Effect of induction strategy on the expression of different recombinant protein synthesized in Escherichia coli under the control of tryptophan promoter

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Efecto de la estrategia de inducción en la expresión de diferentes proteínas recombinantes sintetizadas en Escherichia coli bajo el control del promotor triptófano

Efecte de l'estratègia d'inducció en l'expressió de diferents proteïnes recombinants sintetitzades en Escherichia coli sota el control del promotor triptòfan

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SUMMARY

The proteins gag-24 (HIV-1), and a representative region of the gp-41 (HIV-1), gp-36 (HIV-2), and gp-120 (HIV-1) were expressed in *Escherichia coli* under control of the tryptophan promoter.
The influence of the induction conditions on cellular growth and protein expression were evaluated, and taken into account for establishing the final culture conditions. Different concentration of 3-ß Indoleacrylic Acid (IA between 0 to 60 μg/ml) and tryptophan (from 0 to 100 μg/ml) on growth and expression level, was evaluated for the four expressed recombinant proteins, using the same plasmid, culture medium, and growth conditions at shake flasks level.
The expression level achieved for each recombinant protein depended on the induction conditions.
The results suggest that there is not a predetermined rule about the induction conditions to achieve high level expression of recombinant proteins expressed under tryptophan promoter. Finally we decided to carry out the production of the proteins by derepresion of the expression system, without the use of tryptophan in the cultivation medium. The levels of expression obtained with these clones were higher than 15% for each one of the tested proteins. These results are very superior to those reported previously for the several proteins of the HIV. These procedures could be also applied to evaluate the expression of other recombinant proteins expressed in *E. coli* under the control of tryptophan promoter.

Key words: *E.coli*, 3-ß Indoleacrylic Acid, Induction strategy, Recombinant proteins, Tryptophan promoter.

RESUMEN

Las proteínas gag-24 (VIH-1) y una región representativa de la gp-41 (VIH-1), gp-36 (VIH-2) y gp-120 (VIH-1) fueron expresadas en *Escherichia coli* bajo control del promotor del triptófano.
La influencia de las condiciones de inducción en el crecimiento celular y la expresión de las proteínas fueron evaluadas y tomadas en cuenta para el establecimiento de las condiciones de cultivo final. Se evaluaron diferentes concentraciones de ácido 3-ß Indolacrílico (entre 0 a 60 μg/ml) y triptófano (de 0 a 100 μg/ml) sobre el nivel de crecimiento y de expresión para las cuatro proteínas recombinantes expresadas, utilizando en todos los casos las mismas condiciones (plasmídio, medio de cultivo y condiciones de crecimiento) a nivel de zaranda.
El nivel de expresión alcanzado para cada proteína recombinante dependió de las condiciones de inducción.
Los resultados sugieren que no existe una regla predeterminada sobre las condiciones de inducción para alcanzar altos niveles de expresión de las proteínas recombinantes expresadas bajo el promotor del triptófano. Finalmente decidimos llevar a cabo la producción de las proteínas por derepresión del sistema de expresión, sin el uso de triptófano en el medio de cultivo. Los niveles de expresión obtenidos con estos clones fueron superiores a 15% para cada una de las proteínas evaluadas. Estos resultados son muy superiores a los reportados previamente para las proteínas del VIH. Estos procedimientos también podrían aplicarse para evaluar la expresión de otras proteínas recombinantes expresada en *E. coli* bajo el control del promotor triptófano.

Palabras clave: *E. coli*; ácido 3-ß indolacrílico; estrategia de inducción; proteínas recombinantes; promotor triptófano.

RESUM

Les proteïnes gag-24 (VIH-1) i una regió representativa de la gp-41 (VIH-1), gp-36 (VIH-2) i gp-120 (VIH-1) van ser

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expressades en *Escherichia coli* sota control del promotor del triptòfan.
La influència de les condicions d'inducció en el creixement cel·lular i l'expressió de les proteïnes van ser avaluades i considerades per a l'establiment de les condicions del conreu final. Es van avaluar diferents concentracions d'àcid 3-ß Indolacrílic (entre 0 i 60 μg/ml) i del triptòfan (entre 0 i 100 μg/ml) en relació al nivell de creixement i d'expressió per a les quatre proteïnes recombinants expressades, utilitzant en tots els casos les mateixes condicions (plasmidi, medi de cultiu i condicions de creixement) a nivell de garbell.
El nivell d'expressió assolit per a cada proteïna recombinant depenia de les condicions d'inducció.
Els resultats suggereixen que no hi ha una regla per defecte sobre les condicions d'inducció per assolir alts nivells d'expressió de les proteïnes recombinants expressades sota el promotor del triptòfan. Finalment vam decidir dur a terme la producció de proteïnes per repressió del sistema d'expressió, sense l'ús de triptòfan en el medi de cultiu. Els nivells d'expressió obtinguts amb aquests clons van ser superiors al 15% per a cadascuna de les proteïnes avaluades. Aquests resultats són molt superiors als reportats prèviament per les proteïnes del VIH. Aquests procediments també es podrien aplicar per avaluar l'expressió d'altres proteïnes recombinants expressada en E. coli sota el control del promotor triptòfan.

Paraules clau: *E. coli*; àcid 3-ß indolacrílic; estratègia d'inducció; proteïnes recombinants; promotor triptòfan.

INTRODUCTION

Achieving high expression levels of target protein is one of the main objectives in the cultivation of recombinant cells. This objective may be accomplished through to two complementary approaches, namely, genetic engineering and optimal induction. However, combined effects (medium composition, induction strategy, etc), that result in optimal expression or product stability, may not be predictable based upon host strain type or plasmid construction and may be achieved using widely differing fermentation conditions.
Regulated promoters are most commonly used to allow control of the cloned-gene expression level by manipulation of environmental parameters. Different ways of inducing the expression of heterologous proteins cloned in *E.coli* under the ptrp system have been reported in the specialized literature. The most commonly used method is the use of competitive agents analogous to tryptophan, such as of 3-ß Indoleacrylic Acid (IA) and Indolyl-3-propionic acid (1, 2). The ptrp promoter can also be induced by derepression when the tryptophan present in the culture medium is depleted during the fermentation process (3).
The trp promoter (tryptophan) is a strong and widely used. It is negatively regulated by repressor trp protein complex and the derrepresion is produced by the absence of tryptophan in the culture medium or the addition of an inductor, 3 β-Indoleacrylic acid (4).
The tryptophan synthesis in bacteria such as *E. coli* is primarily controlled through feedback inhibitions of the end product tryptophan on the enzymes at metabolic level and repression of the trp operon at transcriptional level. Previous studies revealed that termination of the operon transcription by attenuation also occurs at a lower intracellular concentration of tryptophan (5). Attenuation is the conjunction of processes of transcription and translation and brings about a finer control for the regulation of transcription in the tryptophan biosynthetic pathway. It is a tryptophan-sensitive timing mechanism that determines whether transcription is attenuated or allowed to continue. The trp operon attenuation is defined by the concentrations of charged tryptophan tRNA (Trp-tRNATrp). When the tryptophan concentration is high, the concentration of Trp-tRNATrp is also high. Translation will follow closely on the heels of transcription, proceeding rapidly past the Trp codons; the attenuator structure is formed and transcription is halted. When tryptophan concentrations are low, however, the ribosome stalls at the two Trp codons because charged tRNATrp is unavailable; a base paired structure is formed and prevents attenuation; transcription continues to proceed but translation stops as a result of a lack of tryptophan (6). The joint action of these three regulation mechanisms including feedback inhibition, repression and attenuation is believed to give rise to complicated dynamic behaviors such as oscillations (7).
In some genetic constructions used for the expression of recombinant proteins under the promoter trp it is necessary to add 3-ß Indoleacrylic Acid for the induction of the promoter. This inductor competes with the present tryptophan for the repressor and the complex oppressor-indoleacrylic is not recognized by the operator, which will increase the transcription of the gene (8).
Several heterologous proteins have been cloned under the control of tryptophan promoter: Human Epidermal Growth Factor (1), ß-Galactosidase (9), Calf Chymosin (10), but in very few reports, the authors studied the effect of the induction conditions on the cellular growth and the protein expression kinetics (11).
In this report, the effect of the induction conditions (IA and tryptophan concentration) on the production of four different recombinant proteins expressed in *E. coli* under the control of tryptophan promoter, using the same plasmid, culture medium, and growth conditions were studied.

MATERIALS AND METHODS

E coli host strains and expression plasmid
The following *E. coli* recombinant strains were used: HB-101 (pHIVCA-1), W-3110 (pHIVTA-1), C-600 (pHIVTA-2), and W-3110 (pIT 120) (12). These strains have been used for the expression of gag-24 (HIV-1), and a representative region of the gp-41 (HIV-1), gp-36 (HIV-2) (13), and gp-120 (HIV-1) proteins (14), under the control of the tryptophan promoter. The pFP15 was used as basal plasmid to all constructions (13).
Media composition and culture conditions
Cultures were performed in shake flasks (G-25, New Brunswick Scientific Co, INC, E.U.), using M9 medium (15) supplemented with glucose 20 g/*l*, casein hydrolysate 10 g/l, and ampicillin 50 μg/ml. Cultivations were performed at a temperature of 37°C and stirrer speed of 250 rpm. The pH was not controlled and culture time was 12 hours. The effect of IA concentration was determined by adding 20 μg/ml of tryptophan to the culture medium, and different concentrations of IA (between 0 to 60 μg/ml) were added after 1hour of cultivation. The influence of the initial tryptophan concentrations (from 0 to 100 μg/ml) on protein

expression was studied in the absence of IA in the culture medium.

Analytical methods

Cell growth was determined by measuring optical density at 530 nm using a spectrophotometer GENESYS 10UV (Electron Corporation, USA).

The total protein concentration present in whole cell extracts was determined by the Lowry and collaborators method using as pattern protein albumin from bovine serum (Merck) (16).

Polyacrylamide gel electrophoresis was performed by the method described by Laemmli (1970) (17). In all cases were applied 20 ug of total protein in each line of electrophoresis. The samples were processed in the buffer Tris-HCl 125 mM pH 6.8, 4% (w/v) SDS, glycerol 20% (w/v), 125 mM DTT, to 100°C for 15 minutes and were applied in a polyacrylamide gel 15% finally stained with a solution of Coomassie Blue. As patterns of molecular weight were used: phosphorylase b (97 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (21 kDa) and lysozyme (14 kDa).

In all cases expression level (%) of each protein present in whole cell extracts was determined by densitometric analysis of SDS-PAGE using a Densitometer GS-800, BioRad (SoftWare ¨Quantity One 4.6.5¨, The Discoverys Series).

In all cases expression level (%) of each protein was determined by comparing the percentage that represents the band-specific of the recombinant protein expressed in each case with the total of proteins (100%) detected by the Densitometer in each line of electrophoresis.

All reagents used in the preparation of the solutions for the different processes, were of quality for analysis.

In all experiments at least 3 assays were carried out.

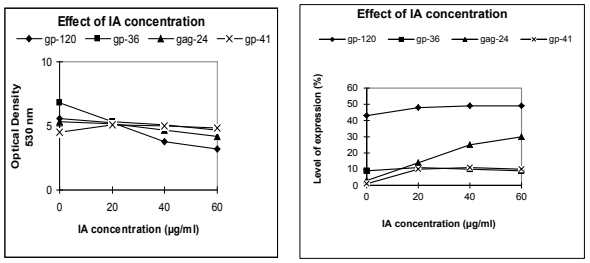
RESULTS AND DISCUSSION

In this work the tryptophan promoter was used, considering the literature reports that the expression of genes under this promotion signal leads to high expression levels in a relatively simple form (18). As has been reported in the literature, the behavior of the expression of recombinant proteins expressed under the control of the tryptophan promoter depend on a series of factors, like for example, the composition of the cultural medium used, the conditions of cultivation including the aeration, the agitation level, the temperature, and the pH of growth. (1, 11, 19).

The effect of inductor concentration on the production of four different recombinant proteins in shake flasks was determined. In all cases, when the inductor concentrations was increased, only a slight reduction on cellular growth was observed (Figure 1a), and optical density was maximal in absence of IA. Figure 1b shows that in the case of the gp-120 and gp-36 the level of expression (49% and 10%, respectively), did not increase in the presence of different IA concentrations.

The expression level of gag-24 is a function of the amount of inductor utilized; using 60 μg/ml of IA, 30% of expression was obtained. When the process was done in the absence of IA, the expression of gp-41 was almost not detected, while using concentrations higher than 20 μg/ml the expression was constant at a 10 % level. In all cases, when expression was evaluated in the absence of IA, levels decreased in comparison with values reported when the inducer of the system was used. The initial amount of tryptophan used in the culture medium (20 μg/ml) had inhibiting effects.

Analyzing the influence of the concentration of the inductor on the cellular growth and the level of expression (figure 1), it was demonstrated that a general only rule for predicting the results doesn't exist. Using similar expression vectors, cultivation medium and conditions of similar growth in all the cases, the obtained results are different for each of the analyzed proteins. Using concentrations higher than or equal to 20 μg/ml of IA the level of obtained expression turned out to be independent of the concentration of the inductor used (Figure 1b). An exception is the gag-24 protein, which doesn't correspond with the previous results reported in the literature, asserting that a concentration higher than 25 μg/ml decreases the productivity and the expression of the recombinant proteins drastically (20, 21, 22, 23, 24). These points to evidence those others factors could be influencing in the process.



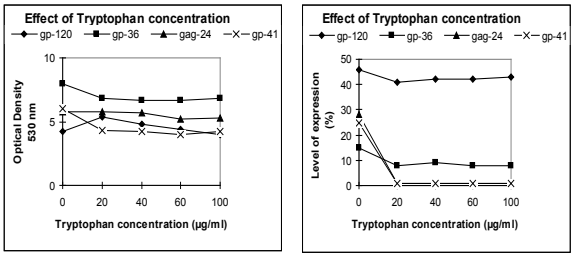
A B

Figure 1. *Effect of different concentration of IA on growth (A) and expression level (B). Cultures were performed in shake flasks, using M9 medium (15) supplemented with glucose 20 g/l, casein hydrolysate 10 g/l, and ampicillin 50 μg/ml. Cultivations were performed at a temperature of 37°C and stirrer speed of 250 rpm. The pH was not controlled and culture time was 12 hours. The effect of IA concentration was determined by adding 20 μg/ml of tryptophan to the culture medium, and plus different concentrations of IA (0 to 60 μg/ml), added after 1hour of cultivation.*

In some proteins, like for example, the gp-41 and the gag-24, when the process was carried out in absence of the inductor, was observed that the level of expression fell drastically, because an initial concentration 20 μg/ml of tryptophan in the cultural medium had a totally repression effect of the tryptophan promoter (25, 26). This agree with the previous reports of other authors (1), while the expression of the gp-120 and gp-36 proteins was only affected slightly, for what in some cases the tryptophan promoter behaved like constitutive and in other cases like regulated (19). This evidences that the influence of the inductor concentration in the production of recombinant proteins obtained through a genetic construction, in which the gene of interest is inserted in a plasmid under the control of the tryptophan promoter, depends on diverse factors, fundamentally on the particular characteristics of the cloned proteins, as well as of the host strain used (11, 25).

The expression level of different proteins was studied in the absence of IA. Initial tryptophan concentration did not have a great influence on cellular growth (Figure 2a). For all cases in the interval between 20 to 100 μg/ml of tryptophan, optical density was almost constant, while in the interval of 0 to 20 μg/ml, cell growth had a slight reduction for gp-36 and gp-41. The percentage of expression of gp-36 and gp-120 decreases slightly (to 10% and 40%

respectively), as the initial concentration of the repressor in the culture medium is increased from 0 to 20 μg/ml (Figure 2b). Concentrations above this level, however, do not influence the protein expression appreciably. In the cases of gag-24 and gp-41, the absence of tryptophan in the culture medium led to the highest levels of expression (more than 25%). Addition of 20 μg/ml of tryptophan is enough to reduce drastically the total amount of synthesized protein. In all cases, the best yields were obtained when the expressions of the recombinant proteins were performed in the absence of IA and tryptophan.



A B

Figure 2. *Effect of different concentration of tryptophan on growth (A) and expression level (B). Cultures were performed in shake flasks, using M9 medium (15) supplemented with glucose 20 g/l, casein hydrolysate 10 g/l, and ampicillin 50 μg/ml. Cultivations were performed at a temperature of 37°C and stirrer speed of 250 rpm. The pH was not controlled and culture time was 12 hours. The influence of the initial tryptophan concentrations (0 to 100 μg/ml) on protein expression was studied in the absence of IA in the culture medium.*

Upon analyzing the influence of the concentration of the repressor on the expression level, it was demonstrated that in general terms, in absence of tryptophan, high values of expression in all cases take place (Figure 2b), compared with those obtained while carrying out the induction using IA**.** On the other hand, when high concentrations were used, the level of expression was affected in different degree, depending on the expressed protein. In the case of the gp-36 and gp-120 proteins, high levels of expression were observed in presence of high concentrations of tryptophan, since it is known that the repression of the tryptophan promoter is incomplete (11).

Otherwise, when using high concentrations of tryptophan (20-100 μg/ml), the level of expression was affected totally in the case of the proteins gp-41 gag-24 and only slightly in the case of the gp-36 and gp-120 proteins.

The levels of expression obtained with these clones were higher than 15% for each one of the tested proteins. These results are very superior to those reported previously for the several proteins of the HIV, like for example, the gp-41 and the gp-36 proteins (27), the gp-120 (28) and the gag-24 (29).

Finally we decided to carry out the production of the proteins by derepresion of the expression system, without the use of tryptophan in the cultivation medium.

When the tryptophan promoter is used for the expression of recombinant proteins in *E. coli*, although similar conditions could be used in the case of the expression vector, medium and cultivation conditions, it is essential to analyze case by case the influence of the inductor's and repressor's concentrations on the production of the protein of interest, in order to select the most favorable induction conditions. Finally, it was demonstrated that for each protein, there is a defined concentration of IA and tryptophan, which maximizes its production. These procedures could be also applied to production of other recombinant proteins expressed in *E. coli* under the control of tryptophan promoters.

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REFERENCES

1. Shimizu, N., Fukuzono, S., Harada, Y., Fujimori, K., Gotoh, K., and Yamazaki, Y.: Mass production of human epidermal growth factor using fed-batch cultures of recombinant *Escherichia coli.* Biotechnology and Bioengineering, 38, 37-42 (1991).
2. Pulliam T.R., Winston S., Bentley W.E.. Tryptophan regulated expression and aqueous two-phase separation of recombinant HIV-fusion peptides. Enzyme Microbial Technology, 20: 46-51 (1997).
3. Iijima S., Kawai S., Mizutani S., Taniguchi M., Kobayashi T.. On-off regulation of gene expression from tryptophan promoter with cross-flow filtration. Applied Microbiology and Biotechnology. 26: 542-545 (1987).
4. Yanofsky, C.; Crawford, I. P. The tryptophan operon. In *Escherichia coli* and Salmonella typhimurium: Cellular and Molecular Biology; Neidhardt, F. C.; Ingraham, J. L.; Magasanik, B.; Low, K. B.; Schaecter, M.; Umbarger, H. E., Eds.; American Society for Microbiology: Washington, DC, Vol. 2, pp 1453-1472, (1987).
5. Yanofsky, C.; Horn, V. Role of regulation features of the trp operon of *Escherichia coli* in mediating a response to a nutritional shift. J. Bacteriol., 176(20), 6245-6254, (1994).
6. Lehninger, A. L.; Nelson, D. L.; Cox, M. M. Principles of Biochemistry, 2nd ed.; Worth Publishers: New York, (1993).
7. Xiu, Z.-L.; Zeng, A.-P.; Deckwer, W.-D. Model analysis concerning effects of growth rate and intracellular tryptophan level on the stability and dynamics of tryptophan operon and tryptophan biosynthesis in bacteria, J. Biotechnol., 58, 125-140, (1997).
8. Shimizu N., Fukuzono S., Nishimura N., Odawara Y., Fujikawa K.. Cultivation of *Escherichia coli* harbouring hybrid plasmids. Journal Fermentation Technology. 65: 7-10 (1987).
9. Shimizu N., Fukuzono S., Fujimori K., Nishimura N., Odawara Y.. Fed-batch culture of recombinant *E. coli* with inhibitory substance concentration monitoring. Journal of Fermentation Technology. 66: 187-191 (1988).
10. Morales, J., Muzio, V., Torrens, I., Jimenez, V., Silva, A., Santos, A., Quiñones, Y., Narciandi, R.E., Herrera, L.. Expression of the chymosin gene in *E. coli*. Interferón y Biotecnología. Vol 6, N 3, 1989: 242-250 (1989).
11. Narciandi, R.E. and Delgado, A.: Production of the recombinant p36 protein from HIV-2 in *Escherichia coli* using the trp promoter. Biotecnology Letter, 14, 1103-1108 (1992).
12. Sambrook J, Russell DW. Molecular Cloning. A Laboratory Manual. New York, NY, USA., Cold Spring

Harbor Laboratory Press, 999 p.ISBN 0879695773, (2001).
13. Novoa, L.I., Machado, J.A., Fernandez, J.R., Benitez, J.V., Narciandi, R.E., Rodriguez, J.L., Estrada M.P., Garcia, J., Herrera, L.S.. Method for the expression of heterologous proteins produced in fused form in *E. coli*, use thereof, expression vectors and recombinant strains. European Patent Application No. 90202108.8 (1994).
14. Duarte C.A., Montero M.N., Saralena A., Valdes R., Jimenez V., Benitez J., Narciandi R.E., Madrazo J., Padrón G., Sanchez G., Giljian G., Persson K., Ojeda S., Caballero A., Miranda A., Dominguez M., Wahren B., Menendez A.. Multiple polypeptide containing epitopes of HIV-1 envelope induces neutralizing monoclonal antibodies against the V3 loop. AIDS Research Human Retroviruses 10: 233-241 (1994).
15. Miller, J.H.: Experiments in molecular genetics, p 431-432. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York (1972).
16. Lowry, O.H., J. Rosebrough, A.L. Farr and R.J. Randall, R.J. J. Biol. Chem. 193, 265-275, (1951).
17. Laemmli U.K. Cleavage of structural proteins during assembly of the head of bacteriophage T4. Nature 227: 680-685 (1970).
18. De Boer H.A., Comstck L.J., Yansura D.G., Heyneker H.L.. Promoter Structure and Function. (R.L. Rodriguez and M.J.Chamberlain, eds.), Praeger, New York. 462-467 (1982).
19. Narciandi R.E. High-Level Production of p36 from HIV-2 in Fed-Batch Culture of Recombinant *Escherichia coli*. Journal of Fermentation and Bioengineer ing. 81: 360-362 (1996).
20. Shimizu N., Fukuzono S., Nishimura N., Odawara Y., Fujikawa K.. Cultivation of *Escherichia coli* harbouring hybrid plasmids. Journal Fermentation Technology. 65: 7-10 (1987).
21. Calcott P. H., Kane J.F., Krivi G. G., and Bogosian G.. Parameters affecting production of bovine somatropin in *E. coli* fermentations. Developed Industrial Microbiology. 29: 257-266 (1988).
22. Salazar-Cavazoz E., Santillan M. Optimal performance of the tryptophan operon of *E. coli*: a stochastic, dinamical, mathematical-modeling approach. Bull Math Biol., 76 (2): 314-334. (2014).
23. Bhartiya S, Rawool S, Venjatesh KV. Dynamic model os *Escherichia coli* tryptophan operon shows an optimal structural design. Eur J. Biochem., 270 (12): 2644-2651, (2003).
24. Xiu ZL, Chang ZY, Zeng AP. Nonlinear dynamics of regulation of bacterial trp operon: model analysis of integrated effects of repression, feedback inhibition, and attenuation. Biotechnol. Prog. 18 (4): 686-693, (2002).
25. Narciandi R.E., Garcia J., Motolongo J., Machado J.A., Benitez J., Novoa L.I , Herrera L. Produccion, aislamiento y semipurificacion de la proteína Transmembranica gp-41 sintetizada en *Escherichia coli*. Biotecnologia Aplicada, Volumen 10, No 1, (1993).
26. Narciandi R.E., Garcia J., Motolongo J., Machado J.A., Benitez J., Novoa L.I , Herrera L. Production and Purification of a Fused Recombinant Protein gp-36 (HIV-2) from *Escherichia coli*. Journal Chernical Technology and Biotechnology, 66, 1- 6. (1996).
27. Coia G., Hudson P.J., Lilley G.G.. Construction of recombinant extended single-chain antibody peptide conjugates for use in the diagnosis of HIV-1 and HIV-2. J. Immunology Method. 192: 13-23 (1996).
28. Smythe J.A., Nardelli P.C., Gallo R.C., Gershoni J.M.. Production of linear polymers of HIV gp-120 binding domains. Prot. Eng. 7: 145-147 (1994).
29. Hausdorf G., GewieB A., Wray V., Porstmann T.. A recombinant human immunodeficiency virus type-1 capsid protein (rp24): its expression, purification and physico-chemical characterization. Journal Virology Method. 50: 1-10 (1994).