RAPID AND SIMPLE PROCEDURE TO MULTI-COPY CLONES DETERMINATION IN YEAST

Nelson Santiago and Eduardo Martínez

Center for Genetic Engineering and Biotechnology, PO Box 6162, Havana, Cuba.

Recibido en junio de 1993. Aprobado en Septiembre de 1993.

Key word: Yeast; Multi-copy.

SUMMARY

A procedure for the screening of integrative multi-copy heterologous gene in yeast is presented using cells that have been grown in 96 well microtitre-plates.

RESUMEN

Se presenta un procedimiento que permite la determinación del número de copias de genes heterólogos integrados en levaduras, utilizando el crecimiento de los clones en placas de 96 pocillos.

INTRODUCTION

High level expression associated with multiple integration events has been reported for *Pichia pastoris* (Sreekrishna *et al*, 1989) and *Kluyveromyces lactis* (Van Den Berg *et al.*, 1990). It can be obtained using vectors containing multiple expression cassettes (Thill *et al.*, 1990) or for very high copy number by screening the multiple integration events from transformations, using DNA fragments designed for single copy transplacement (Clare *et al.*, 1991).

As multi-copy transplacement events occur at a low frequency, in order to obtain high-copy integrants a mass screening is necessary, colony hybridization has been routinely used (Clare *et al.*, 1991). Here we report a single procedure for the isolation and estimation of the copy number consisting on dot blot methods of intact whole cells which were grown in microtitre wells and then lysed on nitrocellulose.

MATERIALS AND METHODS.

The restriction enzymes were purchased from HEBER BIOTEC, (Cuba); the radioactive isotopes were from Amersham Int.(U.K); peptone,yeast extract, sorbitol and dextrose were from OXOID (U.K); mercaptoethanol, NaOH, SDS, DTT, SSC, Zimolyasa and

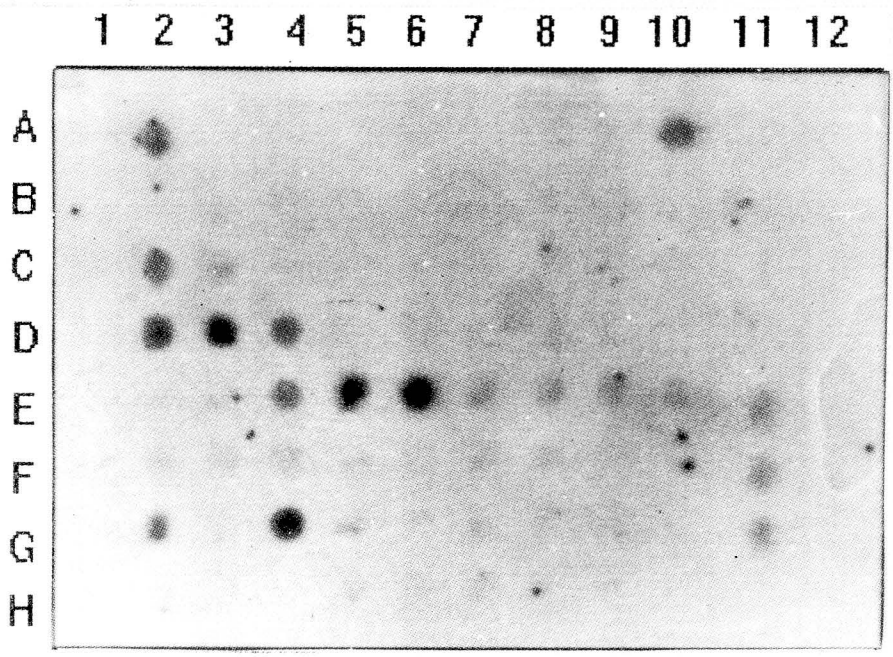

Fig.1 Dot Blot Analysis. A1, B1, C1, D1, E1, F1, G1, H1: Cultured medium. A12, B12, C12, D12, E12, F12, G12, H12: Negative controls. E11, F11, G11: Positive controls (one copy gene). The others are screening transformed clones

EDTA were from SIGMA (USA); nitrocellulose membrane was from Schleicher & Schuell(Germany).

The plasmid pPPc316-4 (Morales *et al*, 1992) was transformed according to Cregg *et al* (1985), in the *Pichia pastoris* MP36 strain (Yong *et al*., 1992).

Independently selected transformants were inoculated into 150 μl of YPD (Peptone 2 %, yeast extract 1 % and dextrose 2%) in 96 well microtitre dishes. After incubation for two days at 30°C, the optical density was measured using a Titertek Multiskan Plus equipment (ICN.FLOW) at 492 nm. Cells from 100 μl aliquots of these cultures were filtered onto nitrocellulose membranes using a dot blot manifold (BIORAD).

These cells were prepared for *in situ* hybridization by placing the membranes sequentially, at room temperature, on filter papers soaked in:(I) 2.5 % mercaptoethanol/ 0.2 M NaOH, 0.1% SDS, 15mM DTT (20 min) (II) 2X SSC (2X 5min) (III) 2 mg/ml Zimolyasa, 1M Sorbitol, 0.1M EDTA ph7.5 (37°C Over night) (IV) 1% SDS (20 min) (V) 0.1 M NaOH/1.5M NaCl (5 min) (VI) 2X SSC (2X 5 min).

The membranes were air dried, baked at 80°C for 1 hour and hybridized according to Maniatis *et al*, (1989), with a chymosin gene probe which was obtained by cutting the plasmid pPPc316-4 with NcoI-XbaI.(figure 1). The dot intensity signals were measured on the X-Ray films using a 2202 ULTROSCAN Laser Densitometer equipment (LKB)at 633nm.

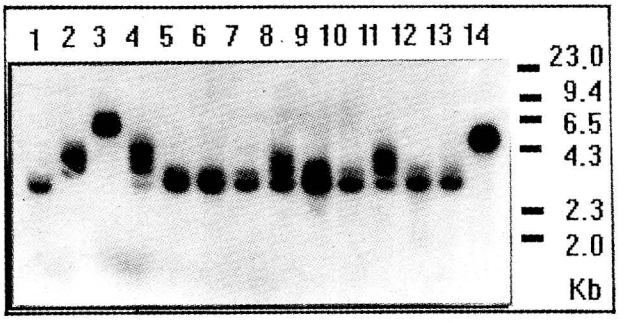

Fig 2 Southern Blot Analysis. Line 1. E11 Control 1 copy. Line 2. D2 4 copies. Line 3. E6 8 copies. Line 4. D4 4 copies. Line 5. E4 1 copy. Line 6. D6 1 copy. Line 7. E 1 copy. Line 8. A2 3 copies. Line 9. E5 7 copies. Line 10. C2 1 copy. Line 11. A10 3 copies. Line 12. F11 Control 1 copy. Line 13. G11 Control 1 copy. Line 14. G4 10 copies.

Table 1.
Values of intensity densitometer, optical density and copy number.

	1	2	3	4	5	6	7	8	9	10	11	12
A	- 0.000 -	13.7 0.820 2.98	- 0.706 -	- 0.909 -	- 0.936 -	- 0.950 -	- 0.856 -	- 0.866 -	- 0.900 -	15.72 0.936 2.99	- 0.920 -	- 0.756 -
B	- 0.000 -	- 0.877 -	- 0.852 -	- 0.902 -	- 0.772 -	- 0.815 -	- 0.921 -	- 0.766 -	- 0.899 -	- 0.915 -	- 0.990 -	- 0.888 -
C	- 0.000 -	4.80 0.850 1	4.190 0.750 1	- 0.881 -	- 0.792 -	- 0.664 -	- 0.884 -	- 0.979 -	- 0.786 -	- 0.766 -	- 0.815 -	- 0.905 -
D	- 0.000 -	20.02 0.855 4.05	45.63 1.264 6.44	20.67 0.923 4.00	- 0.866 -	- 0.925 -	- 0.920 -	- 0.872 -	- 0.733 -	- 1.023 -	- 0.970 -	- 0.877 -
E	- 0.000 -	- 0.970 -	- 0.923 -	5.07 0.903 1	37.63 0.966 6.9	40.58 0.905 8	0.980 0.905 1	5.19 0.980 0.945	5.20 0.843 1.101	5.57 0.996 1.1	5.57 0.996 1.1	- 0.815 -
F	- 0.000 -	4.99 1.104 0.807	- 0.803	5.19 0.854 1.09	4.32 0.966 0.798	- 0.801 -	3.85 0.732 0.940	4.00 0.915 0.780	- 0.886 -	- 0.855 -	5.20 0.930 0.99	- 0.915 -
G	- 0.000 -	25.6 0.912 5	- 0.950 -	56.17 0.966 10.36	4.99 0.930 0.958	3.80 0.708 0.909	3.97 0.860 0.782	- 0.992 -	- 0.966 -	- 0.937 -	5.03 0.899 0.99	- 0.833 -
H	- 0.000 -	- 0.995 -	- 0.966 -	- 0.924 -	4.05 0.863 0.795	4.00 0.930 0.769	4.02 0.935 0.769	- 0.766 -	- 0.895 -	- 0.855 -	- 0.940 -	- 0.815 -

Note: In each column:
-Optical density. =633nm. Densimometer
-Optical density. =492nm. Multiskan
-Copy number.

A1, B1, C1, D1, E1, F1, G1, H1: Cultured medium (non-innoculated)
A12, B12, C12, D12, E12, F12, G12, H12: Negative controls (MP36)
E11, F11, G11: Positive controls (one copy gene)
Intensity values of transformants lower than the value of one copy gene control are shown as a dash.

To calculate the copy number we used the following formula.

$$\text{Copy number} = \frac{O.D(\lambda 633nm)/O.D(\lambda 492nm)}{\underset{(1\ copy)}{O.D(\lambda 633nm)}/\underset{(1\ copy)}{O.D(\lambda 492nm)}}$$

where O.D(λ633nm) is determined by laser densitometry and O.D(λ492nm) is determined by Multiskan equipment.

The DNA selected from the high copy number integrants was purified according to Ferbeyre *et al* 1993. This DNA was cut with PstI enzyme and was analyzed by Southern blot according to Manniatis *et al.*, (1989) and hybridized with the same probe (figure 2)

RESULTS AND DISCUSSION

Transformant intensity values were lower than the value of the one copy gene control as shown with a dash (Table 1).

We obtained a rate factor of 5.59 between the intensity of a Dot blot hybridization signal and the values from optical density of the one copy control. Then, from twenty one clones with value higher than the rate factor in densitometer, we determined the number of copies.

Sixty seven independent transformants were screened for multi-copy event as here described under Materials and Methods.

Optical density of the transformants grown into 96 microtitre wells is shown in table 1. In dot blot analysis, around 21 samples gave signals with an intensity equal or higher than the single copy control (figure 1). The values of intensity measured in densitometer are shown in table 1.

The formula shown under the heading Materials and Methods was used to determine high-copy transformants and to calculate their copy number. Seven samples gave values higher than these for single copy control.

Southern blot analyses showed that clones obtained by this method have a pattern of bands that can only be explained by multy-copy transplacement models. We observed a variation in the levels of chymosin protein expression due to a different gene copy number (data not shown).

We concluded that this procedure can be used as a rapid and simple method to obtain and estimate transformants with a high gene copy number.

REFERENCE

CLARE, J. RAYMENT, F. BOLLONTINE, S. SVEEKRISHMO, K. and ROMANOS, M. (1991). High-level expression of *Tetanus* toxin fragment C in *Pichia pastoris* strains containing multiple tandem integrations of the gene . *Biotechnology* **9**:455-460.

CREGG, J. K.BARRINGER, Y.HESSLER, (1985). *Pichia pastoris* as a host system for transformation . *Mol. Cell.Biol.* **5**: 3376-3385.

FERBEYRE, G. and J. MORALES, (1993). A rapid procedure for preparing high molecular weight DNA from yeast for southern analysis *Biotechniques* **14**:386-389.

MANIATIS, T., J. SAMBROOK; E. FRITSCH, (1989). Molecular cloning. A Laboratory Manual.

MORALES, J.; G. FERBEYRE; E. MARTINEZ; J. AGUIAR; V. MARTÍNEZ; A. SOSA; A. VILLAREAL; E. AMADOR; A. SILVA; L. HERRERA, (1992) Producction of Chymosin from the yeast *Pichia pastoris*. Book of short reports of the congress *Biotecnologia* Habana 92.

SREEKRISHNA, K.; L. NELLES, R. POTENZ, J. CRUZE, P. MAZOFERRO, W. FISH, F. MOTOHINO, K. HOLDEN, D. PHELPS, P. WOOD, and K. PORKER, (1989). High-level expression, purification and characterization of recombinant human Tumor Necrosis Factor sinthesised in the methylotrophic yeast *Pichia pastoris. Biochemistry* **28**:4117-4125.

THILL, G.; G.DAVIS, C. STILLMAN, G. HOLTZ, R. BRIERLEY, M. ENGEL, R. BUCKHOLTZ, J. KINNEY, T. VEDVICK, AND R. SEIGEL, (1990). Positive and negative effects on multi-copy integrated expression vectors on protein expression in *Pichia pastoris*. In Heslot, H.; Dovies, J.; Florent, J.; Bobichon, L.; Durand, G. and Penasse, L. (Eds), Proceeding of the 6 th International Symposium on genetics of microorganism. Vol II . Societé Francaise de microbiologie, Paris, France, pp. 477-490.

VAN DEN BERG, J.; J. VAN DER LAKER, A. VAN GAYEN, T. RENNIERS, K. RIETVELD, A. SCHORP, R. BISHOP, K.SCHULTZ, D.MAYER, M. RICHMON, and J.SHUSTER, (1990). *Kluyveromyces* as a host for heterologous gene expression, Expression and secretion at prochymosin. *Biotechnology* **8**:135-139.

YONG, V.; M. GONZALES, L. HERRERA, and J. DELGADO, (1992). El gen *his* 3 complementa una mutación *his*⁻ de *Pichia pastoris. Biotecnologia Aplicada* 9:55-61.