A strategy for quickly identifying all unique two-hybrid or library plasmids within a pool of yeast transformants

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When performing a variety of molecular genetic analyses in yeast, including two-hybrid screens, it is frequently necessary to determine how many different plasmids are present within a particular population of selected clones. For example, all fission and many budding yeast plasmid libraries in common use are maintained at high copy number, which allows the isolation, by complementation of a mutant phenotype, of not only the wild-type copy of the mutated gene, but also multi-copy suppressors. Traditionally, this has been accomplished by isolating plasmids from all surviving colonies, removing contaminants that reduce the transformation efficiency of plasmids isolated from fission yeast (1), and amplifying each plasmid in bacteria. Plasmids are compared using either Southern blot analysis or restriction digest patterns, a complicated process when analyzing genomic clones in which variable length regions flank the same gene. As an alternative, we reasoned that any plasmid identified on the basis of complementation or interaction in a two-hybrid screen could be used in a plasmid-based hybridization assay to identify all other strains containing the same plasmid. By exclusion, this approach would also identify strains that contained a different plasmid. After isolation of this second plasmid, the process can be repeated until no new plasmid is found.

To facilitate these experiments, we have adapted a slot blot quantitation (SBQ) technique originally developed to monitor gene copy number in mammalian cells (2) for use in quantitating gene copy number in both fission and budding yeast. The technique does not require extraction of DNA, which may result in differential recovery of plasmid or mitochondrial DNA, and is much less labor-intensive than quantitative Southern blot analysis using either purified DNA or whole cells embedded in agarose plugs (3,4). In the SBQ technique, an alkaline lysate of whole cells picked directly from colonies on plates is blotted onto a nylon membrane, probed with the sequence of interest and normalized for the amount of cell lysate by subsequent hybridization of the same membrane with radiolabeled total genomic DNA. The ratio of the signal from the sequence-specific probe to the total signal is then compared to the same ratio from a strain known to carry a single copy of the gene of interest in the chromosome. Because the signal from the genomic probe is generally 50–1000 times greater than that from the specific probe, sequential hybridizations can be carried out without stripping the

first probe, with good retention of the cellular DNA on the filter and hybridization efficiency. As a test of the SBQ technique we used it as follows to identify strains containing different plasmids within a population of rescued colonies.

Initial attempts to clone the *pim1* gene in fission yeast by complementation of the temperature-sensitive *pim1-d1^{ts}* mutation (5) yielded 64 colonies. A small scale analysis of the plasmid DNA recovered from nine colonies revealed that all encoded the same gene which, by integrative transformation, was found to be the high-copy suppressor, *spil* (5), and not the wild-type copy of the mutated gene. The *pim1* gene was ultimately cloned by two alternative methods that resulted in lower level expression of *pim1* (5,6). We subsequently found that overexpression of the *pim1* cDNA from a regulatable promoter is lethal (5) and assumed that our previous attempts to clone the gene using genomic libraries was unsuccessful because expression from the *pim1* promoter on a multi-copy plasmid was also lethal. Nevertheless, the question remained whether any yet-unidentified gene existed among the remaining rescued colonies. We reasoned that strains complemented by *spil* would contain one chromosomal and several plasmid-borne copies of this gene, whereas strains rescued by a different gene would contain only the single chromosomal copy.

Approximately 2×10^6 cells were pelleted by centrifugation at 14 000 g for 1 min. The cell pellet was resuspended by vortexing in 150 μ l 0.4 M NaOH, and incubated at 80°C for 10 min. Alternatively, ~ 1 mm³ of cells from a colony was added to the alkaline lysis solution and resuspended by vortexing prior to incubation at 80°C. Cell debris was cleared by centrifugation at 14 000 g for 2 min. A 125 μ l portion of the supernatant was applied by capillary action in the absence of vacuum in a slotblot manifold to GeneScreen Plus hybridization membrane (Dupont), that had been previously soaked in 0.4 M NaOH and air-dried. Each well was rinsed with 125 μ l 0.4 M NaOH. The blot was rinsed twice with excess 2 $\times$ SSC for 5 min, and allowed to air-dry. The DNA was cross-linked to the membrane using the auto-crosslink setting of a UV Stratalinker 2400 (Stratagene). The membrane was then hybridized with randomly primed *spil* DNA, and washed as previously described (7). The hybridization signal was quantitated using a Model 400E PhosphorImager (Molecular Dynamics). To normalize the amount of DNA in each slot, the same blot, without

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Figure 1. *pim1-d115* strains rescued by a high copy number plasmid library (5) were analyzed for *spiI* copy number by SBQ.

removing the *spiI* probe, was re-hybridized with randomly primed *S.pombe* DNA, and washed. The hybridization signals were quantitated as before, and the *spiI* copy number was normalized using values obtained from wild-type cells.

Fifty colonies were analyzed by SBQ and 11 had a *spiI* copy number of approximately one, corresponding to the chromosomal copy (Fig. 1). Southern blot analysis of these 11 strains confirmed that they carried a plasmid that contained the *pim1* gene rather than the *spiI* gene (data not shown). The average *spiI* copy number in the other samples ranged from approximately two to four (Fig. 1), comparable to the average plasmid copy number determined by Brun *et al.* (8). These values are lower than previous estimates of plasmid copy number in *S.pombe* (9), possibly due to strain differences, as suggested by Brun *et al.* (8). Nevertheless, there is a perfect correlation between strains with low *spiI* copy number as determined by SBQ and strains that carry complementing plasmids other than *spiI*. It is possible that *spiI* was isolated four times as often as *pim1* in the original complementation test because high-level *pim1* expression is toxic (5). We are currently analyzing the promoter regions of these newly rescued *pim1* containing plasmids to look for truncations that may lower promoter efficiency.

To test whether SBQ will facilitate application of the hybridization/exclusion strategy in *S.cerevisiae*, we have also shown that it can be used to analyze gene copy number of a plasmid for which the copy number can be modulated experimentally. The *leu2-d* allele of plasmid YEpFAT7.5 does not express sufficient LEU2 gene product to confer leucine prototrophy in single copy (10). Thus, the copy number of YEpFAT7.5 can be modulated by the presence or absence of leucine in the medium. Plasmid YEpFAT7.5 also carries the *URA3* gene, which allows plasmid

selection to be maintained when cells are grown in the presence of exogenous leucine. SBQ was performed as described above on cells grown on YC plates with or without leucine, except that the first probe was radiolabeled 445 bp *EcoRV*–*AccI* fragment from pLEU2-0.9 (10), and the second probe was radiolabeled *S.cerevisiae* genomic DNA. For comparison, quantitative Southern blot analysis was performed. In this case, cells were grown in 5 ml cultures of YC(+/- leu) medium. Total genomic DNA, prepared as described (10), and digested with *XhoI*, *SalI* and *NsiI*, was blotted to nylon membrane, and probed with the 445 bp *EcoRV*–*AccI* fragment of pLEU2-0.9 and the signals in the diagnostic chromosomal and plasmid bands (10) were quantitated using a PhosphorImager. As shown in Table 1, the average copy number determined by the two methods is similar.

Table 1. Comparison of *leu2-d* copy number in *S.cerevisiae* in the presence or absence of leucine by SBQ and quantitative Southern analysis

	<i>leu2-d</i> Copy number	
	SBQ	Southern
No plasmid	1 ± 0.2	1
YEpFAT7.5 + Leu	10.4 ± 0.1	6.9 ± 4.4
YEpFAT7.5 – Leu	56.7 ± 21.3	31.4 ± 5.1

SBQ, n = 3.

Southern, n = 2.

We present a strategy for quickly identifying all different plasmids contained within a population of selected clones. To facilitate the analysis, we have modified a technique previously used in mammalian cells for the quick quantitation of gene copy number in fission and budding yeast. SBQ has also been used to identify *S.pombe* strains that carry spontaneous amplifications of the *sod2* gene (Patterson, Nurse and Stark, in preparation), and can be used in any application in which yeast strains contain different numbers of any DNA sequence, either chromosomal or episomal. We foresee its utility in many other applications, including the identification of novel clones in yeast two-hybrid screens.

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REFERENCES

- Hagan, I., Hayles, J. and Nurse, P. (1988) *J. Cell Sci.*, **91**, 587–595.
- McIntyre, P. and Stark, G.R. (1988) *Anal. Biochem.*, **174**, 209–214.
- Guerrini, A.M., Ascenzioni, F., Gallo, R. and Donini, P. (1993) *Nucleic Acids Res.*, **21**, 6047–6048.
- Sazer, S. and Sherwood, S.W. (1990) *J. Cell. Sci.*, **97**, 509–516.
- Sazer, S. and Nurse, P. (1994) *EMBO J.*, **13**, 606–615.
- Matsumoto, T. and Beach, D. (1991) *Cell*, **66**, 347–360.
- Church, G.M. and Gilbert, W. (1984) *Proc. Natl. Acad. Sci. USA*, **81**, 1991–1995.
- Brun, C., Dubey, D.D. and Huberman, J.A. (1995) *Gene*, in press.
- Heyer, W.-D., Spiczki, M. and Kohli, J. (1986) *Mol. Cell. Biol.*, **6**, 80–89.
- Runge, K. and Zakian, V.A. (1989) *Mol. Cell. Biol.*, **9**, 1488–1497.