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Original Research

Rapid amplification system for recombinant protein production in Chinese Hamster Ovary (CHO) Cells

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Abstract: Recombinant therapeutic proteins have changed the face of modern medicine in the present trend and they continue to provide innovative therapies for deadly diseases. This study describes the development of a novel stable expression system for rapid amplification of genes in Chinese Hamster Ovary (CHO) cells. The expression system consists of a host CHO cell line and an expression vector (pUB-PyOri-D-C) which encodes for Polyomavirus Origin of Replication (PyOri) for amplification of integrated genes in the presence of Py Large T Antigen (PyLT) and Dihydrofolate Reductase (DHFR) selectable marker gene for selection in the presence of Methotrexate (MTX). Use of both PyOri/PyLT and DHFR can reduce the number of rounds of selection and amplification required for isolation of high producing clones. The efficiency of pUB-PyOri-D-C was compared with that of pUB-D-C plasmid using Green fluorescent protein (GFP) and Erythropoietin (EPO) as reporter proteins. Our results showed that pUB-PyOri-D-C-EPO can help development of high expressing clone in one round of selection/amplification as compared to multiple rounds of selection/amplification with pUB-D-C-EPO plasmid. CHO-DG44/EPO clone generated using pUB-PyOri-D-C-EPO gave a productivity of 119 mg/L in shake flask.

Key words: Stable expression, episomal replication, CHO, polyomavirus, polyomavirus large T antigen, DHFR.

Introduction

Expression systems utilizing mammalian cell lines for recombinant protein production are able to introduce proper protein folding, post-translational modifications and product assembly, which are important factors for the complete biological activity of the native protein (1). The workhorse of mammalian protein production in a biopharmaceutical industry is CHO cells, due to their relative ease of use and long antiquity of regulatory acceptance for the production of Biopharmaceuticals (2). Biopharmaceutical products are clinical reagents, vaccines and drugs produced using modern biotechnology for *in vivo* diagnostic, preventive and therapeutic uses.

It is estimated that hundreds of new recombinant proteins and monoclonal antibodies (mAbs) enter preclinical and clinical development each year (3,4). There are over 30 monoclonal antibodies that are FDA approved for a variety of diseases ranging from malignancies to autoimmune diseases to macular degeneration (5). Concomitant global competition in biologics manufacturing has put immense pressure to shorten the time to market. Stable cell line development is a very tedious and time taking process. Existing expression systems such as DHFR/CHO, GS/CHO rely on the stepwise increase in the concentration of drugs such as MTX and MSX for gene amplification. However, they suffer from the drawbacks of inefficient amplification and require 6-12 months for cell line development. Some of the issues have been addressed with the use of weak promoter driving selectable marker gene (6), DHFR variants for efficient selection (7) and high-through put methods for identification of high expressing cell line (8,9). In spite of the above advances in cell line development, DHFR based selection, amplification still remains to be inefficient and time taking process. This is due to (a) drug resistant mechanisms (10), (b) lack of gene stability (11) and (c) number of rounds of selection amplification steps (12).

The current study is focused on reducing the number of rounds of selection, amplification using DHFR as a selectable marker gene and Polyoma virus elements (PyOri, PyLT) for replication of integrated genes. To this end, we have constructed pUB-PyOri-D-C-GFP (Hybrid vector) and pUB-D-C-GFP (conventional vector) plasmids to compare the efficiency of hybrid vector with that of a conventional vector for gene amplification. pUB-PyOri-D-C was further tested for productivity using EPO as a model protein.

Materials and Methods

Cells and Media

The parental CHO-DG44 (NCCS, Pune) cells were cultured in Hams F12/DMEM (Life Technologies) supplemented with 10 % fetal bovine serum (FBS) (Life Technologies). CHO-DG44 stable clones were adapted to growth in suspension in CD-CHO-DG44 media (Life Technologies).

Transient Transfection

Cationic lipid-mediated transfections were performed using the reagent Lipofectamine 2000 (Invitrogen,

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Rockville, MD). Briefly, cells in mid-log phase were seeded at 4 × 10^5 cells/well in a 6-well plate. On the day of transfection, cells were washed with phosphate buffered saline (PBS) and 1.6 mL of fresh media was added. Lipofectamine 2000 (10 µL) and DNA (5 µg) were mixed in Opti-MEM media (400 µL) before being added to cells (1.6 mL) in a 6-well plate.

Stable Transfection

Stable transfection was carried out using electroporation. 0.8 mL of 1 × 10^7 cells/mL was transfected with 5 µg of linear plasmid. Transfected plates were washed with 5 mL of 1 X PBS and flasked with 15 mL of DMEM/10 % FBS. 24 hr post transfection, cells were selected with 1 g/mL of G418.

Amplification Using MTX Selection

G418 resistant, stable pools were selected with 10 nM MTX. Fifteen to twenty days post selection pools, clones were analyzed by FACS.

Replication Assay

Extra chromosomal DNA was isolated using a modified Hirt extraction method (13) at 48 hr post transfection. Hirt-extracted DNA was digested with D*pnI* (New England Biolabs, Beverly, MA). Digested DNA was then analyzed by PCR based replication assay according to the method (14).

Detection of EPO by Enzyme-Linked Immunosorbent Assay (ELISA)

EPO protein quantification was measured using a sandwich ELISA. Corning 96-well immune assay plates were coated with the capture antibody (R&D Systems, USA), allowing the detection of captured EPO, which was detected with goat anti-rabbit IgG antibody peroxidase conjugate (Sigma-Aldrich, India). Reactions were visualized by TMB substrate solution and absorbance of each well was measured at 450 nm.

SDS-PAGE

SDS-PAGE was performed according to the European pharmacopeia methodology by using the Bio-Rad vertical slab gel unit and gel documentation systems. Electrophoresis was carried out in 12 % hand cast gels and gels were silver stained (15).

Western Blot Analysis

EPO samples, subjected to SDS-PAGE, transferred to nitrocellulose membrane and stained with anti-EPO primary antibody (Rabbit anti human erythropoietin, 1:1000, Make: Sigma-Aldrich) at room temperature for overnight on a shaker. Removed the membrane from the primary antibody solution and washed three times with 0.1 % tween 20 (Make: Merck) solution for 15 min each time. After washing incubated the membrane in a secondary antibody solution (Anti rabbit IgG-Alkaline Phosphatase, antibody produced in goat, 1:5000 dilution, Make: Sigma-Aldrich) for 1 hr at room temperature on a shaker. Discarded the secondary antibody solution and washed the membrane with wash buffer for 45 min by changing the buffer every 15 min on shaker. Discarded the wash buffer and added 25 mL of color development solution and incubated the membrane till color develops in dark. Discarded the color development solution, washed the membrane with water for 5 min and dried the membrane (16).

Vectors used

pUB-D-C

DHFR from pSV2-DHFR (ATCC 37146) was PCR amplified and clone into pGEM-T Easy Vector (Invitorgen). Which was isolated by B*amHI* and B*glII* digestion of pGEM-T-DHFR was ligated to B*amHI* linearized pUB-C plasmid (Usha Biotech Ltd, Hyderabad).

pUB-PyOri-D-C

PyOri from pPyA3-1 (ATCC 45018) was PCR amplified and cloned into pGEM-T Easy Vector (Invitrogen). PyOri, which was isolated by B*amHI* digestion of pGEM-T-PyOri (Usha Biotech Ltd, Hyderabad) was ligated to B*amHI* linearized pUB-D-C vector.

pUB-D-C-GFP, pUB-PyOri-D-C-GFP

EGFP from pBOS-H2B-GFP (Invitrogen) was PCR amplified and cloned into pGEM-T Easy Vector. EGFP was digested from pGEM-T-GFP using *NotI* and *NheI* and ligated into pUB-D-C and pUB-PyOri-D-C vectors.

pUB-D-C-EPO, pUB-PyOri-D-C-EPO

Synthetic EPO Gene (accession no.: NM_000799) (Life Technologies, USA) was sub-cloned into pGEM-T Easy Vector. DNA encoding EPO was digested from pGEM-T-EPO and ligated into *KpnI* and *NotI* sites of pUB-D-C and pUB-PyOri-D-C vectors.

pUB-C-PyLT

PyLT from pPyLT-1 (ATCC 41043) was PCR amplified and cloned into pGEM-T Easy Vector. DNA encoding PyLT was digested from pGEM-T-PyLT and ligated into K*pnI* and N*otI* sites of pUB-C plasmid.

Flow Cytometry Analysis

All data presented were collected on a BD FACS caliber. Forward angle and side-scatter light gating were used to identify viable populations whilst doublets were excluded using forward angle and pulse-width scatter gating. EGFP emission (whose emission maxima occurs at 508 nm) was detected on FL4 using a 510/23-band pass filter. PMT voltages were adjusted to ensure autofluorescence associated with untransfected controls described a Gaussian distribution within the first log-decade. Analysis was maintained at an event rate not exceeding 600 cells per sec and a total of 20,000 events were acquired per sample.

Results

A Replicable and Amplifiable Expression System

Selection of high expression clone is a result of gene amplification due to stepwise increase in concentration of drug. During selection, cells tend to amplify genes (~50 kb fragment) surrounded by amplifiable selectable marker gene. In DHFR based amplification system, cells initiate replication in DHFR genes forming onion skin model (17). The gene at that state becomes instable leading to the formation of double minute (DM) chromosomes or reintegrate into adjacent or dif-

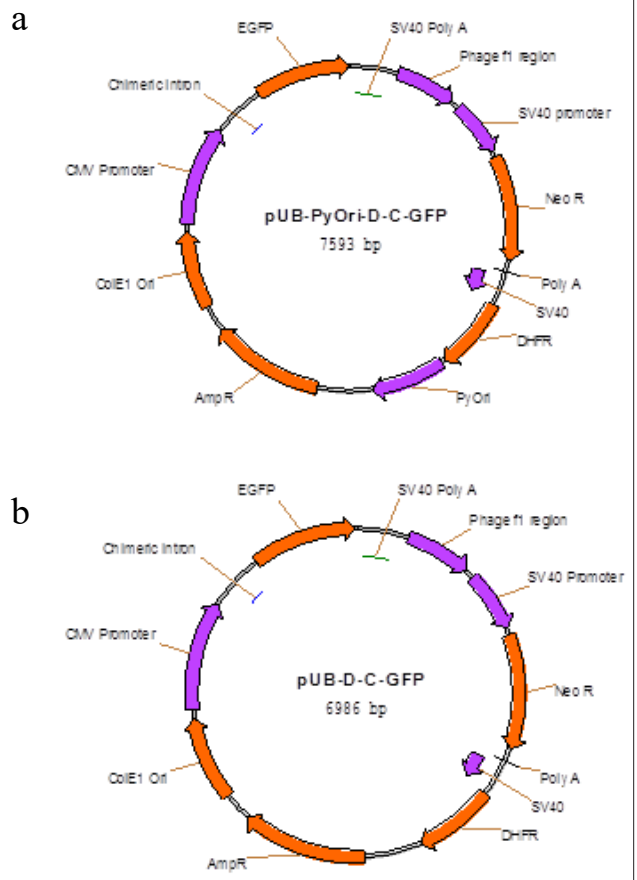

Figure 1. Plasmid Maps. **(a)** pUB-PyOri-D-C-GFP, It encodes for PyOri, DHFR, CMV Promoter and GFP and **(b)** pUB-D-C-GFP, It encodes for DHFR, CMV Promoter and GFP.

ferent regions of chromosomes. However, cell tends to carry out such amplification under stressed condition, which is a random event, making the process very inefficient and time taking process. To improve the process of gene amplification we have designed a hybrid stable expression system. To this end, we have constructed pUB-PyOri-D-C-GFP plasmid. The plasmid encodes for PyOri, DHFR and CMV promoter and GFP reporter protein. Replication of integrated plasmids requires (a) a viral origin of replication (PyOri), (b) the associated transacting protein to initiate DNA replication (PyLT) and (c) permissive host-specific factors and selection of amplified cells is carried out using DHFR/MTX.A control plasmid (pUB-D-C-GFP) was also constructed to compare the efficiency of pUB-PyOri-D-C-GFP.

pUB-PyOri-D-C-GFP (Fig.1a) and pUB-D-C-GFP (Fig.1b) vectors were constructed as described in methods. The functionality of PyOri was tested by Co-transfection of pUB-PyOri-D-C-GFP and pUB-C-PyLT into CHO-DG44 cells using lipofection method. Untransfected CHO-DG44 cells and CHO-DG44 cells transfected with pUB-D-C-GFP were used as controls. 48 hr post transfection extra chromosomal DNA was isolated by the Hirt extraction method and tested for replication of pUB-PyOri-D-C-GFP using PCR based replication assay (Fig. 2). The PCR based replication assay is based on amplification of D*pnI* resistance genes. Plasmids purified from dam$^+$ strains usually possess dam$^+$ methylation and is sensitive to D*pnI* digestion. Plasmids when replicated in eukaryotic cells loose dam$^+$ methylation and become resistant to D*pnI* digestion. From the Fig. 2, it was clear that PyOri is functional in

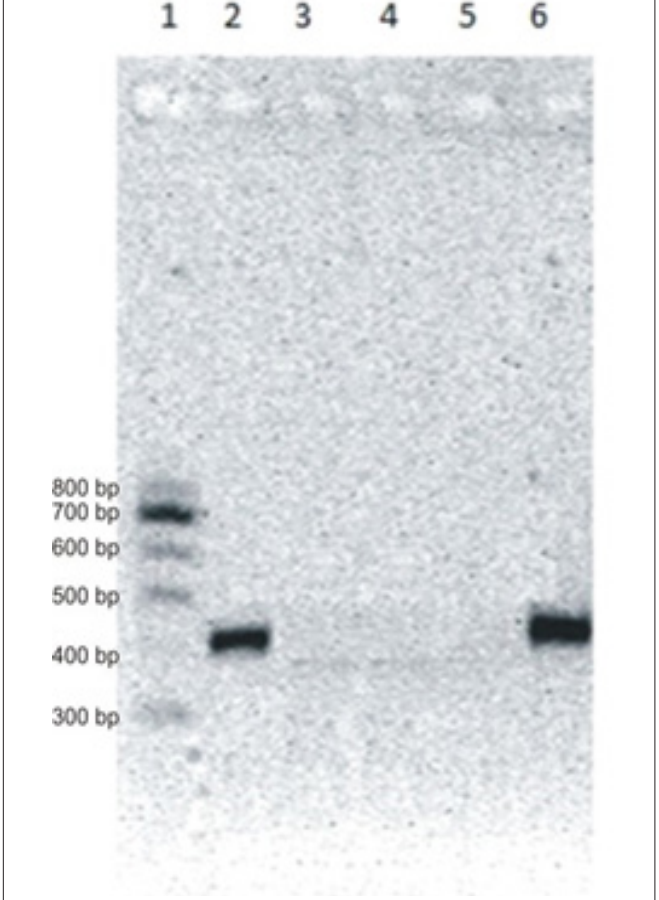

Figure 2. Replication Assay. CHO-DG44 cells were co-transfected with pUB-PyOri-D-C-GFP and pUB-C-PyLT in 6-well plates, 48 hr post transfection extra chromosomal DNA was isolated by the Hirt extraction method and tested for replication using PCR based replication assay. Lane 1: 100 bp Ladder, Lane 2: control for replicated plasmid, Lane 3: Untransfected CHO-DG44 cells, Lane 4: CHO-DG44 transfected with pUB-PyOri-D-C-GFP, Lane 5: CHO-DG44 transfected with pUB-D-C-GFP, Lane 6: CHO-DG44 co-transfected with pUB-PyOri-D-C-GFP and pUB-C-PyLT.

pUB-PyOri-D-C-GFP. This is proven by the presence of 400 bp amplification fragment in lane 6 in Fig. 2.

Rapid Amplification and Selection of High Expressing Cell Lines

DHFR based amplification often requires multiple rounds of selection and amplification, making it a time taking process. To reduce the time for cell line development, we developed a hybrid stable expression system (pUB-Py-D-C-GFP) which consists of Polyomavirus elements for initiation of replication for integrated plasmids and DHFR for selection of amplified cells using MTX.

For rapid amplification 5 µg of linear pUB-PyOri-D-C-GFP was transfected using electroporation to generate stable G418 resistant pool. pUB-D-C-GFP stable pool was also generated using similar protocol and is used as a control for the study. Stable pools were analyzed for GFP expression using FACS. G418 resistant pools generated using pUB-PyOri-D-C-GFP and pUB-D-C-GFP showed similar % GFP expressing cells and GFP Mean RFU (Fig. 3).

G418 resistant pools generated using pUB-PyOri-D-C-GFP and pUB-D-C-GFP was further tested for amplification by selecting cells with 10 nM MTX. However,

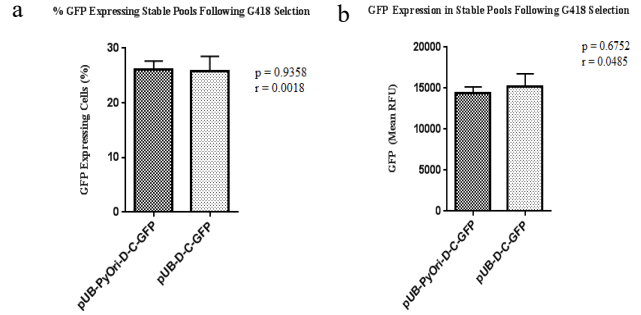

Figure 3. Comparison of GFP Expression in G418 resistant Stable Pools. Stable pools were generated Using pUB-PyOri-D-C-GFP and pUB-D-C-GFP: Five micrograms of pUB-PyOri-D-C-GFP and pUB-D-C-GFP plasmids were transfected into CHO-DG44 cells and selected with 1 mg/mL G418. Twenty day's post selection stable pool was analyzed for GFP expression using FACS. **(a)** % GFP expressing cells (p value is 0.9358, using independent samples t test,) and **(b)** GFP Mean RFU (p value is 0.6752, using an independent samples t test). Overall mean values are shown ± SEM for each group (n = 3).

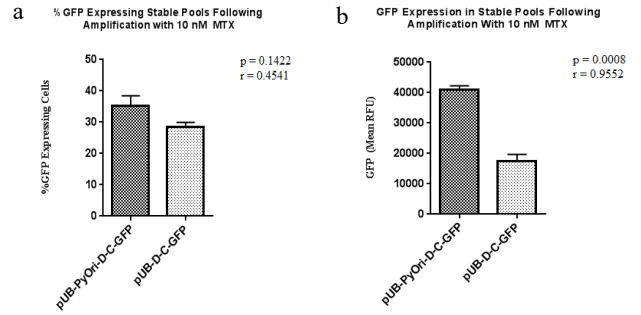

Figure 4. Comparison of GFP Expressing Stable Pools Following Amplification with 10 nM MTX. G418 resistant stable pool was generated using pUB-PyOri-D-C-GFP and pUB-D-C-GFP were selected with 10 nM MTX. Selection of pUB-PyOri-D-C-GFP stable pool was carried out in the presence of PyLT-Ag. Twenty days following selection, 10 nM MTX resistant stable pools were analyzed using FACS. **(a)** % GFP Expressing Stable Pool (p value is 0.1422, using independent samples t test,) and **(b)** GFP Mean RFU (p value is 0.0008, using an independent samples t test). Overall mean values are shown ± SEM for each group (n = 3).

MTX selection of pUB-PyOri-D-C-GFP was initiated after transient transfection of pUB-C-PyLT which transiently expresses and induces replication of integrated plasmids. Following selection, 10 nM MTX resistant stable pools were tested for GFP expression using FACS. pUB-PyOri-D-C-GFP showed 1.2 fold increase in % GFP expressing population (Fig.4a) and 2.3 fold increase in GFP Mean RFU (Fig. 4b) indicating that pUB-PyOri-D-C-GFP can amplify integrated plasmids without the need for a stepwise increase in concentrations of MTX.

Construction of High EPO Expressing Cell Line using Rapid Amplification System

The hybrid expression vector (pUB-PyOri-D-C) which was proven to show high expression at stable pool using GFP as a reporter protein was further studied for its use in therapeutic protein production using EPO as a model protein. pUB-PyOri-D-C-EPO and pUB-D-C-EPO were constructed as described in methods. pUB-PyOri-D-C-EPO and pUB-D-C-EPO were transiently transfected into CHO-DG44 cells using lipofection. Forty eight hours, post transfection supernatant was analyzed for EPO using both qualitative (SDS-PAGE (Fig. 5a), Western blot (Fig. 5b)) and quantitative (ELISA, (Fig.6)) methods. Both the methods confirmed the identification of EPO.

Stable expression studies were carried out by transfection of linear pUB-PyOri-D-C-EPO and pUB-D-C-EPO into CHO-DG44 cells. G418 resistant stable pools generate using pUB-PyOri-D-C-EPO was further selected with 10 nM MTX in the presence of PyLT. Twenty

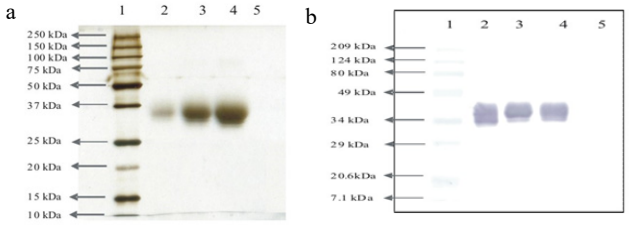

Figure 5. Characterization of EPO Using SDS-PAGE and Western Blot Analysis: pUB-PyOri-D-C-EPO and pUB-D-C-EPO were transiently transfected into CHO-DG44 cells in a 6-well plate. 48 hr post transfection EPO was analyzed by SDS-PAGE and Western Blotting. **(a) SDS-PAGE:** Culture supernatants were analyzed on 12 % acrylamide gel. Lane 1: protein marker, Lane 2: EPO standard, Lane 3: CHO-DG44 transiently transfected with pUB-PyOri-D-C-EPO, Lane 4: CHO-DG44 transiently transfected with pUB-D-C-EPO, Lane 5: Negative control (CHO-DG44 culture supernatant). **(b) Western Blot Analysis:** EPO samples analyzed on SDS-PAGE were transferred on transferred to nitrocellulose membrane and analyzed using goat anti-EPO antibody. Lane 1: Protein marker, Lane 2: EPO standard, Lane 3: CHO-DG44 transiently transfected with pUB-PyOri-D-C-EPO, Lane 4: CHO-DG44 transiently transfected with pUB-D-C-EPO, Lane 5: Negative control (CHO-DG44 culture supernatant).

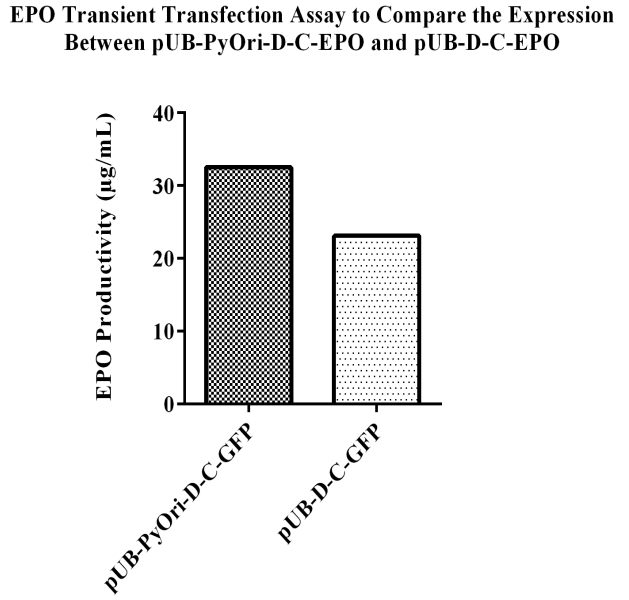

Figure 6. EPO Transient Transfection Assay to Compare the Expression between pUB-PyOri-D-C-EPO and pUB-D-C-EPO. pUB-PyOri-D-C-EPO and pUB-D-C-EPO were transiently transfected into CHO-DG44 cells in a 6-well plate. 48 hr post transfection EPO was analyzed by ELISA.

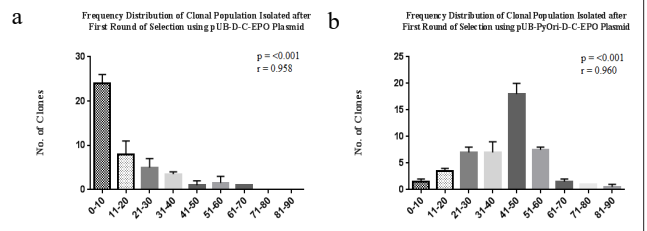

Figure 7. Frequency Distribution of EPO Producing Clones Following Selection with 10 nM MTX. CHO-DG44 cells were transfected with pUB-PyOri-D-C-EPO and pUB-D-C-EPO and selected with 1 mg/mL G418. pUB-PyOri-D-C-EPO stable pool was amplified with 10 nM MTX in the presence of transiently expressed PyLT antigen. pUB-D-C-EPO stable pools were also amplified with 10 nM MTX. Single clones from amplified pools were isolated by limiting dilution method. Clones were analyzed for EPO productivity in a 6-well plate. Frequency distributions of clones with varying productivity were as shown in Fig. **(a)** p value is <0.001, using ANOVA analysis and **(b)** p value is <0.001, using ANOVA analysis. (n = 2).

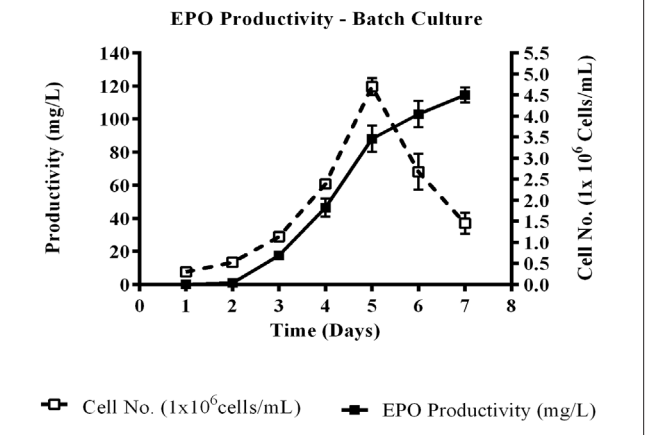

Figure 8. Batch Study of EPO Producing Clone Developed Using pUB-PyOri-D-C-EPO: Cells were seeded at 3×10^5 cells/mL and cultured for 7 days. A viable cell count was performed daily and culture supernatant was assayed for productivity on day 7 using EPO ELISA.

days following selection cells were plated by limiting dilution to isolate single clones. 10 nM MTX selection and clonal isolation were also carried out with pUB-D-C-EPO stable pool without PyLT. EPO productivity in 47 stable clones with CHO-DG44/pUB-PyOri-D-C-EPO and 48 stable clones with CHO-DG44/pUB-D-C-EPO were tested in 6-well plates. Data of which was shown in Fig. 7. From the Fig. 7, it was clear that pUB-PyOri-D-C-EPO gave rise to more number of high producing clones as compared to pUB-D-C-EPO. It is also clear that use of PyOri and PyLT in stable expression system can reduce the number of rounds that are required for development of high producing clones. The Clone (P2E8) with the highest productivity (89 mg/L) was tested for productivity in 250 mL shake flaks with a working volume of 100 mL.

Batch study was carried out in a 250 mL shake flask with a working volume of 100 mL. Cells were seeded at 3×10^5 cells/mL and cultured in batch mode for 7 days. Cell density and productivity were estimated every 24 hr (Data shown in Fig. 8). From the study it was clear that P2E8 maintained normal doubling times indicating that amplification and selection at 10 nM did not affect its doubling time. EPO productivity was found to be 119 mg/L.

Discussion

The results described in this report indicate that MTX-resistant stable high expressing CHO cells can be obtained within one round of selection/amplification. The rapid isolation of high expressing cells could be due to an increase in activity of DHFR, an increase in the rate of synthesis of this protein, an increase in the copy number of genes coding for this function. Thus the general properties of these CHO cells isolates are similar to the properties of high MTX-resistant murine (18) or other Chinese hamster Ovary Cells (19).

DHFR based gene amplification often involves several rounds of selection/amplification with a stepwise increase in the concentration of MTX. The stepwise increase in concentration of MTX is often preferred to avoid drug resistance mechanisms (20). During gene amplification cells tend to initiate replication in DHFR gene forming onion skin structures (21). These structures are quite unstable leading to the formation of double minute chromosomes, which remain as episomes or re-integrate into the chromosome forming tandem repeats (21). These natural amplification processes are very slow and inefficient.

This report describes a novel hybrid stable amplification system which brings in both replication and selection together. To this end, we have constructed pUB-PyOri-D-C plasmid which encodes for the Polyoma virus origin of replication, which helps in replication of integrated genes in the presence of PyLT and DHFR gene which helps in the selection of amplified cells. Polyoma virus is also known to fire replication several times during one cell cycle (22). This unique feature helps rapid amplification of integrated gene, thereby minimizing the number of rounds of selection amplification. Continuous replication of integrated hybrid plasmid can also lead to instability of amplified genes leading to loss of productivity during long term culturing. This is overcome by restricting Polyoma virus based replication for a very short period by transient transfection of PyLT plasmid.

Our results showed no difference in % GFP expressing cells and Mean GFP expression in the absence of PyLT. However, in the presence of PyLT pUB-PyOri-D-C-GFP showed a 1.2 increase in the % GFP expressing population and 2.3 fold increase in Mean GFP expression after 10 nM MTX selection. The increase in % GFP expression and Mean GFP could be due to increase in copy number due to gene amplification. EPO expression studies showed that pUB-PyOri-D-C-EPO resulted in more number of high expressing cells as compared to conventional expression vector pUB-D-C-EPO. Clone (P2E8) had given 119 mg/L productivity after scalability up to 100 mL shake flask (Batch study).

We developed a novel hybrid stable amplification system (pUB-PyOri-D-C). The expression system brings Polyomavirus elements (PyOri/PyLT) and DHFR together for rapid amplification of integrated genes. With the use of pUB-PyOri-D-C the number of rounds of selection/amplification required for genera-

tion of high expression clone was brought down to one. Making this system robust and efficient compared to any other DHFR based expression systems.

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