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# SEAP expression in transiently transfected mammalian cells grown in serum-free suspension culture

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## Abstract

A transient transfection process was established using a novel 'in-house' developed transfection reagent, Ro-1539. It allows rapid production of large quantities of various recombinant proteins. Here we describe the transient expression of the secreted human placental alkaline phosphatase (SEAP) by HEK293EBNA and CHO cells in serum-free suspension culture. Unexpectedly, high expression levels of SEAP (150 $\mu$g/ml) were found 3–4 days post-transfection when placental alkaline phosphatase (AP) was used as the reference enzyme. To confirm these data, an SDS–PAGE analysis was performed and the visible SEAP protein band (MW of 65 kDa) was compared with co-migrated purified placental AP protein as reference. The scanning analysis of the gel showed that SEAP, a truncated form of AP, has a higher specific activity than the purified placental AP. A correction factor was introduced permitting a direct comparison of placental AP activity with the expression levels of SEAP. Scale-up of the transfection system from spinner flask to bioreactor was simple and straightforward, resulting in similar yields of SEAP. Finally, the effectiveness of Ro-1539 was compared to that of other transfection reagents.

## Introduction

Viral and non-viral gene delivery systems in transient gene expression of recombinant proteins are of great importance (Boulikas 1998; Ledley 1995). The main advantage of these systems is that they speed up the process of producing milligram amounts of protein (1–100 mg). This is necessary in pharmaceutical research (rapid proof of concept) but it is also essential in genomics/proteomics (i.e., target validation). Additionally, it is often the only way to express 'difficult' proteins. Recently, a new generation of monoclonal antibodies – fully humanized antibodies – has been identified as potential therapeutics for a variety of diseases (Chadd and Chamow 2001). They can be generated using state of the art technologies such as transgenic mice or the phage display system. For both technologies, a highly efficient transfection system is needed to express milligram amounts of human antibodies for initial characterization and profiling (Davis et al. 1999).

Furthermore, transient gene expression has been successfully used in establishing cell-based high throughput screening assays (HTS) (Durocher et al. 2000; Mutel et al. 2000).

In addition to the type of delivery method, the choice of suitable host cell lines and optimal cell culture conditions are important factors for achieving high expression levels (Schlaeger 2000).

Non-viral gene delivery can be achieved using cationic lipids, polymers or peptides to form transfectable complexes with DNA (Baker et al. 2000). In the present work we used Ro-1539, a novel transfection reagent developed in-house, which is a peptide based compound (Schlaeger et al. 1999). This system is a two component mixture (A+B) exhibiting high synergism. The novel gene delivery system showed maximum expression using the optimal weight ratio of

DNA (0.1–0.2 $\mu$g/ml) and both Ro-1539 reagent components (A: 0.15 $\mu$g/ml, B: 0.5 $\mu$g/ml) (Schlaeger et al. 1999). By using Ro-1539, we were able to establish a simple, reproducible and scaleable transient gene delivery system with HEK293 cells. During the optimization of a large-scale transfection process we noticed the importance of cell culture conditions such as medium composition, growth characteristics and transfection protocol for obtaining high expression levels (Schlaeger 2000).

To assess the transfection efficiency and the expression levels of secreted recombinant proteins directly depending thereupon in more detail, we used the secreted form of the human placental alkaline phosphatase (SEAP) (Berger et al. 1988; Cullen and Malim 1992; Yoon et al. 1988). The glycosylated placental alkaline phosphatase (AP) is normally associated with the cellular membrane (Kain and Ganguly 1996). Human placental AP, a heat stable enzyme with resistance to L-homoarginine, hydrolyses a wide range of substrates (orthophosphoric monoesters) at alkaline pH (Cullen and Malim 1992).

SEAP is the truncated form of the human placental alkaline phosphatase in which the coding region for the membrane localization has been altered. It is one of the most sensitive and convenient reporter molecules available.

We used the inexpensive and highly sensitive multiwell-chemiluminescence enzyme assay to determine the amount of SEAP in the culture supernatant (Bronstein et al. 1994).

Here, we describe the transient expression of SEAP in serum-free HEK293EBNA and CHO suspension cultures using the novel transfection reagent Ro-1539. In addition, the scale-up potential of this transient production method was assessed.

## Materials and methods

### *Chemicals and expression vector*

The peptide component A of the reagent Ro-1539 (a modification of dioleylmelittin) was synthesized in-house and dissolved in acetonitrile/water (1:1). It is stable under $N_2$ at 5 °C for up to 1 month (Legendre et al. 1996; Padmaja et al. 1996). The cationic polymeric compound B was dissolved in water to a concentration of 0.9 mg/ml.

The pCMV/SEAP plasmid encoding the human placental alkaline phosphatase was purchased from Tropix. Inc., MA, USA. Plasmid preparation was performed using a commercially available kit (Nucleobond Ax, Machery-Nagel AG, Switzerland). The plasmid concentrations were determined spectrophotometrically and confirmed by agarose gel electrophoresis using pUC18 DNA as standard (Pharmacia Biotech, Zürich, Switzerland).

### *Cell culture*

Human embryonic kidney cells HEK293EBNA were adapted to serum-free growth in a calcium-reduced and fortified version of the original HL-medium (Schlaeger and Christensen 1999; Schumpp and Schlaeger 1990). The fortified version of the HL medium is used for many different human cell lines grown in adherent state as well as in suspension culture. CHO-dhfr$^-$ cells were cultured using serum-free DHI medium as described earlier (Schlaeger 1996). The cells were routinely grown in spinner flasks (Bellco, Inotech AG, Dotlikon, Switzerland) at an agitation rate of 105 rpm using about 70% of the recommended working volume. The large scale culture was performed in a 12-l bioreactor equipped with a draft tube and pitched blade stirrer (MBR, Zürich, Switzerland). Temperature, stirrer speed, and pH-value were maintained at 37 °C, 85 rpm, and 7.2, respectively. The dissolved oxygen level was maintained at 30% DO by sparging an oxygen–air mixture.

### *Transfection procedure*

For transfection experiments, cells were cultured to a density of 8–15×10e5 cells/ml, centrifuged for 2 min at 1200 rpm (280×*g*) Labofuge 400 Heraeus (Kendro, Switzerland) washed once with heparin-free HL medium, and resuspended in heparin-free HL medium as previously described (Schlaeger and Christensen 1999). The cell concentration was adjusted to 5.5–6× 10e5 cells/ml and the culture was incubated for at least 6 h before transfection took place. After addition of the transfection complexes, the cells were incubated for 1–5 days without medium replacement, but with addition of a concentrated feed solution containing amino acids, glucose, and vitamins on days 2, 3 and 4 as described earlier (Schlaeger 1996).

### *Preparation of transfection complexes*

The DNA complexes were formed in 1/10 of the

culture volume in HL medium at room temperature. Under optimized gene delivery conditions for HEK293EBNA cells, 0.2 $\mu$g DNA (quantity for 1 ml cell culture) was added to 0.1 ml of fresh medium and mixed gently. After 2 min 1 $\mu$l Ro-1539 was added and incubated for 15 min at room temperature. 0.1 ml of the transfection complexes were transferred to 1 ml of prepared cells, followed by gentle shaking and incubation at 37 °C. For CHO cells ($0.8\times10^6$ cells/ml), 0.5 $\mu$g DNA and 2 $\mu$l Ro-1539 were used. For each of the transfection reagents FuGENE 6 (Roche Diagnostics, Switzerland, Lipofectamin 2000 and DMRIE-C (Invitrogen, Switzerland) the optimal ratio of DNA to reagent was tested first. The final transfection experiments were performed using 1 $\mu$g DNA and 4 $\mu$l reagent. For the rest similar conditions as described for Ro-1539 were used.

*Product analysis*

After 3–4 days of incubation, culture supernatant from transfected cells (or from control cells) was centrifuged and the supernatant was frozen at −80 °C. The SEAP activity was quantitatively determined by using a commercially available chemiluminescence assay following the supplier's protocol (Roche Molecular Biochemicals Lucerne, Basel). The samples were not heat-treated. The assay is based on the use of the substrate CSPD [3-(4-methoxyspirol-[1,2-dioxetane-3,2(5′-chloro)-tricyclo(3.3.1.1. decane]-4-yl)phenyl phosphate] which is dephosphorylated by alkaline phosphatase. This results in an unstable dioxetane anion, which decomposes and emits light with its maximum activity at a wavelength of 477 nm.

The light signal was quantified in a TopCount™ Microplate Scintillation System (Canberra Packard S.A.) and was linear up to five orders of magnitude and proportional to the concentration of alkaline phosphatase. Some experiments were performed using the single tube measurement (Lumat LB9501, Berthold AG, Regensdorf, Switzerland). No different results between microplate and single tube measurement were obtained. In the case of quantification of SEAP, the reference enzyme was not pre-treated by heat as for the culture samples.

Gel electrophoresis was performed with 4–20% gradient SDS–PAGEs (Novex Electrophoresis GmbH, Frankfurt, Germany) and scanning of the gels was performed using the FluorChem 8000 Imager (Alpha Innotech, USA).

**Results and discussion**

In order to establish the highly sensitive SEAP chemiluminescence assay, we first prepared a calibration curve by diluting alkaline phosphatase (positive control in the assay kit) in the dilution buffer (Figure 1). In a second experiment, we studied the kinetics of the enzyme activity (reference AP) in order to assess the time window we could use to measure the light signal (Figure 1). The signal remained constant for at least 60 min. Endogenous alkaline phosphatases of mammalian cells are described to be heat-sensitive in contrast to the heat-stable human placental AP (Bronstein et al. 1994). In order to avoid an interference of SEAP activity with the endogenous AP, a heat inactivation step of the cell-free culture medium is recommended by the assay supplier. The culture supernatant from SEAP transfected HEK293EBNA cells were analyzed with and without heat inactivation (Figure 1). The kinetics clearly describe a heat-stable SEAP activity. Heat treatment of the samples did not greatly reduce the chemiluminescence signal, indicating low levels of endogenous AP. Thus a heat treatment of the samples was omitted.

Earlier transfection experiments to determine the optimum ratio of DNA to Ro-1539 showed that low amounts of the novel reagent and low DNA concentrations were needed to obtain high expression levels for various intracellular proteins (Schlaeger et al. 1999). However, we found also out previously that it was necessary to readjust the DNA: Ro-1539 ratio to achieve a successful and high expression of different recombinant proteins.

To determine the DNA concentration for optimal SEAP secretion we performed DNA dose response kinetics in the presence of low Ro-1539 concentration using HEK293EBNA cells in serum-free suspension culture. The results from the chemiluminescence enzyme assay are shown in Figure 2 as relative light units (RLU) per ml of culture supernatant. The maximum expression of SEAP activity was obtained at about 0.2 $\mu$g DNA/ml cells within 3 days post-transfection. This is similar to luciferase expression in these cells. Higher DNA concentrations together with increasing amounts of transfection reagent did not significantly enhance the expression of recombinant phosphatase activity (data not shown).

We next studied the time kinetics of the expression of SEAP activity into the culture supernatant using the same transfection conditions (0.2 $\mu$g DNA/ml cells and low concentration of Ro-1539 (1 $\mu$l)). Using

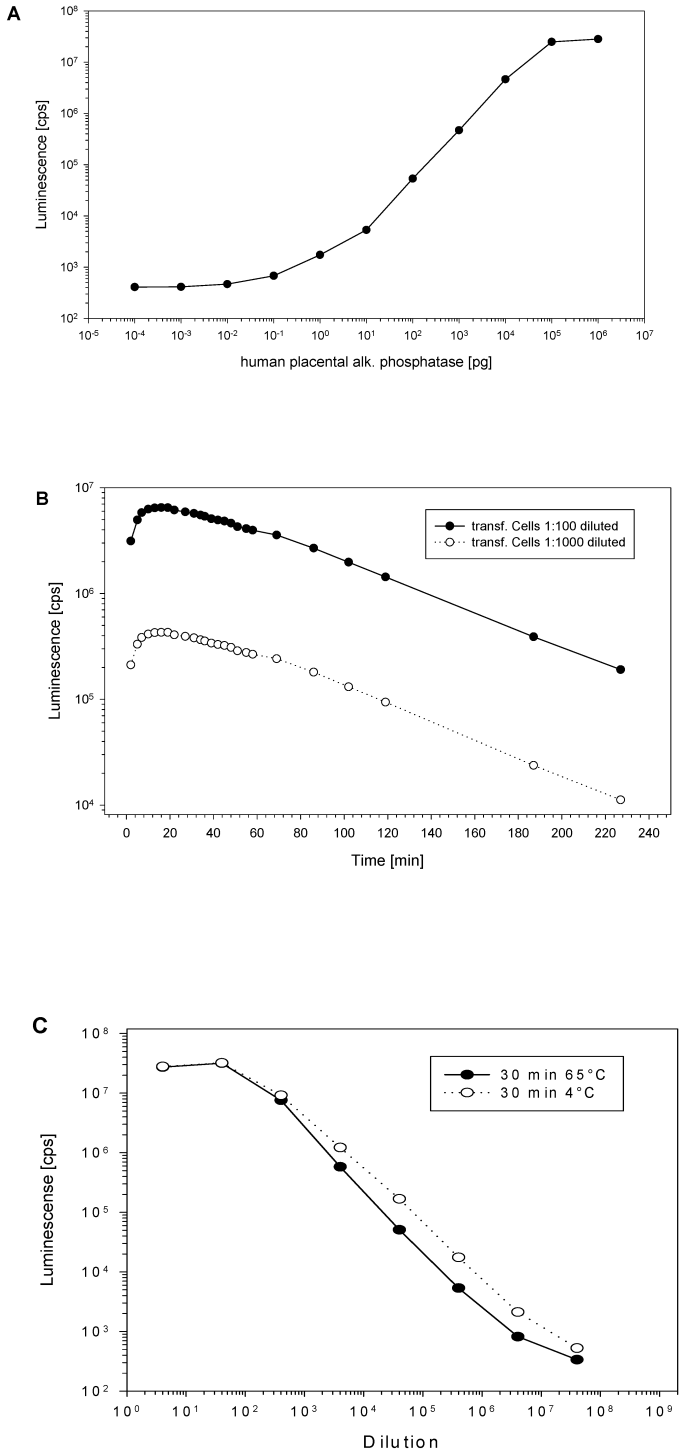


*Figure 1.* Characteristics of human alkaline phosphatase. (A) Human placental alkaline phosphatase calibration curve. (B) Kinetics of light reaction. (C) Heat inactivation curve.

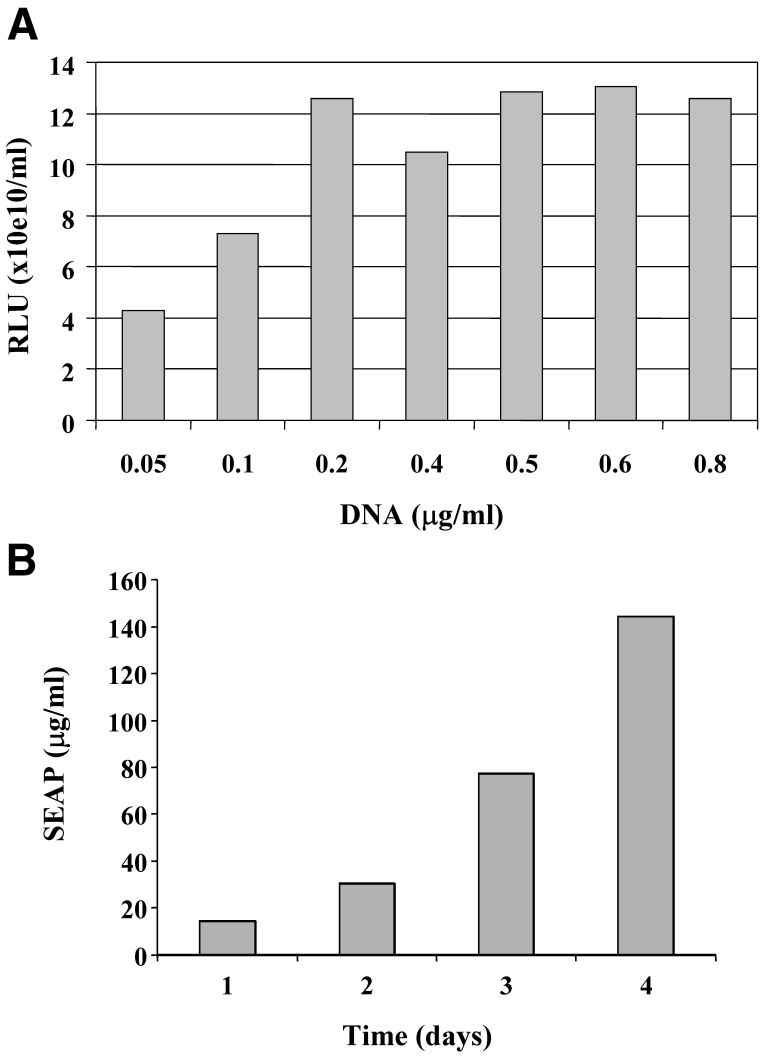


*Figure 2.* SEAP expression in HEK293EBNA. (A) DNA dose response: 15 ml HEK293EBNA cells ($5\times10^5$ cells/ml) were transfected at low Ro-1539 concentration (1 $\mu$l/ml cell suspension) with increasing amounts of DNA and incubated in spinner flasks 3 days post-transfection. SEAP was measured in the culture supernatants by the chemiluminescence SEAP reporter gene assay and expressed as relative light units (RLU) per ml culture. (B) Time kinetics of transient SEAP expression in HEK293EBNA cells. The cells were transfected as described for Figure 2A. The yield of the recombinant enzyme was expressed as $\mu$g SEAP per ml culture using the positive control from the assay kit as a reference enzyme.

human placental AP from the assay kit as a reference, we correlated the measured chemiluminescence signal of the enzyme activity with the amount of SEAP in the culture supernatant (Figure 2). Comparing the data with yields of other secreted recombinant proteins and also with the expression levels from stable producing HEK293 cells (e.g., 5–50 $\mu$g/ml), the produced amount of SEAP (at 4 days post-transfection) appears to be too high.

In order to confirm the expression levels of SEAP in transiently transfected HEK293EBNA cells we analyzed the culture supernatant from the experiment in Figure 2 using gel electrophoresis. We expected a strong Coomassie blue staining intensity, which should correlate with the corresponding amount of the co-migrating reference enzyme. However, we realized that the reference placental human alkaline phosphatase was stabilized by (protecting) proteins, which did not allow visualization of the corresponding band of the enzyme (data not shown).

Further SDS–PAGE analysis was performed, after obtaining BSA-free alkaline phosphatase purified

from human placenta from the SEAP assay kit supplier. The protein gel is shown in Figure 3. Serum-free HL culture medium contains transferrin and insulin as the only protein components. Only transferrin could be detected as the major band, which did not change in intensity during the 4 days post-transfection. Insulin is too small and the concentration in the culture medium is probably too low to render it visible. SEAP is the second dominant band in the gel and increased significantly from day 1 to day 4 post-transfection. It showed a weaker staining intensity than transferrin, the dominant band. From additional studies we know that the staining capacity with Coomassie of human transferrin is significantly higher compared to that of alkaline phosphatase.

The high level of enzyme activity determined by the chemiluminescence assay did not correspond to the Coomassie-blue stained recombinant SEAP protein band migrating at 65 kDa in the SDS gel. Therefore, we suggested that recombinant SEAP protein has a higher specific enzyme activity than placental AP enzyme.

The background staining of proteins with different molecular weights increased slightly at day 4. This was due to an increase of cellular proteins released from damaged and dead cells.

Scanning analysis of the stained gel was performed to further quantitate SEAP expression (FluorChem 8000 Imager).

The accumulated amount of recombinant SEAP in the supernatant within 4 days post-transfection was determined to be 22.6 $\mu$g per ml culture. The yield was approximately 7-fold lower than that found by the chemiluminescence assay (Figure 2). The average values obtained from several separately performed expression kinetics ranged between a factor of 6.5 and 7. A correction factor of 7 was used in all subsequent SEAP enzyme assays in order to obtain good agreement with the data obtained by SDS–PAGE analysis. It should be noted that using a truncated form of a protein can result in a modified activity: For example, the truncated form of interferon showed a significant higher antiviral activity than the original full-length protein (Doebeli et al. 1988).

Finally, we tested the efficiency of SEAP expression in HEK293EBNA and in CHO cells cultured in serum-free media in suspension comparing different commercially available transfection reagents.

First, we evaluated the optimal DNA:reagent ratio for the different transfection reagents in HEK293EBNA and CHO cells. During this evaluation, we measured the SEAP expression levels with the transfection reagents Ro-1539, DMRIE-C, Lipofectamin 2000 and FuGENE 6. The determined

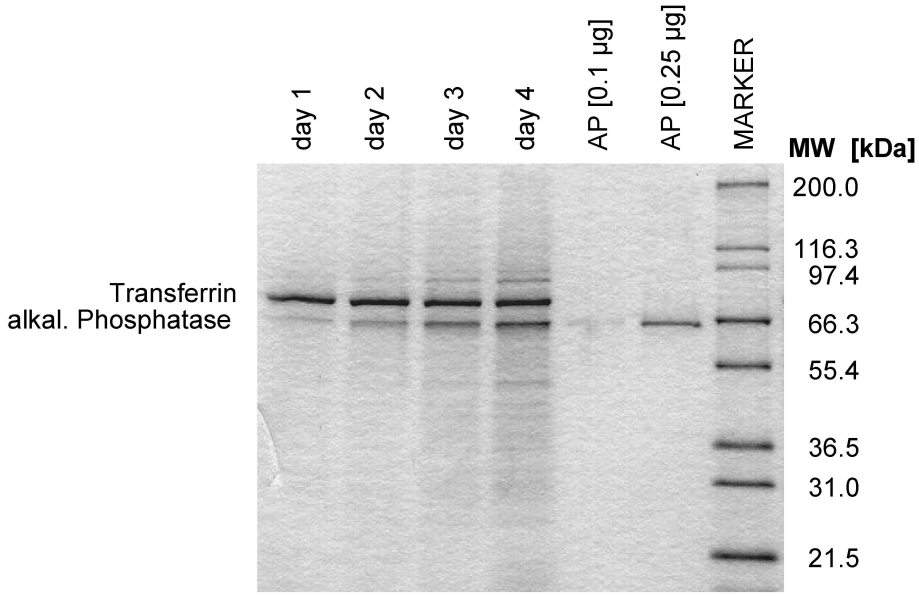


*Figure 3.* SDS–PAGE of culture supernatant from transfected HEK293EBNA cells. (A) Lanes 1–4: 15 $\mu$l supernatant from day 1 to 4 post-transfection. Lanes 5 and 6: Purified AP at 0.1 and 0.25 $\mu$g. Lane 7: MW protein marker.

SEAP activities were corrected by the correction factor 7 and plotted as $\mu$g/ml SEAP. Figure 4 shows the SEAP yields in HEK293EBNA cells and in CHO cells. Under our test conditions, the amount of SEAP protein produced in HEK293EBNA cells was significantly higher than in the rodent CHO cells (Figure 4). It is notable that a low DNA concentration (0.2 $\mu$g/ml) was necessary for HEK293EBNA cells only in the presence of Ro-1539. For all other transfection complexes 1 $\mu$g plasmid and 4 $\mu$l transfection reagent were used for 1 ml HEK293EBNA culture.

Our results showed that Ro-1539 and Lipofectamin 2000 are highly efficient transfection reagents. Both reagents showed comparable efficiency for plasmid delivery, however, cell toxicity was significantly lower when Ro-1539 than when Lipofectamin 2000 was used (microscopic observations). In SDS–PAGE analysis of the HEK293EBNA culture supernatant, a strong protein background staining was observed from the samples transfected with Lipofectamin 2000, thus supporting the microscopic observation data on Lipofectamin 2000 associated cell toxicity (not shown).

To demonstrate the scaling-up potential and further utility of this novel transfection system in suspension culture, we performed a transient SEAP expression experiment in a stirred bioreactor (12 l). HEK293EBNA cells grown in spinner culture were transferred to the bioreactor in heparin-free media. After 1 day of incubation, the cells were transfected

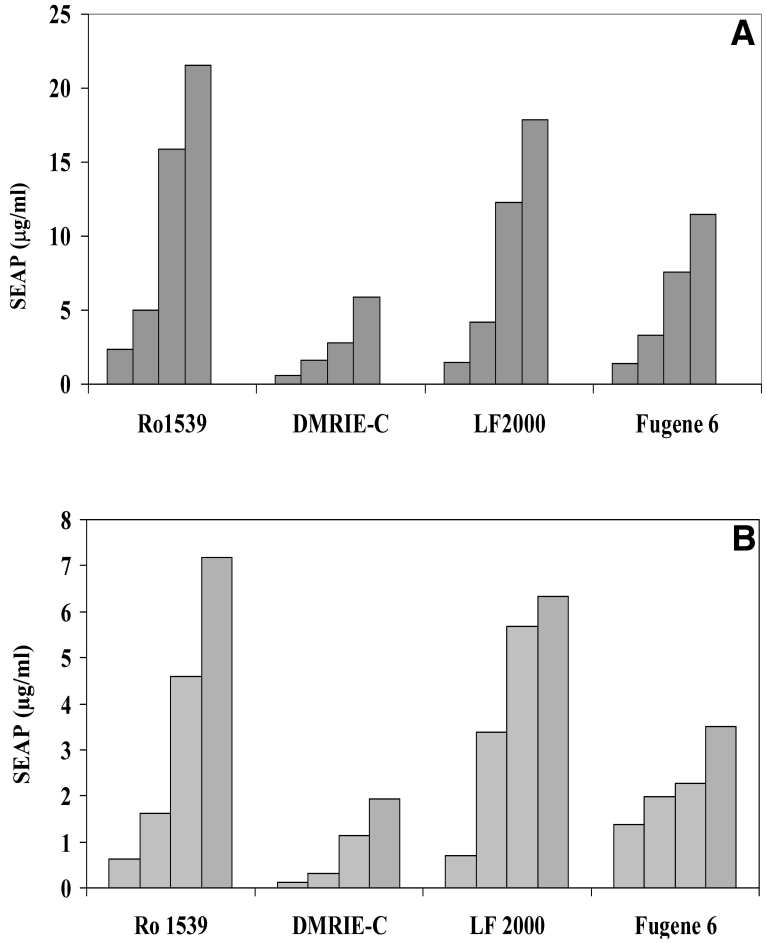


*Figure 4.* SEAP expression using different transfection reagents. The SEAP expression, which was corrected by the factor 7, was monitored from day 1 to 4 post-transfection. (A) HEK293EBNA: 15 ml cells were transfected with Ro-1539, DMRIE-C, Lipofectamin 2000 and FuGENE 6. Ro-1539 was used at 1 $\mu$l/0.2 $\mu$g DNA. All other transfection reagents were used at 4 $\mu$l/1 $\mu$g DNA. (B) CHO:15 ml cells were transfected using Ro-1539 at 2 $\mu$l/0.5 $\mu$g DNA. All other transfection reagents were used at 4 $\mu$l/1 $\mu$g DNA.

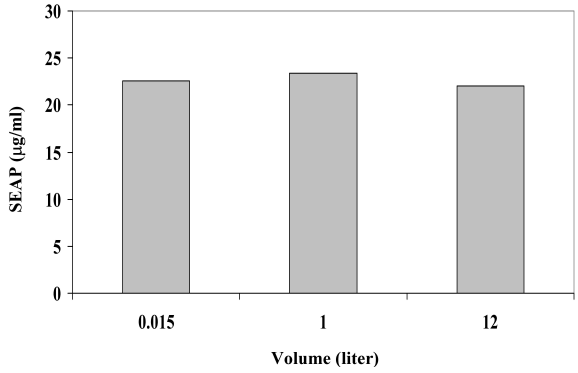


*Figure 5.* Comparison of SEAP expression in spinner flasks and in a 12-l bioreactor. HEK293EBNA cells were washed and transferred into a 12-l bioreactor at a cell density of $3\times10^5$ cells/ml. The cells doubled within 24 h. Before transfection 1 l of cell suspension (at $6\times10^5$ cells/ml) was taken and transferred into spinner flasks (0.015 l and 1 l). The cells in the fermentor and in the spinner culture were then transfected as described in Materials and methods. Three days post-transfection the SEAP activity was measured and corrected by the factor 7 to get its concentration in $\mu$g/ml.

by adding the DNA-Ro-1539 complexes to the bioreactor as described in Material and Methods. As controls, 15 ml and 1 liter cell culture from the bioreactor were transfected simultaneously and grown in spinner flasks. The SEAP activity was determined 3 days post-transfection using the enzyme assay and expressed after correction (as described above) as $\mu$g SEAP/ml cell culture supernatant. Figure 5 shows clearly that similar yields of SEAP can be obtained in small-scale spinner flasks and a 12-l bioreactor. Thus the transfection system with Ro-1539 is easy to use for small scale as well as for large-scale production.

Using the novel Ro-1539 transfection system other recombinant proteins, such as human antibodies can also be transiently expressed in both HEK293EBNA cells and CHO cells grown in liter scale as well as in a 12-l bioreactor (data not shown).

## Conclusions

The expression of SEAP protein in HEK293EBNA and CHO cells grown in serum-free suspension culture using the novel transfection reagent Ro-1539 has been described. We could show that the truncated form of the human placental alkaline phosphatase possesses a seven times higher specific activity than the full-length enzyme. Thus, to obtain the quantity of enzyme produced (in $\mu$g/ml), we introduced a correction factor of 7 using the unmodified AP enzyme as a reference.

Furthermore, in a comparative study with other transfection reagents Ro-1539 showed the best results with highest expression levels at low DNA amounts together with the highest percentage of viable cells for both, HEK293EBNA and CHO cells.

Using the determined correction factor, the average expression of SEAP in suspension culture within 3–4 days was determined to be between 20 and 30 $\mu$g/ml in HEK293EBNA and approximately 7–8 $\mu$g/ml in CHO cells.

Finally, the scale-up to a 12-l reactor scale of the transient Ro-1539 based transfection/expression system in HEK293EBNA cells was simple and straightforward.

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