selection of monoclonal populations from BHK-EGFP-HTLVLTR luc)*

BHK-EGFP cells were plated and stably transfected with 5 µg linearized pGL4LTRLuc plasmid using the same procedure for the first transfection. Two days after transfection, the cells were cultured in complete medium containing 0.2 mg/ml hygromycin and 1 mg/ml genitcin (G418) (Invivogen, USA) for 3-4 weeks. Stable colonies were isolated by culturing them at limited dilution in wells of a 96-well plate for three cycles. Subsequently several single clones of the G418-resistant colonies were taken under luciferase assay with following procedure. After seeding  $10^4$  cells into 96-well plates for 24 hr, BHK-EGFP-HTLV-1Luc cells were transfected with 0.2 µg Tax expression plasmid using Polyfect reagent (Qiagen, 493277090A012 Germany). Transfection was normaliaized as previously described. After additional 48 hr incubation, we performed luciferase by using One Glo system assay (Promega-Inc.) according to the manufacturer's instructions onto a Synergy4 luminometer (Biotek, USA). The cells with high constitutive luciferase expression were removed. Furthermore 27 colonies were selected for the lowest background and high *Luc* expression upon co-culture with  $10^4$ Hut-102 cells for 48 hr.

### *Evaluating the sensitivity and of reporter cell line using different number of effector cell*

In order to evaluate the sensitivity of this system, a matrix including different numbers of effector cells (Hut-102, HTLV-1 producing cell) and reporter cell (BHK-EGFP-HTLVLTR-Luc cell) was prepared and co-cultured in a 96-well plate according to the Table-1. In a similar experiment, a Jurkat cell, as negative viral control, was co-cultured with the reporter cell simultaneously. In addition, three non-co-cultured wells were considered for measuring background expression. The experiments were replicated in different days (in triplicate format). In all different experiments, similar conditions were applied for both cultivation of cells and measurement of Luciferase expression.

#### *A. Investigating the application of reporter cell in drug screening against HTLV-1 virus*

First, for determining toxicity of the medicines used in this study, a number of  $2 \times 10^4$  reporter (reporter) cells were co-cultured with  $3 \times 10^4$  Jurkat cells in duplicate format in a 24-well plate. It was then exposed to different densities of Arsenic trioxide (0.5, 1, 1.5 and 2 µmol/ml; Sina Daru, Iran) and Interferon alpha 2 (500, 1000, 1500, 2000 Iu/ml; Pooyesh Daru, Pdferon, Iran) (29). After 72 hr, viability percentage of the cells was easily calculated, by using flow - cytometry. This value was also computed in separated 24-well plate cell culture for reporter and Jurkat cells with hemocytometer and 0.4% Trypan Blue solution

(w/v). To implement cytometry, 1 µg/ml PI was added to the cells and viability and number of treated reporter and Jurkat cells versus control cells were studied, using control cells. Fluorescence expression in reporter cells was read in FL1 channel, and dead cells or cells in the final stage of apoptosis (the presence of PI in the cell) were read in FL3 channel. FloMax 2.7 was employed for analyzing the outputs.

Subsequently, a number of  $3 \times 10^5$  HuT-102 and MT2 cells were cultivated separately in a 6-well plate in RPMI1640 media, containing two pharmaceutical compositions, namely (As: 1 µmol/ml), (IFn: 1000 Iu/ml), versus control media. After 72 hr, cells were counted and the number and percentage of their viability were determined. Then, 50 ul HuT-102 and MT2 were added (from control and treated cells, separately) to the reporter cell, cultivated the day before, in 96-well plate in triplicate format. EGFP and luciferase expression were measured after 60 hr. Then luciferase expression (the effect of medicine on the virus and effector cell) and EGFP expression (the effect of medicine on BHK-21 cell and its number) were calculated and modified using the following formula.

$$\text{Luc Test} / \text{EGFP Test} / \text{Luc control} / \text{EGFP control} = \text{Corrected luciferase expression}$$

A.1. Comparison between the luciferase expression level and the amount of produced p19 antigen, using ELISA

After 72 hr exposure to the effector cells, in the presence or absence of pharmaceutical composition in the A-stage, sampling from cell culture supernatant media was done and ELISA for p19 antigen of HTLV-1 was performed according to kit (ZeptoMetrix .Buffalo, NY). Next, it was read in ELISA reader at 450 nm wavelength.

A.2. Comparison between luciferase expression and HTLV-I tax mRNA expression

HuT-102 and MT2 cells were centrifuged (at 300g) in the A-stage after 72 hr. The supernatant was removed and washed three times with phosphate buffer. Total RNA was then extracted using RNX™ (Plus) (CinnaGen Inc.) according to its Protocol. After cDNA synthesis, using QuantiTect Reverse Transcription Kit (Qiagen, Germany), Real-time PCR test was done, using HotTaq EvaGreen® qPCR Mix (CinnaGen Inc.) according to the following protocol, 1- Primery denaturation at 95 °C for 10 min, 2- Contain 40 cycles with following steps: 3-Denaturation at 95 °C for 10 sec, 4- Extension at 60 °C for 60 sec, Melt curve: 55-99 °C (with 1°C increase during every 5 sec) and with the presence of Tax gene specific primer:  
TaxF: 5'-GGATACCCAGTCTACGTGT-3'  
TaxR: 5'-GAGCCGATAACGCGTCCATCG-3'

As internal control, GAPDH primer (housekeeping gene) was used (15). The Real-time PCR test was done by using Corbett (65HO).

**Statistical analysis**

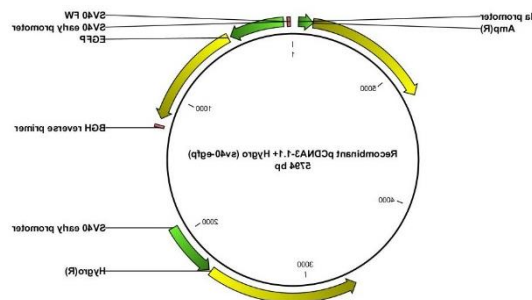
Statistical analysis was done with SPSS16.0, by using Pearson's correlation test, t-test and one-way ANOVA.

**Results**

**The creation of pCDNA3.1+Hyg SV40-EGFP recombinant as the second vector**

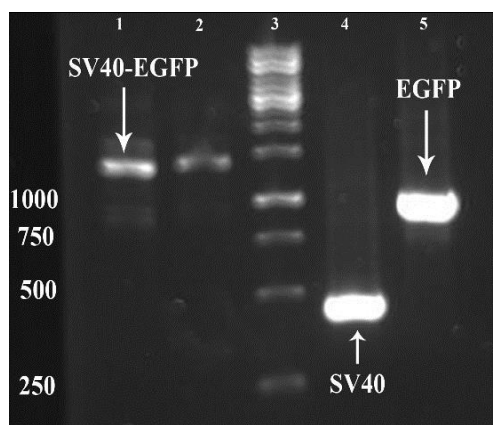
By using the SOEing PCR, the integration of SV40 promoter was done at the EGFP gene upstream. Then, it (SV40-EGFP) was cloned, at the same time when the CMV promoter was removed, in pCDNA3.1+Hyg between the XbaI- BglIII sites. The SV40 promoter was selected due to its weaker performance to CMV, so the cell line permanent EGFP expression could exert less potential negative effect on the activity of the first vector (pGL4LTRLuc) in the cell.

The proper performance of the cloned SV40-EGFP segment was concluded by the transiently transfection of BHK-21 cells with the EGFP reporter vector and the observation of EGFP protein in the cell, after 24 hr, using a fluorescence microscope. The proper performance of the luciferase reporter vector (pGL4-LTRLuc) was previously shown with the transiently transfer to the cell (26).

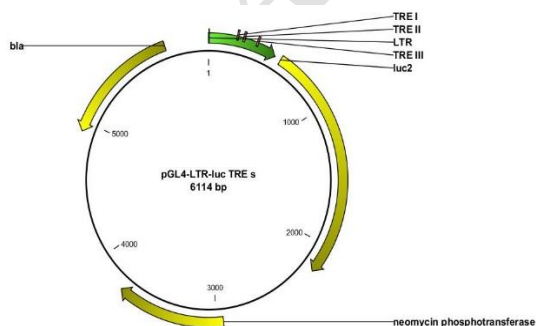


(c)

**Figure 1.** (a) SOEingPCR; On the left, SV40-EGFP fragment that was double digested between XbaI- BglIII sites, and undigested SV40-EGFP fragment can be seen on the second lane. Lane 3- 1 kb lambda marker bands, lane 4- amplified SV40 promoter from pCDNA3.1+Hyg containing 455 bp, lane 5- amplified EGFP gene from pEGFP-N1 containing 948 bp. (b) and (c): structure of pGL4LTRLuc and pCDNA3.1 SV40-EGFP Hyg+ vectors, respectively



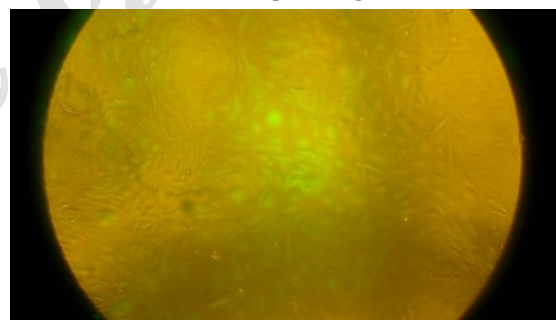
(a)



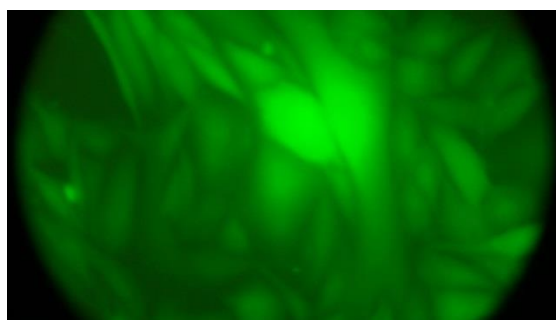
(b)

**Establishing the BHK-EGFP-HTLVLTR-Luc reporter cell line**

In order to build this cell line, the EGFP expressing vector was stably transferred to the BHK-21 cell (Figure 2b). The fluorescence expression level in different clones (Figure 3a) and the accuracy of relationship between the fluorescence expression and the number of cells have been shown (Figure 3b). In the second stage, the luciferase reporter gene carrying vector was stably transferred to the EGFP expressing clones.

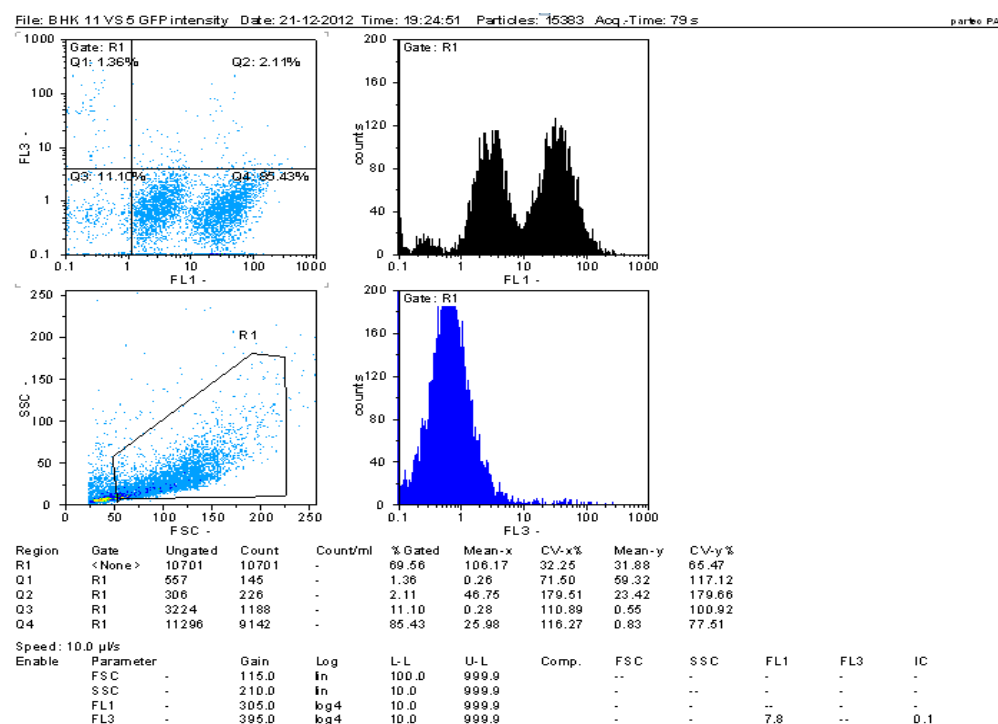


(a)

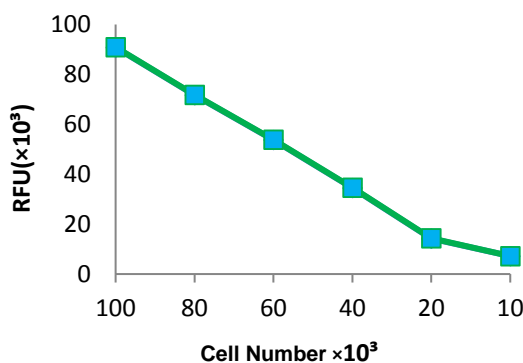


(b)

**Figure 2.** Fluorescence microscopic examination: Samples of (a) transiente and (b) stable transgenic EGFP expressing single-clones BHK-21, isolated by the serial dilution test, can be seen



a)



b)

**Figure 3. (a)** Using a flowcytometer, a suspension was prepared from two clones, i.e. 11 (with lower fluorescence) and 5 (with higher fluorescence), and then the Propidium iodide (PI) color was added to it. The comparison between the PI colored, dead (FL3) cells and the two Fluorescence (FL1) producing cell populations are presented in the upper left panel. The fluorescence intensity of the first clone (close to the center of the curve), a base-10 logarithmic number, is lower than the second clone. The correlation between the EGFP activity and the cell viability of the cell suspension was conveniently evaluated by flow cytometry using PI staining comparing with the dye exclusion method as described elsewhere(28). **(b)** The linear relationship between the cell numbers and the expression level of EGFP (relative fluorescence unit) is obvious where there are more than  $20 \times 10^3$  cells. The experiment was done in triplicate format and the level of the expression was measured by the means of the flowmeter (85% sensitivity)

With inducing 236 isolated single-clones by the Tax expression vector, the luciferase expression examination was done to obtain the best clone in terms of the expression level and the relative expression of luciferase. Unfortunately, in many colonies, either luciferase expression was turned off or the difference between the luciferase expression and the background expression levels was 2 times or less. Finally, 27 colonies with more than 2 times (up to 5.9x) expression were selected. Followed by the investigation of their reproducibility, two clones which exhibited

strong transactivation of the integrated HTLV-1LTR-Luc gene after co-cultured with HTLV-1infected cells were finally confirmed. Behind the reproduction and the stock preparation from these colonies, experiments were continued with one of them.

**Investigating the sensitivity of the reporter cell line luciferase expression based on the numbers of effector to reporter cells**

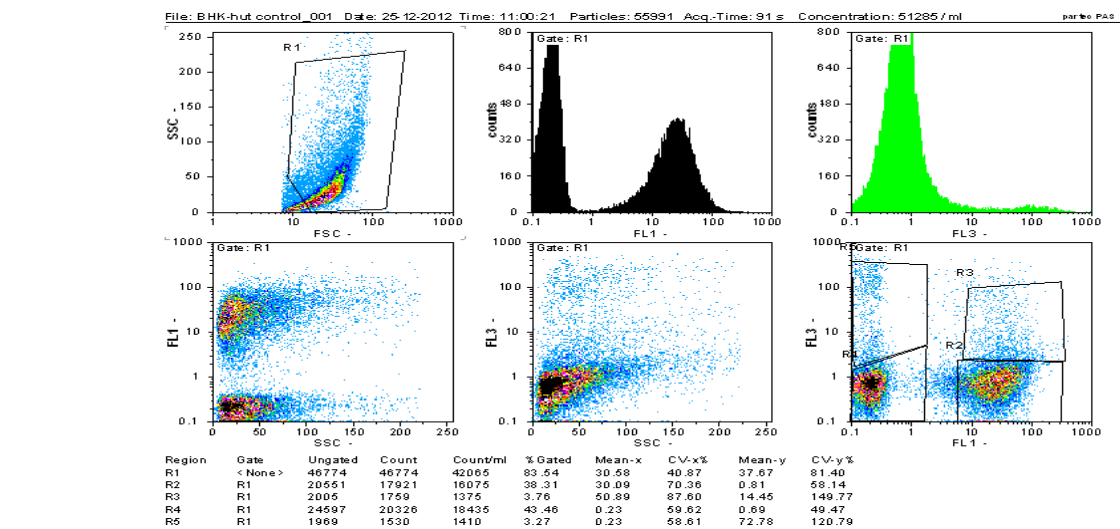
In order to obtain the expression level based on the cell and quantitative estimation, the induction effect of

different numbers of effector (HTLV-1 producer) on the reporter cells was used instead of using different virus numbers according to Table 1. Then the luciferase expression level (1.a) and the ratio of luciferase expression (1.b) were measured. The highest and the best expression levels under the condition of  $1.5 \times 10^4$  effector to  $1 \times 10^4$  reporter cells ratio (1.5/1) were obtained. The more increase in effector cell number, the higher expression level was observed. This relationship was faded with an increase in the number of the effector cells to more than 15.000 where a decline in luciferase level was also observed.

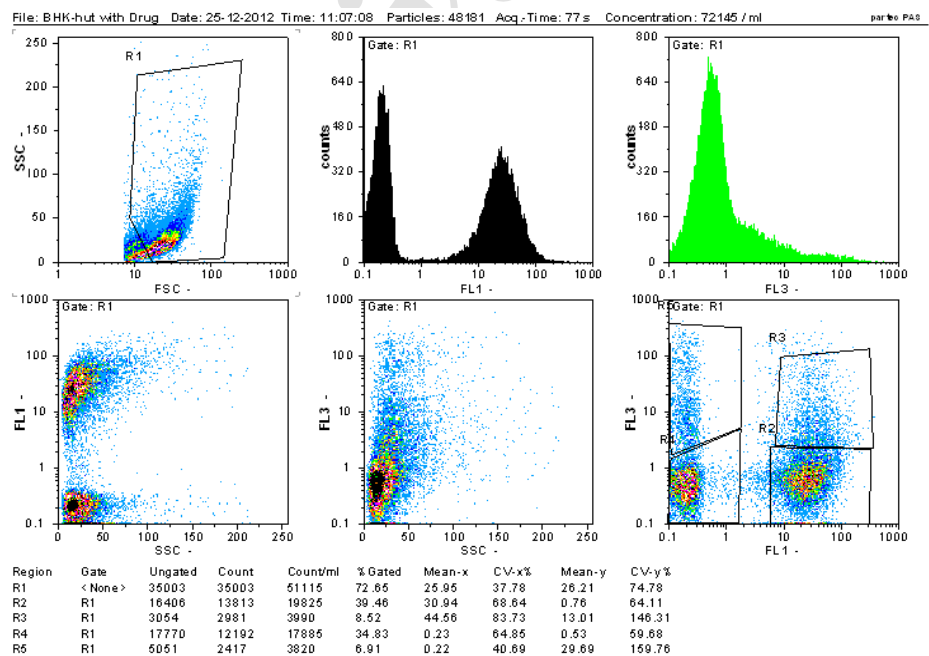
A matrix containing the reporter cell with the effector cells and with the Jurkat cell (as the negative control)

separately was cultivated. After 48 hr incubation, the luciferase expression level (a) and the ratio of luciferase expression (b) were measured. a) The mean of the relative luciferase expression level ( $RLU \times 10^3$ ) from the three experiments in two different days was presented. The best luciferase expression was bolded. b) The test to control the luciferase expression ratio (of Table 1.a experiments) was calculated.

The mean of luciferase expression level by reporter cell versus HTLV-1 (MT2 and HuT-102) producing cell, HTLV-1 free cell (Jurkat), was measured. HuT-102 and MT2 effector cells caused an increase in the luciferase expression level by 3x and 2.5x, respectively, while the Jurkat cell decreased it by 0.9 x.



a)



b)

Figure 4. The viability of reporter and Jurkat cells were measured after 72 hr co-cultured in the absence(a) and presence(b) of drugs Arsenic (1 μmol/ml), and Interferon α<sub>2</sub> (1000 Iu/ml) using flow cytometry method

**Table 1.** Optimization of the expression level, affected by different numbers of effector-to-reporter cells

a)

Effector → Reporter ↓	5000	10000	15000	20000	25000	30000
5000	469 ± 21	653 ± 43	798 ± 24	802 ± 27	710 ± 28	566 ± 33
10000	553 ± 36	895 ± 44	968 ± 27	862 ± 38	808 ± 32	605 ± 61
15000	674 ± 28	917 ± 41	939 ± 34	768 ± 26	822 ± 47	575 ± 69
20000	733 ± 32	699 ± 26	732 ± 49	765 ± 22	663 ± 48	618 ± 30

b)

Effector → Reporter ↓	5000	10000	15000	20000	25000	30000
5000	1.59 ± 0.07	2.4 ± 0.18	2.78 ± 0.09	2.82 ± 0.1	2.54 ± 0.11	2.1 ± 0.14
10000	1.72 ± 0.12	3 ± 0.16	3.2 ± 0.1	2.86 ± 0.14	2.76 ± 0.12	2.24 ± 0.26
15000	1.93 ± 0.09	2.7 ± 0.14	2.82 ± 0.11	2.3 ± 0.09	2.45 ± 0.16	1.84 ± 0.25
20000	1.84 ± 0.09	1.81 ± 0.07	1.86 ± 0.14	2 ± 0.06	1.73 ± 0.14	1.67 ± 0.09

### Evaluating the application of the reporter cell in drug screening against HTLV-1 virus

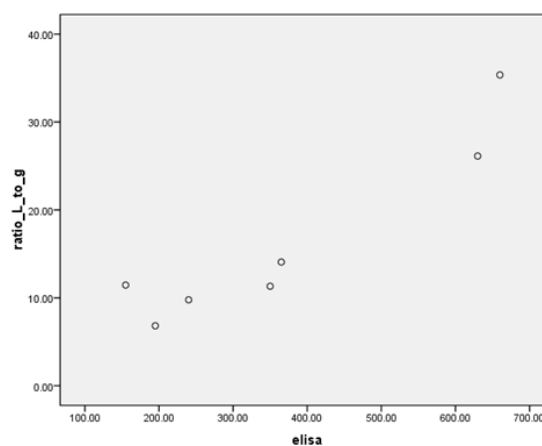
The effect of different values of Arsenic trioxide (0.5-2 µmol/ml) and Interferon alfa (500-2000 Iu/ml) on the reporter and Jurkat cells, after 72 hr, was calculated based on the cell counting and the corrected viability percent of the cells to the control. Those drug densities, in which more than 90% of cells remained viable, were selected for the next test (i.e. the effect of which on the HTLV-1 infected cell and on the virus itself), and subsequently for the luciferase expression. These densities included: Arsenic (1 µmol/ml), and Interferon (1000 Iu/ml). By adding the (above) mentioned densities of these two medicines to the cellular cultivation media, the hybrid effect of them on the reporter, Jurkat, and effector cells was measured by using the flow cytometry method (Figure 4).

### The effect of the new combination therapy against HTLV-1 on the luciferase expression of the reporter cell

The luciferase expression level after co-culture of the 72 hr treated effector cells (Hut-102, MT2) with the reporter cells, considerably decreased in the presence of drugs ( $P$ -value = 0.005). In terms of the Hut-102 and MT2 cells, these expressions decreased to less than half (from 32×5 to 14.1×3) and about 0.68 (from 12.9×1.4 to 8.7×1.7), respectively, in comparison to the cells which received no drugs.

### The correlation between the luciferase expression and the HTLV-1 p19 antigen

P19 antigen is a HTLV-1 matrix antigen which determines the quantitative measurement of the viral particle and its reproduction. Therefore, by seeking this antigen in cellular cultivation, the effect of the drug on the formation of viral particle versus the control cell can be quantitatively observed. The level of p19 antigen, after the cultivation of the effector cells (MT2, Hut-102), considerably decreased in the presence of drugs ( $P$ -value = 0.005 &  $P$ -value = 0.032). Furthermore, in terms of Hut-102 and MT2, this level declined to less than 0.3 (from 645 × 21 Pg/ml to



**Figure 5.** The correlation between ELISA p19 and luciferase expression (instead of luciferase expression, the ratio of Luciferase to GFP expressions was used to normalize the luciferase expression, shown on vertical axis) ( $P$ -value = 0.002, and  $R$  = 0.932)

172×25 Pg/ml) and lower than 0.55 (from 357 Pg/ml to 195 Pg/ml), respectively, in comparison with the cells which received no drugs. Meanwhile a strong correlation between ELISA and the luciferase expression was observed ( $P$ -value = 0.002, and  $R$  = 0.932) (Figure 5).

### Assessment the amount of the intercellular HTLV-1 RNA, using the real-time RT-PCR

The level of RNA expression after culturing the effector cells (Hut-102 and MT2) in the presence of medicine was measured, by using the Tax gene specific primer. For normalization of the experiment, GAPDH primers (Housekeeping gene) were used. By incorporation of the  $\Delta\Delta C_t$  technique, a decline in the expression level was observed in MT2 cells (1.22 cycles). However, this decline was not observed in Hut-102 cells.

## Discussion

Due to the necessity of developing a biological system for HTLV-1, a unique HTLV-1 reporter cell line with two reporter vectors was designed in order to monitor HTLV-1 infectivity and the cell viability

simultaneously. At first, these two reporter vectors were constructed and tested, and then BHK-21 cell was stably transfected by them. The EGFP reporter vector and the luciferase reporter vector confer Hygromycin and Genitacin (G418) resistance to the transfected cells respectively. Therefore, the selection clones of the BHK-EGFP-HTLV-1-Luc cells were performed by culturing them in the presence of hygromycin and genitacin.

By investigating the 236 G418 resistance clones, it was unfortunately determined that in most cases, luciferase gene or the virus promoter was turned off. This can be due to the vector integration in heterochromatin regions of the genome. There are reasons such as methylation in the critical regions of the promoter, the removal of the vector from the cell for any reason, or aberrant vector cut off at the time of integration can also be mentioned. In addition, it is said that in the spontaneous selection, the reporter resistant cells grow faster than the resistance gene carrying cells. Unfortunately, other groups of clones were the cells that despite of very high luciferase inductive expression had the high background level of expression too. This caused a very small difference between the test and control groups, leading to an insignificant test interpretation. As mentioned earlier, the virus promoter can be affected by the cellular signaling pathways. On the other hand, regarding the similarity between TRE and cyclic AMP response element (aka CRE)(30), there is a possibility that the virus promoter gets affected by the cellular signaling pathways (31). This possibility can be associated with the luciferase background expression without an induction by the Tax protein. However, the background level of expression in stabilized cells was to the extent that the expression difference between the test and control cells declined from 50 folds, in temporary transfer (32), to about 3 folds, in permanent transfer. This is due to the fact that in Tax protein, transactivation of the promoter is done through binding to CBP/P300, CREB, and P/CAF proteins. Okada and Jeang showed that the different values of these proteins had so greater impact on the activity of the promoters in stabilized cells than in cells to which the virus promoter was temporarily transferred (31).

The first reporter cell line for HTLV-1 was established using HeLa cell by Copeland *et al.*, based on the HTLV-1LTR (33). The induction of the  $\beta$ -galactosidase expression in this cell was done with the Tax protein, but simultaneous cultivation of this cell with MT2 did not result in reporter expression. While, an increase was observed in reporter expression in the cell established in this design, in the presence of the Tax protein, as well as MT2 and Hut-102 cells. After Copeland *et al.* Astier-Gin *et al.* developed LTR-LacZ based cells for HTLV-1(21). They performed the experiments in a 25cm<sup>2</sup> cell cultivation flask with, at least,  $1.5 \times 10^5$  reporter cells. Statistically, he reported

the minimum number of effector cells required for the induction of the reporter cell equal to a ratio of 13-22 to 1 (13-22/1). However, in this study, the best ratio of the effector to reporter was achieved equal to  $1.5 \times 10^4 / 10^4$  (i.e. 1.5/1), although, the sensitivity of the established cell was observed with less than 2000 effector cells (data not shown). The developed cell was easily used by its cultivation in 96-well micro plate, using  $10^4$  cells. This is the greatest advantage of using this cell vastly in order to achieve such goals as drug screening (22).

In contrast to HIV, in HTLV-1 infection, viremia is essentially a cell-based cytoviremia (40, 41). Therefore, to develop the infection, co-culture of the virus producing cells is used (42). In order to investigate the specificity of the cells which were developed in this study, two HTLV-1 producing cell lines were used. Moreover, Jurkat cell was employed as a negative control and for normalization of the co-culture of two cells. The co-culture of Hut-102 and MT2 cells, like Tax protein, increased the luciferase expression; while, the co-culture with Jurkat cell decreased the background level of expression. This decline could be due to the consumption of nutrients by this cell and the subsequent reduction of the reporter cell metabolism, leading to a decreased level of luciferase expression.

In all of the mentioned studies on HTLV-1, the established cells included only one reporter. In this research, a second EGFP-based reporter is permanently expressed, using the SV40 promoter. In addition, EGFP expression can be a criterion for the cell number in the tests. As it was shown, the expression of this reporter was in direct, linear relationship with a cell number over  $20 \times 10^3$  cells. The application of this specification could be advantageous in the drug screening trials, where other effects of drug on the reporter cell could be distinguished from the effects on the effector cell or virus.

Although, there was a number of commercial methods for drug resistance phenotyping in the case of HIV (34), Chiba-Mizutani *et al.* developed a system capable of virus tracking and drug-induced cytotoxicity, using a murine leukemia virus (MaRBLE) transformed T-lymphocyte cell line, as well as two luciferase reporter genes. The introduction of luciferase to the second reporter is an advantage of that cell compared to the method used in the present study; however, in this design, the SV40 promoter was used, instead of CMV, to ease the effect of the first reporter on the cellular energy. Regarding that there are confirmed anti-HIV drugs, his group had more maneuverability for evaluating the established system (17). In this study, since there were no confirmed anti-HTLV-1 drugs (35, 36, 40-42), the medicine which was used in a previous research was employed (29, 37, 38).

The effects of drug on the virus and the effector cell were measured by investigating the luciferase expression in the reporter cell (considering the EGFP expression



and the number of viable cells), in comparison with ELISA p19 antigen of HTLV-1, and Real-time RT-PCR. The results suggested a strong correlation between ELISA p19 and luciferase expression ( $P$ -value= 0.002, and  $R= 0.932$ ). This means that the effect of drug on the virus reduced the extracellular particles (ELISA, p19), and so this decline was also seen in the luciferase expression because the reporter cell had been affected by the lower virus number. The results from the real-time RT-PCR test were different, in a way that although a slight decline was seen in the level of viral RNA of MT2 cell, this decline was not observed in Hut-102 cells. In other words, pharmaceutical composition had no effect on the Tax expression, and in fact on the transcription of the intercellular provirus regarding its comparison with GAPDH gene, which is a function of drug effects on the cell. In result, these drugs, especially in terms of Hut-102 cell, have not caused transcription decline, but it seems they have mostly decreased the virus release, attachment, penetration, virus insertion, and/or reverse transcription and integration mechanisms in the reporter cells. Diagnosis of the drug impact on these last mechanisms is not possible with ELISA, indicating the superiority of this system over ELISA. As mentioned before; drugs caused a decrease in the number of the virus released from the cell. Regarding that the interferon was a drug used and previous studies showed its role in releasing the virus from the cell, this was an expectable effect (39). On the other hand, these results revealed the superiority of the present study over the molecular methods from different dimensions.

## Conclusion

Despite this, although the medium-throughput constructed system are cost effective and advantageous for performing basic and pharmaceutical studies, it is associated with a number of disadvantages such as the need for facilities including cellular cultivation laboratory and costly devices like luminometer.

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