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Optimization of Fermentation Conditions for Production of Recombinant Human Interleukin-2 in *Escherichia coli*

Lee, In Young, Myung Kuk Kim, Doe Sun Na, Kyung-Soo Hahm, Moon H. Han and Sun Bok Lee*

Genetic Engineering Center
Korea Advanced Institute of Science and Technology
P.O.Box 131, Cheongryang, Seoul 130-650, Korea

대장균에서의 재조합 인체 인터루킨-2 생산을 위한 발효조건 최적화

이인영·김명국·나도선·함경수·한문희·이선복*

한국과학기술원 유전공학센타

For optimal production of recombinant human interleukin-2 (IL-2) in *E. coli* the effect of fermentation conditions on cell growth, IL-2 production, and stability of recombinant cells were investigated. Among the complex nutrients tested in this work, yeast extract, peptone and corn steep liquor were found to be effective for recombinant cell growth. The recombinant cells were maintained stably under repression condition (30°C), but the stability of recombinant cells were drastically reduced upon induction of IL-2 expression (42°C) even under the selection pressure. Addition of antibiotics to the culture medium resulted in the cell growth inhibition without significant improvement in recombinant stability. When the expression of IL-2 gene was induced at different growth phases, highest IL-2 production was achieved by the induction of IL-2 at the middle-exponential growth phase. It was found that the production of IL-2 significantly inhibited the cell growth and the expression of other genes in the plasmid.

Interleukin-2 (IL-2) is an inducible lymphokine produced by T cells upon antigenic or mitogenic stimulation. Two distinct effector cell function, T cell cytotoxicity and natural killer activity, have been shown to be enhanced by IL-2. These actions establish the rationale for using IL-2 in the treatment of immunodeficient patients (1,2). Therefore, the human IL-2 is an attractive product for biotechnological research, and recently human IL-2 cDNA has been successfully cloned and expressed in *E. coli* at Genetic Engineering Center, KAIST (3,4).

While many reports on the cloning and expression of recombinant DNA (rDNA) have been published, studies on the fermentation process of recombinant cells are extremely limited. For successful development of rDNA gene products, however, not only rDNA technology but also bioprocess engineering technology is required. Although many had underestimated the importance of bioprocess technology during the early period of biotechnological development, it is now widely recognized the importance of biotechnology process engineering and the scarcity of related knowledge base.

In recombinant fermentation, basically the same process variables that are used in the traditional fermentation processes have to be considered. These include parameters related to media design and optimization, fermentor operation, and the

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*Corresponding author

state and control variables involved in the recombinant fermentation processes(5). When the recombinant fermentation process is to be optimized, however, both the microbiological process variables and fermentation process variables should be carefully studied and evaluated since both variables are highly interactive(5). In order to maximize gene product productivity, therefore, fermentation process variables have to be optimized depending on the characteristics of a given recombinant strain.

In view of this background, we have made an effort on the establishment of optimal fermentation conditions for the production of recombinant human IL-2 in *E. coli*. In this study, the effect of medium composition on cell growth and IL-2 production, the stability of recombinant cells with and without selection pressure, and the determination of optimal induction time have been investigated.

Materials and Methods

Recombinant plasmids

Plasmid pNK-2 was construted by inserting the coding sequence of native human IL-2 into the *Bam*HI site of pAS1 vector, as described elsewhere (3). Plasmid pNKM-21, which was obtained through a site-specific mutagenesis of plasmid pNK-2, produces IL-2 mutein in which the cysteine residue at amino acid 125 of native IL-2 has been replaced with serine.

Plasmids pILBR and pILBT were derived from pNK-2 in order to introduce Tc^r gene. Details of construction methods are described elsewhere (4). Restriction maps of the recombinant plasmids used in this study are shown in Fig. 1.

Escherichia coli M5248 (λ *bio 275 cI 857* Δ H1) was used as a host strain, which contains the cI 857 gene on its chromosome. Expression of recombinant human IL-2 is under control of the P_L promoter of bacteriophage lambda.

Culture medium and fermentation

Luria (LB) medium, composed of 10g yeast extract, 10g tryptone, and 10g NaCl per liter, was used for seed culture of recombinant cells. Fermentation (F) medium contains per liter: 50g glucose, 10g NH_4Cl, 1.2g sodium citrate, 1.0g $MgCl_2 \cdot 6H_2O$, 3.0g K_2HPO_4, 0.4g K_2SO_4, 15mg $FeCl_3 \cdot 6H_2O$, and 10m*l* of trace element solution (3μM MoO_3, 400μM H_3BO_3, 10μM $CuSO_4$, and 80μM $MnCl_2$, 80μM $ZnSO_4$). F medium supplemented with 1.0g yeast extract was designated as FYE 1 medium. Antibiotics such as ampicillin and tetracycline were added to the medium when required. Glucose and other medium components were sterilized separately at 121°C for 15 min and the initial pH of media was adjusted to 7.0 with 4N NaOH.

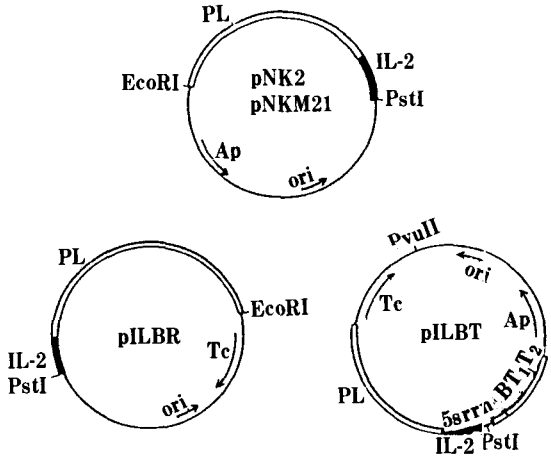

Fig. 1. Physical maps of the recombinant plasmids used in this study.

Jar fermentation of recombinant cells was carried out in a 5*l* fermentor (Shin-Young Engineering Co.) equipped with an DO analyzer and a pH controller. One-hundred m*l* of seed culture cultivated at 30°C for 16 hrs in shake flasks was transferred into a fermentor containing 1.9*l* of culture medium. Air flow rate and agitation speed were 1.5 vvm and 400 rpm, respectively.

The pH was controlled automatically at 7.0 with 4N NaOH. For IL-2 induction, the culture temperature was shifted from 30°C to 42°C at appropriate culture time. Fermentation in a jar fermentor was carried out in a fedbatch mode operation; when the concentration of glucose in the medium was reduced to about 10g/*l*, 30g glucose and 1.5g K_2HPO_4 was added intermitantly.

Analytical methods

Glucose concentration was determined by DNS method (6). Cell growth was monitored by measuring optical density (O.D.) of culture broth at 540 nm with a spectrophotometer (Spectronic 20 Baush & Lomb). Dry cell weight and the viability of recombinant cells were determined as described else-

where(4). Protein assay was followed by Lowry method(7).

Stability of recombinant cells was expressed as the ratio of plasmid-harboring cells to total recombinant cells and determined by replica-plating method. Colony isolates grown in LB agar plate were tooth-picked into LB agar plate containing antibiotics (ampicillin, tetracycline or ampicillin plus tetracycline). After incubating for two days at 30 °C, the fraction of plasmid-harboring cells was determined by counting the number of antibiotics-resistant cells.

Ampicillin concentration was determined by bioassay method using *Micrococcus luteus* 9341 as a test organism. The activity of β-lactamase during cultivation of recombinant cells was measured by the iodine-titration method (9).

The content of IL-2 was determined by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) according to the method of Laemmli (8). SDS-PAGE was carried out using 14% polyacrylamide gel. Protein band corresponding to IL-2 (M.W. 14.4Kd) was identified from SDS-PAGE of purified IL-2 and marker proteins of known size. Purification method of IL-2 and details of SDS-PAGE are described elsewhere (10). The fraction of IL-2 to total intracellular protein was determined by scanning of SDS-PAGE gels using a densitometer (Sebia, France).

Results and Discussion

Effect of fermentation medium

The optimization of fermentation medium is one of the most important tasks in the production of recombinant gene products. Fermentation medium for recombinant cells should provide the precusors and energies required not only for the host-cell metabolism but also the production of gene product.

The effect of various complex media on cell growth and IL-2 production was first examined in shake-flask culture using a recombinant strain *E. coli* M5248/pNK-2. Complex media such as yeast extract, malt extract, beef extract, bacto-peptone, casamino acid, casein, and corn steep liquor were added to F medium, which has been optimized previously for high-cell density culture of non-recombinant *E. coli* cells (11), and effective complex medium components were screened. Among the complex nutrients tested, yeast extract, peptone, and corn steep liquor were found to be very effective for IL-2 production, and therefore the effect of nutrient concentration was further examin-

Table 1. Effects of Medium Composition on IL-2 Producton[a]

Basal Medium	Additional Nutrient		Cell Conc. (OD)	IL-2 content (%)	Relative total IL-2 production level
	Medium Component	Concentration			
F	–	–	1.3	20.2	1.0
F	Yeast extract (YE)	2.0 g/l	2.6	19.4	1.9
F		5.0 g/l	3.1	16.4	1.9
F		10.0 g/l	4.0	15.4	2.3
F	Corn steep liquor (CSL)	2.0 ml/l	1.6	16.8	1.0
F		5.0 ml/l	2.2	17.8	1.5
F		10.0 ml/l	3.3	19.0	2.4
F	Peptone (PT)	2.0 g/l	2.2	19.9	1.7
F		5.0 g/l	3.0	20.4	2.3
F		10.0 g/l	3.4	20.7	2.7
LB	Glucose	5.0 g/l	2.8	20.9	2.2

(a) Interleukin-2 production was induced for 3 hrs by shifting the culture temperature to 42 °C after 4 hr cultivation of recombinant cells at 30 °C in shake flasks.

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As shown in Table 1, higher cell growth was achieved as the concentration of complex nutrients was increased while the intracellular IL-2 content was not significantly affected. Since total IL-2 production is determined by both recominant cell concentration in the fermentor and the IL-2 content in the cell when considered on the basis of culture volume, the productivity of IL-2 was found to be enhanced with increasing the concentration of complex nutrients.

When the recombinant cells are cultivated in a large-scale, less expensive medium is required and thus lower concentration of complex medium components would be desirable. In order to compare the efficiency of less nutritious medium with that of rich medium in the production of IL-2, recombinant cells were cultivated in a jar fermentor using LB medium (10g/*l* yeast extract, 10g/*l* tryptone) and FYE1 medium (F medium supplemented with 1.0g/*l* of yeast extract). As shown in Fig. 2, the rate of cell growth in LB medium was faster than that in FYE1 medium. But the maximum cell concentration and the intracellular IL-2 content were almost the same in both cases. Since total IL-2 production level in FYE1 medium was not significantly different from that observed in LB medium (Fig. 2), less expensive FYE1 medium was chosen as production medium of IL-2. It should be mentioned,

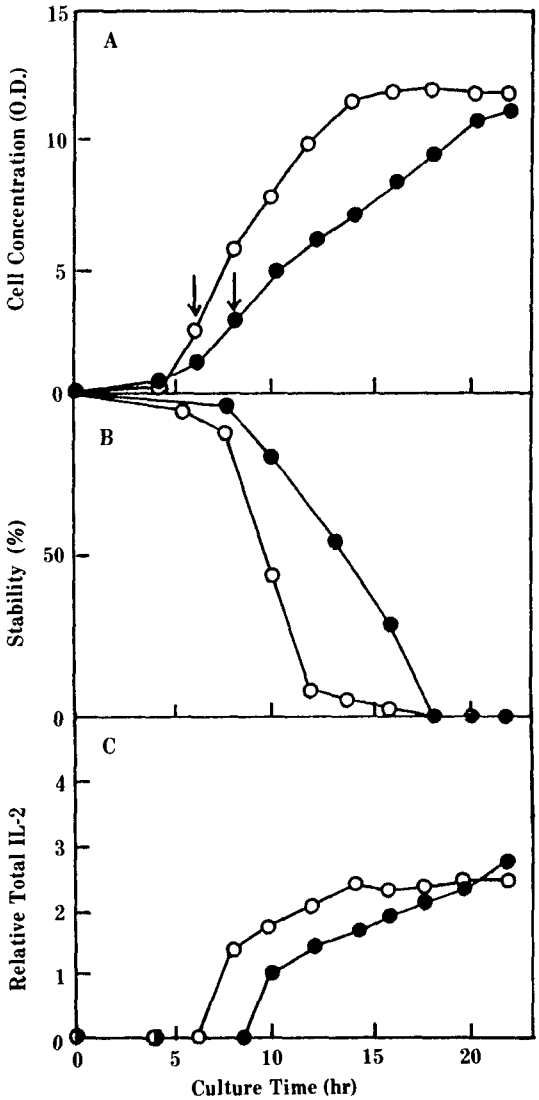

Fig. 2. Effect of culture medium on (A) cell growth, (B) recombinant stability and (C) total IL-2 production in a jar fermentor. (●) FYE1 medium. (○) LB medium.
Arrows in (A) indicate the induction time.

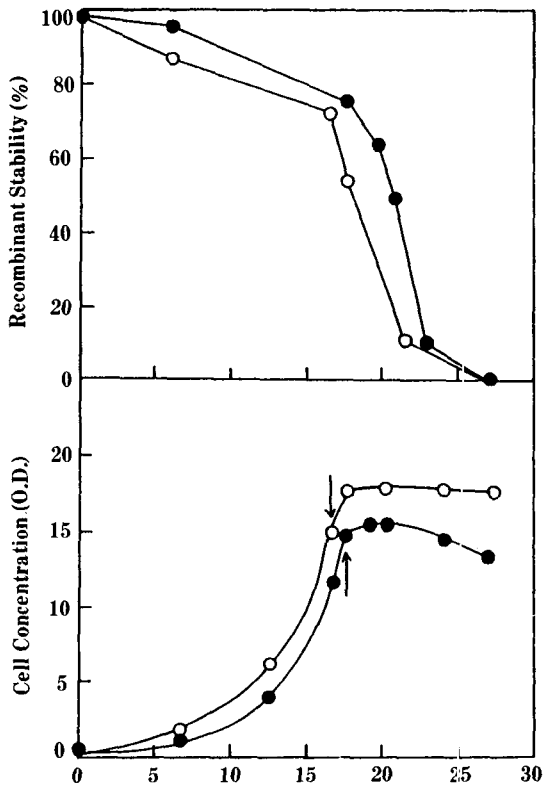

Fig. 3. Effect of selection pressure on stability and cell growth. (○) no selection pressure; (●) in the presence of ampicillin (100mg/*l*).
Arrows indicate the induction time.

however, that the productivity of IL-2 is higher in LB medium than in FYE1 medium.

Stability of recombinant cell

Among a number of methods employed for stable maintenance of recombinant cells(5), antibiotics are widely used to prevent the growth of the plasmid-free cells because of thier convenience.

With a recombinant cell *E. coli* M5248/pNK-2, which carries an ampicillin-resistance gene in the plasmids, the recombinant stability during inoculum preparation stage was examined with and without addition of ampicillin to the culture medium. In the presence of 50 mg/*l* of ampicillin more than 95% of recombinant cells showed ampicillin resistancies after cultivation of 15 hr in shake flasks. In the absencee of selection pressure, however, the stability of recombinant cells was about 75%.

In order to further examine the effect of selection pressure on the stability of recombinant cells under IL-2 production condition, *E. coli* M5248/pNK-2 was cultivated in FYE1 medium with and without addition of ampicillin (100 mg/*l*). For IL-2 induction, in this experiment, culture temperature was shifted to 42°C after cultivation of recombinant cells at 30°C. As shown in Fig. 3, the plasmids were rapidly lost upon induction of IL-2 even in the presence of ampicillin. (Ampicillin was hardly detected in the culture broth after logarithmic growth phase, which implies that the degradation by β-lactamase encoded in the plasmid is significant). Fig. 3 also indicates that ampicillin adversely affects the growth of recombinant cells.

Since ampicillin could not prevent the formation of plasmid-free cells effectively under expression conditions, we further examine the efficiency of tetracycline as selective pressure using recombinant plasmids pILBR and pILBT. The addition of tetracycline, however, significantly reduced the cell growth with marginal improvement in recombinant stability (Table 2). Total IL-2 production of pILBR and pILBT was lower than that of pNK-2 (data not

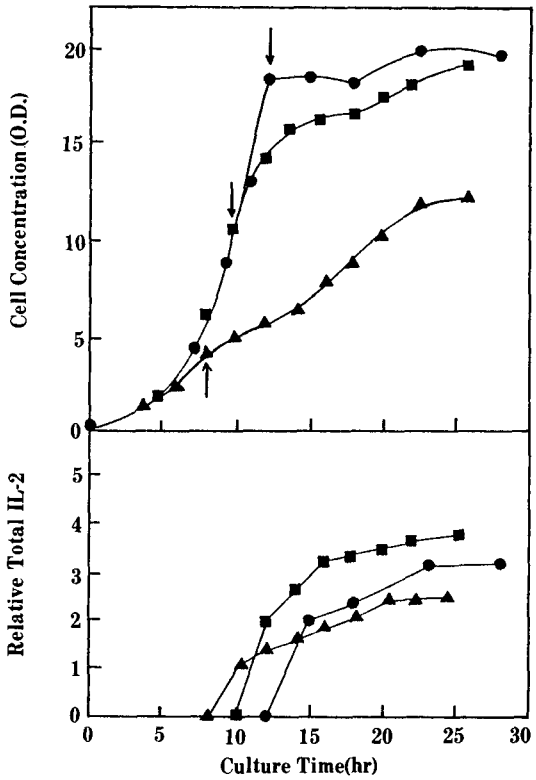

Fig. 4. Effect of induction time on cell growth and total IL-2 production. (▲) early induction; (■) middle induction; (●) late induction.
Arrows indicate the induction time.

Table 2. Effect of Selection Pressure on Cell Growth and Stability of Recombinant Cells[a]

Plasmids	Culture Time (hr)	In the absence of selection pressure		In the presence of selection pressure[b]	
		cell conc. (O.D)	Stability (%)	cell conc. (O.D)	Stability (%)
pILBR	0	0.06	99	0.06	99
	4	1.58	84	0.30	94
	10	2.98	0	0.38	16
pILBT	0	0.06	96	0.06	96
	4	1.85	92	0.73	96
	10	3.15	23	1.25	36

[a] Culture temperature was shifted to 42°C after cultivation of recombinant cells at 30° for 4 hr in shake flasks.
[b] Selection pressure: 15 μg/ml tetracycline (pILBR) or 15 μg/ml tetracycline + 50 μg/ml ampicillin (pILBT)

shown) because of the severe growth inhibition by tetracycline.

Effect of induction time

Determination of an optimal induction time is important in optimizing the recombinant fermentation process. Since the plasmids are rapidly lost during the IL-2 production phase as mentioned earlier, induction of IL-2 gene expression should be done after cultivation of recombinant cells for an appropriate period under repressed state.

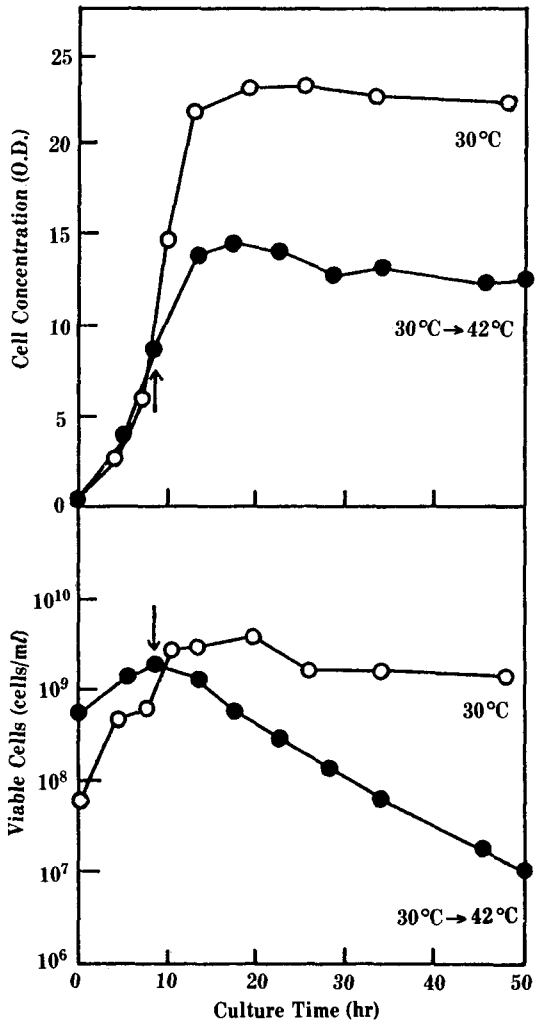

Fig. 5. Effect of IL-2 production on the growth and viability of recombinant cells.

E. coli M5248/pNKM-21 was cultivated either at constant temperature (30 °C) without induction (○) or with a temperature shift (30 °C→42 °C) at the time indicated by arrows (●).

To examine the effect of induction time on cell growth and IL-2 production, the logarithmic growth phase was subdivided into early-, middle-, and late- logarithmic phase based on the growth curve of recombinant cells at 30 °C, and the expression of IL-2 gene was induced at three different growth states. Experimental results shown in Fig. 4 indicate that total IL-2 production level (determined from protein concentration and IL-2 content) is highest when IL-2 production is induced at the middle-logarithmic growth phase.

Recently Botterman *et al.* (12) have shown that the induction of gene expression during the logarithmic phase for production of the EcoRI restriction enzyme resulted in five-fold increase in productivity as compared to that of late induction. Our experimental results shown in Fig. 4 are consitent with there observations.

Effect of IL-2 production on cell growth and β-lactamase activity

In general, the growth rate of recombinants is adversely affected by the expression of rDNA which causes stress on the metabolism of the host organism(5). Exceptions to this general rule have also been found. In the case of T4 DNA ligase production, for example, a slight increase in growth rate was observed after the gene is expressed through a temperature shift (13).

To examine the effect of IL-2 production on cell

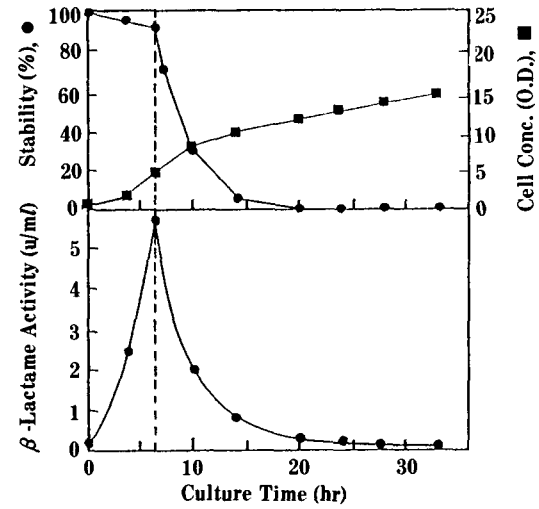

Fig. 6. Effect of IL-2 induction on cell growth, recombinant stability and β-lactamase activity.

Induction time was shown as a broken line.

growth, optical density of culture broth and the viability of recombinant cells were measured during cultivation of *E. coli* M5248/pNKM-21. As shwon in Fig. 5, induction of IL-2 expression severely inhibited the growth of recombinant cells. The decrease in cell viability during IL-2 production phase was even more profound as compared with the cells grown under repressed state throughout the fermentation period. Overproduction of IL-2 also affects the expression of other genes in the plasmid. Fig. 6 shows that the activities of β-lactamase in plasmid pNKM-21 are significantly decreased upon induction of IL-2. It was also observed that the degree of inhibition of β-lactamase synthesis is higher as IL-2 production levels are increased (data not shown).

요 약

대장균을 이용한 재조합 인체 인터루킨-2(IL-2) 생산의 최적화를 위하여 발효조건이 세포성장과 IL-2의 생산 및 재조합 세포의 안정성 등에 미치는 영향을 조사하였다. 복합배지의 경우에는 yeast extract, peptone, corn steep liquor 등이 재조합 세포성장에 좋은 효과를 나타내었다. 재조합 세포의 안정성은 IL-2 발현 억제 조건(30℃)에서는 안정하게 유지되었으나 IL-2 생산 조건(42℃)에서는 selection pressure를 주었을 때 조차도 현격하게 감소함을 알 수 있었다. 한편 배양배지에 항생제를 첨가한 경우에도 안정성 유지에는 별로 도움이 되지 못하고 세포성장만 억제됨을 알 수 있었다.

유전자의 발현은 대수 증식 중기에서 유도했을 때가 IL-2 생산에 가장 좋은 것으로 나타났으며 IL-2 생산은 세포성장과 플라스미르내의 다른 유전자들의 발현을 상당히 저해시킴을 알 수 있었다.

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