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Determination of plasmid copy number in yeast transformants by means of agarose plugs

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The determination of plasmid copy number in *Saccharomyces cerevisiae* transformants containing circular or linear plasmids is currently performed with total yeast DNA extracts (1) obtained from cultures grown under selection. The determination is based essentially on quantitative Southern hybridization (2, 3) of an appropriate probe to a sequence present on both plasmid and chromosomal DNA in digested or undigested samples run out on conventional agarose gels. The DNA extraction procedure calls for treatment of the cell lysates with organic solvents that could entail systematic losses of either plasmid or chromosomal DNA thus producing artifactual results.

We propose here a method based on the assumption that quantitative analysis of plasmid and chromosomal DNA extracted from yeast cells embedded in agarose plugs will furnish more reliable results. With this procedure the cells are lysed *in situ*, thus avoiding possible losses of material, and the chromosomes and plasmid DNAs, trapped within the agarose matrix, can be separated by pulse field gel electrophoresis.

We have determined the plasmid copy number of two yeast transformants with the proposed method and compared the results with those obtained by means of the traditonal method mentioned above. One of the yeast clones contained a linear plasmid of about 6 kb, called p7, and the other a dimerized version of p7 of about 12 kb, called p14 (manuscript in preparation). The plasmids are derivatives of YRP17 (4) and thus contain the ARS1 and TRP1 DNA sequences that map on chromosome IV of *S.cerevisiae*. Cultures of the two yeast transformants were grown to a concentration of about 1×10^7 cells/ml in 20 ml of minimal medium containing 40 μg/ml of all the amino acids except tryptophane (MMaa). The cells were then collected and used to prepare agarose plugs and to determine the percentage of plasmid-bearing cells. In fact, in order to measure plasmid copy number it is essential to determine the percentage of plasmid-bearing cells in the culture under study. This task is not effortless and we have previously described (5) a laborious method for the correct determination of the percentage of cells that contain plasmid in a yeast culture growing under selection. With the yeast clones used in this study we were able to obtain comparable results by growing and assaying the cells in MMaa. Aliquots of the cultures were spread on plates containing either complete medium or MMaa and the colony counts on the two medium were compared. The percentage of plasmid-containing cells in the experiment described here, was 85% for p7 and 90% for p14.

Agarose plugs were made with spheroplasts prepared essentially as described by Beggs (6). The formation of spheroplasts was monitored by microscope observation and when about 90% of the cells had been converted to spheroplasts, the suspension was centrifuged and gently resuspended in 250 μl 1 M sorbitol, 50 mM phosphate buffer, pH 7.5. The spheroplasts were then mixed with 350 μl of 1% low melting agarose prepared in 125 mM EDTA and kept warm at 50°C. After mixing, the suspension was quickly poured into a mold and set on ice. The plugs were then incubated overnight at 50°C in 4–5 volumes of lysis buffer (0.5 M EDTA, 10 mM Tris–HCl pH 8, 1% Sarkosyl, and 2 mgr/ml Proteinase K). After this time the plugs were rinsed with 50 mM EDTA at 50°C and stored in 50 mM EDTA at 4°C until use. Separation of plasmid from chromosomal DNA was obtained by FIGE, as described in the legend to Figure 1. Under these conditions all the yeast chromosomes migrated as a single band distinct from the plasmid DNA (Figure 1A). To avoid the problem of quantitative hybridization of large DNA fragments due to inefficient transfer onto filters, the gel was dried and hybridized directly (7) under stringent conditions. Control experiments performed with the untransformed yeast strain (data not shown) demonstrated that hybridization to the chromosomal DNA is quantitative. The probe used was a [α-^{32}P]dATP labeled (8) ARS1-TRP1 fragment of YRP17, obtained by digesting this plasmid with *Xba*I and *Cla*I. The hybridization pattern obtained is displayed in Figure 1B. Only two radioactive bands are present, associated with the chromosomal and the plasmid bands. It is to be noted that the wells are totally free of radioactivity, showing that spheroplasts embedded in the agarose plugs were lysed completely. The portions of the gel containing chromosomal and plasmid DNA were cut out and the radioactivity was counted in an LKB scintillation counter. Table 1 gives the values for copy number obtained by the procedure described here along with parallel data obtained using total DNA extracts. The agarose plug method furnishes values significantly lower than those obtained by means of the traditional method, which allows us to infer that loss of chromosomal DNA can occur when the traditional method is employed.

The agarose plug method also gives reliable results when used for determination of copy number of circular plasmids. In this case the agarose plugs must be digested to completion with a restriction enzyme that cuts the chromosomal and plasmid DNA in such a way that the sequences to be used for targeting are contained on only one fragment. Separation of digested DNA can be obtained by conventional electrophoresis or by a suitable CHEF program.

The precise determination of plasmid copy number must be taken into account whenever stabilizing, amplifying, and control sequences are being studied. This is clearly important when

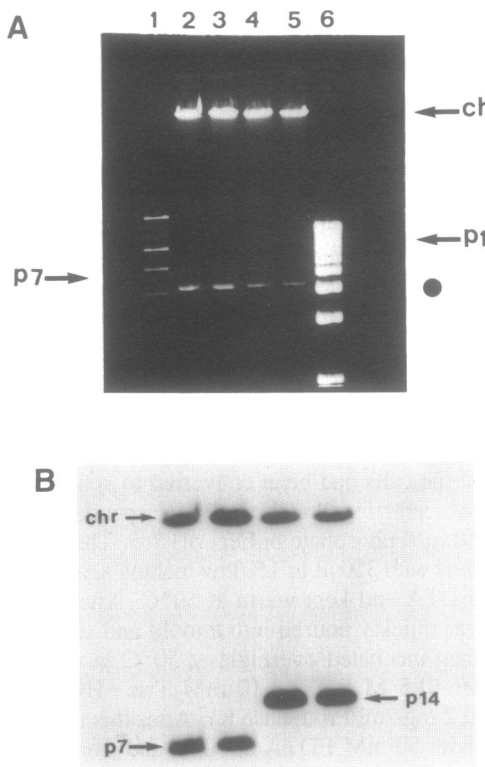

Figure 1. Separation of yeast chromosomes and of linear plasmids by FIGE using a *Bio-Rad* CHEF Mapper System. The conditions were: 1% agarose in 0.5× TBE (1× TBE is 89 mM Tris-base and boric acid, 2 mM EDTA) 14°C, 3 V/cm for 20 h. The forward migration interval varied from 9 s to 60 s with a reverse pulse of 5 s. **A)** ethidium bromide stained gel: lane 1: HindIII digested lambda DNA; lanes 2 and 3: DNA extract containing plasmid p7; lanes 4 and 5: DNA extract containing plasmid p14: lane 6: 1 kb ladder (BRL). The arrows point to the positions of the plasmids and the black dot to that of the yeast killer plasmid. **B)** hybridization of the gel shown in A) using as a probe the yeast *XbaI–ClaI* DNA fragment present on chromosome IV and on the plasmids. The values obtained for the percentage of plasmid-bearing cells were used to correct the radioactivity counts. In the case of p14 the counts were also corrected for the presence of two ARS1–TRP1 fragments in the dimeric molecule.

Table 1. Determination of plasmid copy number in *S.cerevisiae* transformants

plasmid	DNA in agarose plugs* copies/cell	Total DNA extracts* copies/cell
p7	3.8	29
	4.0	18
p14	2.1	8.2
	2.5	7.5

*Results of 2 experiments.

evaluating transformed yeast strains for their potential usefulness in biotechnology.

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