Chapter 20

Determination of Plasmid Copy Number in Yeast

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1. Introduction

Autonomously replicative plasmids are widely employed as vectors for the expression of cloned genes in yeast *(1)*. A number of different kinds of such plasmid vectors exist, which fall into three classes: *ARS* (autonomously replicating sequence) based plasmids, 2-μm-based plasmids, and *ARS* plasmids containing a centromere.

ARS-based plasmids are highly unstable because of maternal bias: this is an unequal inheritance of the vector at cell division, in which the plasmid tends to stay in the mother cell rather than entering the budding daughter cell. This generates plasmid-free segregants, but can also result in very high copy numbers in the plasmid-containing cells. *ARS*-based plasmids, can, in addition, integrate and in this way become stably maintained.

Addition of a centromere (*CEN*) counteracts the instability of *ARS* plasmids, but although these *ARS-CEN* plasmids exhibit greater stability, they are present at only one to three copies per cell.

The most widely employed vectors are those based on the yeast 2-μm plasmid. This is a naturally occurring plasmid present in most strains of *Saccharomyces cerevisiae*. It is normally present in the cell at between 40–100 copies per cell and is stably maintained through mitosis and meiosis by its partitioning system.

Essentially, two types of 2-μm-based vector exist: those containing the entire 2-μm sequences, which approach the stability of the native

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plasmid, and those that incorporate only those sequences required for autonomous replication and stability, *ORI* and *STB*, respectively. In this instance, the *trans*-acting factors required for 2-μm partitioning need to be provided by endogenous 2-μm plasmids within the host cell. Unequal segregation occasionally can still occur during mitosis, and such vectors are therefore very highly, but not completely, stable.

The 2-μm *ORI-STB*-based plasmid vectors are often present at around 20 copies per cell, although this can vary greatly. A vector containing a *leu2-d* marker, for example, which has a defective promoter, can drive the copy number of the plasmid up to around 100 copies per cell, when it is grown under selection.

Since most plasmid vectors are not 100% stable, any copy number determination must also involve the estimation of the stability of the vector. Two copy number values can therefore be derived: the average copy number per cell in the population and the copy number per plasmid-containing cell.

The expression level of a plasmid-borne gene cannot be interpreted meaningfully without knowing the plasmid copy number, as gene expression level often directly reflects gene dosage. Regulatory or other factors may determine whether the level of expression of a particular gene is linearly related to its copy number. Often, particularly at high copy numbers, the relationship does not hold true, indicating limiting factors affecting expression. In some cases, the gene product may have a toxic effect on the cell, and this often is manifested by decreased plasmid copy number and stability.

The method employed for plasmid copy number determination in yeast involves the use of blotting-hybridization techniques to determine the amount of a plasmid-borne yeast gene (normally a selectable marker) relative to the corresponding single copy chromosomal gene, as outlined in Fig. 1. Total DNA is isolated and then restricted with an endonuclease that cleaves the DNA on either side of both the plasmid-borne and chromosomal marker. This digestion must be arranged to generate marker fragments that are dissimilar in size from the plasmid and chromosome (usually the plasmid marker fragment is larger). After subsequent agarose gel electrophoresis, the genomic DNA gel is Southern blotted and hybridized with a [^{32}P]-labeled marker fragment. This probe will hybridize to both the single copy chromosomal fragment and the marker gene from the plasmid. After autoradiography, two differently sized bands will

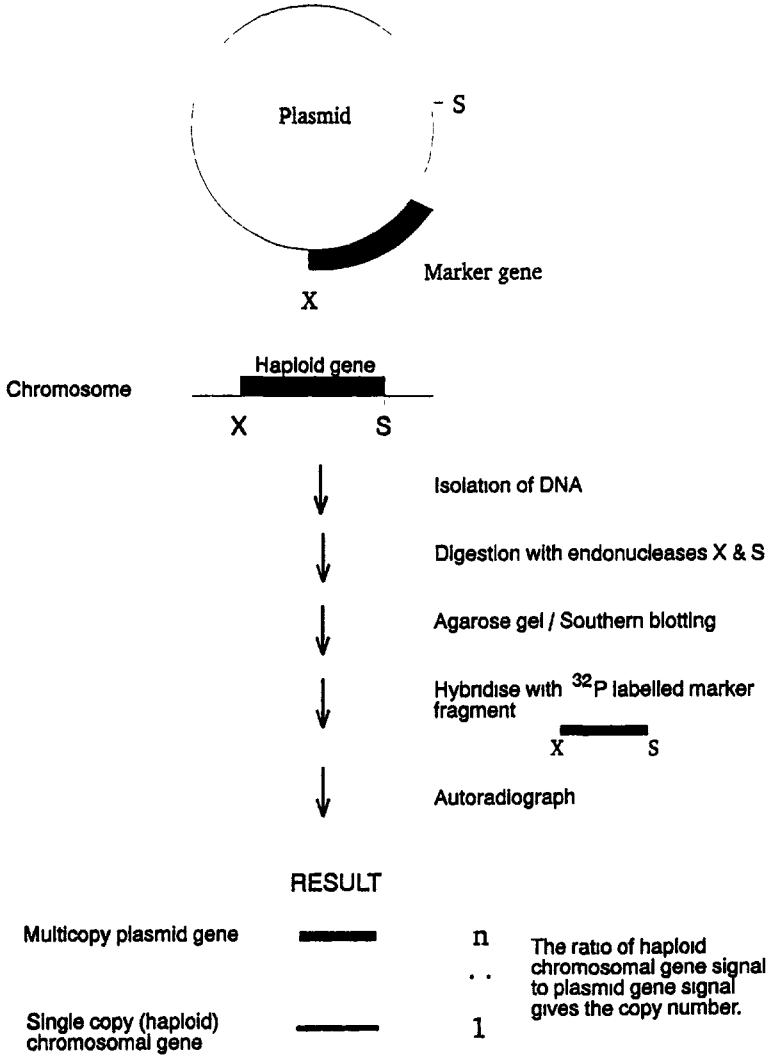

Fig. 1. A schematic representation of the method employed for copy number determinations.

be apparent on the X-ray film. The ratio of the amount of bound probe to the chromosomal and plasmid bands gives a measure of the copy number.

There are several important stages in this method. The initial DNA extraction method is perhaps the most important, since it is crucial that the isolation does not cause uneven losses of chromosomal or plasmid

DNA, or the results will be meaningless. Total cellular DNA must therefore be isolated by a method that will not incur such losses. This generally means using a serial extraction/precipitation procedure such as that described by Cashmore et al. *(2),* Piper, or Evans and Bignell, in this volume (Chapters 11 and 18, respectively). A second highly important part of the protocol is obtaining complete restriction of the total DNA. This can be a problem as the DNA obtained by the aforementioned method is not totally pure and, consequently, digestion efficiency of the endonuclease is often significantly reduced. Other stages in the method that demand particular care are:

1. The requirement for an even and complete Southern blot, so that it will be quantitative for the two DNA fragments;
2. Setting up the hybridization to avoid nonspecific background contamination of the Southern blot filter; and
3. An accurate method of measuring the amount of bound hybridization probe to the bands on the filter.

2. Materials

1. Total DNA is required for the method, which has been isolated from the plasmid-containing cells by a procedure that does not lead to uneven loss of plasmid DNA. The method described by Cashmore et al. *(2)* involves the digestion of the yeast cell walls to produce spheroplasts, which are then lysed, followed by phenol-chloroform extraction to remove proteins, and ethanol precipitation to recover total nucleic acids. RNA is removed by RNase treatment. The DNA obtained in this way requires additional treatment to ensure it can be fully restricted. The DNA sample is normally incubated at room temperature for 20 min with 3 U of porcine α-amylase, followed by a 30-min incubation at 55°C with 1 U of amyloglucosidase, isolated from *Aspergillus niger.* These two enzymes incorporate different activities to break down contaminating carbohydrates, which are present in this type of preparation. (Volumes 2 and 4 in the Methods series can also be consulted, and *[5].*)
2. Materials for DNA restriction and agarose-gel electrophoresis. Sample loading buffer at 5X final concentration is composed of 25% (w/v) Ficoll 400, 0.125% (w/v) bromophenol blue, and 0.25% (w/v) xylene cyanol FF, in gel buffer.
3. TE buffer: 10 m*M* Tris-HCl, 1 m*M* EDTA, pH 8.0.
4. Solution O: 1.25*M* Tris-HCl, 0.125*M* $MgCl_2$, pH 8.0 (store at 4°C).
5. dNTPs: 100 m*M* solutions of dATP, dTTP, and dGTP (store at –20°C); [α-^{32}P]dCTP (370 MBq/mL) (e.g., Amersham [Arlington Heights, IL] PB10475).

6. Solution A: 1 mL solution O, 18 µl β-mercaptoethanol, 5 µL each of dATP, dTTP, and dGTP.
7. Solution C: Suspend 50 A_{260} U of random hexadeoxynucleotides (Pharmacia [Uppsala, Sweden] 27-2166-01) in 550 µL TE, to give a concentration of 90 A_{260} U/mL (store at –20°C).
8. OLB buffer: Mix solutions A:B:C in the ratio 10:25:15 (total 50 µL, sufficient for 10 labeling reactions) (store at –20°C).
9. Stop buffer: 20 m*M* NaCl, 20 m*M* Tris-HCl, pH 7.5, 2 m*M* EDTA, 0.25% SDS, 1 µ*M* dCTP.
10. Acetylated bovine serum albumin (BSA) (Gibco [Gaithersburg, MD] BRL 540-5561UA).
11. Sephadex G50, medium grade (Pharmacia 17-0043-01).
12. Polyallomer wool (aquarium filter, available from pet shops).
13. Materials for Southern blotting and hybridization (*see* for example, Chapter 9, ref. *5*, and vols. 2 and 4 of the Methods series). A wide assortment of containers and incubators can be used for hybridizations; however, if possible, it is recommended that dedicated equipment, designed specifically for this task (e.g., Hybaid, Teddington, UK), be used, as this optimizes both effectiveness and protection from irradiation. Hybridization conditions that keep background radioactivity to a minimum are essential. The recommended hybridization solution for this purpose consists of 1% BSA (fraction V), 1 m*M* EDTA, 0.5*M* NaH_2PO_4, pH 7.2 and 7% SDS *(3)*. It is advisable to ensure that the BSA has fully dissolved before the addition of SDS, which is normally added as a 20% solution. The solution normally employed for washing is 4X SSC (35 g sodium chloride, 17.64 g sodium citrate/L in distilled water) containing 0.1% SDS.
14. The estimation of the band ratios requires a scintillation counter set for [^{32}P] and radioactive ink, which is required for markings to enable autoradiographic bands to be aligned with their filter location. The ink is made by the addition of radioactive dCTP to 500 µL writing ink to give approximately 1 × 10^6 cpm. (*See* Note 13 for alternative method.)

3. Method

1. Restrict approximately 5 µg of total genomic DNA with restriction endonucleases that will cleave the chromosomal and plasmid DNA on either side of the yeast marker, so that it liberates a fragment containing the same gene of different size from the chromosome (Fig. 1). Since the DNA must be fully restricted for this method, it is often beneficial to restrict the DNA in a large volume, approx 400 µL, to dilute impurities that are normally present in the DNA sample. Additionally, extra endonucleases can be added, which cut outside the marker region; these do not affect the two

marker fragment sizes, but break down the chromosomal DNA and make it more amenable to digestion by the endonucleases that do count. Furthermore, prolonged digestion, preferably overnight, usually is necessary. In some cases, subsequent addition of further enzyme and further prolonged digestion may be necessary to obtain fully restricted DNA The extent of the digestion is determined by agarose-gel electrophoresis *(see the following)*.

2 Since the restriction is in a large volume, it is necessary to ethanol precipitate the sample to enable it to be subsequently loaded on a gel. This is done by the addition of 40 µL 3*M* sodium acetate, pH 5.6, and 800 µL ethanol; after mixing, the sample is placed in a dry ice/ethanol bath for 5 min. The DNA is pelleted by spinning at 1300 rpm (11,500*g*) for 15 min in a benchtop microfuge. The supernatant is discarded and the pellet washed by the addition of 400 µL of 70% ethanol. It is then repelleted by spinning at 1300 rpm for 5 min, then dried under vacuum, and resuspended in 20 µL of TE buffer.

3. For electrophoresis, to each of the samples a 25% volume of 5X loading buffer is added (resulting in a 1X concentration) and then they are loaded into the wells of a 0.8% agarose gel. Electrophoresis should be sufficient to obtain a good separation between the plasmid and chromosomal marker fragments. Figure 2 illustrates the difference between completely restricted and incompletely restricted total DNA samples following agarose gel electrophoresis

4 The gel is Southern blotted (*see* Note 1 and Chapter 9 as well as vol. 2 in this series, and ref. *5*). Then the blot filter is prehybridized by incubation in the hybridization buffer for 30 min at 65°C, with continuous agitation. The volume of the buffer for prehybridization is not important, provided sufficient buffer is present to keep the filter covered; usually an excessive volume is used at this step.

5. The auxotrophic marker gene present on the plasmid is employed as the hybridization probe. It is liberated from a sample of purified plasmid DNA by digestion with an appropriate restriction endonuclease and then separated on a low-gelling temperature agarose (0.8–1.0% w/v) gel. The fragment is cut out of the gel and placed in a 1.5-mL microfuge tube. Water is added at a ratio of 1.5 mL/g of gel slice. The tube is then placed in a 100°C bath for 7 min to melt the agarose and denature the DNA (*see* Note 2).

6. Afterward, the tube is placed directly into a 37°C water bath, where it can be kept for 10–60 min prior to use for the labeling reaction.

7. An aliquot of the fragment DNA is [^{32}P]-labeled to a high specific activity by the random primer method *(4)*, as follows:
   a. To a microfuge tube, at room temperature, add the following in the stated order: 16 µL DNA fragment (25 ng, made up to volume by addition of water); 5 µL OLB buffer, 1 µL acetylated BSA (10 mg/mL);

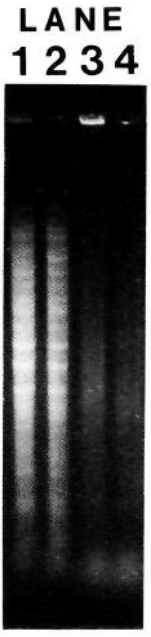

Fig. 2. Restricted genomic DNA samples run on a 0.8% agarose gel. Tracks 3 and 4 show samples that are incompletely restricted and underloaded and are not adequate for a copy number determination. Tracks 1 and 2 show samples that have been fully restricted, but are slightly overloaded.

2.5 µL α-[^{32}P] dCTP (10 µCi/µL = 0.37 MBq/µL); 0.5 µL Klenow (large fragment) DNA polymerase I (2 U).

b. Incubate at room temperature for at least 5 h, or overnight if more convenient.
c. Stop reaction by adding 100 µL stop buffer.

8. The labeled DNA probe fragments are separated from the unincorporated nucleotides (*see* Note 3) by Sephadex G50 minicolumn fractionation, as follows:
   a. Remove the long, narrow end-tip of a glass Pasteur pipet by etching at the "neck" constriction where it first becomes narrow, using a diamond-tipped pen, and then breaking off the long narrow-stemmed tip.
   b. Insert a small amount of sterile polyallomer wool into the top of the residual Pasteur pipet stem and, using the detached narrow tip as a "ramrod," push the wool down to the "neck" constriction, to form a bung. It is important that this bung should not be too tightly compacted, as otherwise liquid will not flow through it; yet it must be tight enough to retain the G50 particles.

c. Vertically clamp the Pasteur pipet to form a column and place a small beaker underneath. Then load the G50 column, as follows: Shake the bottle to suspend the particles; take a new Pasteur pipet, with an attached filling device, and quickly fill the pipet with the suspension; insert the tip of the pipet into the vertical column, right down to just above the polyallomer wool bung; then discharge the G50 into the column, raising the tip of the pipet as the column fills, so that it stays at the surface. It is important that this is done quickly, before the G50 particles settle out. It is also important that no air bubbles are formed in the column material (*see* Note 4). Fill the column to ~1.5 cm from the top. Then wash the column with TE buffer by filling the small 1.5-cm-long reservoir and allowing it to drain away. Repeat twice and check that the buffer flows through the column freely; otherwise, it will not be satisfactory and will have to be redone.

d. Replace the beaker under the column with a rack containing 1.5-mL microfuge tubes, numbered 1–15. Add the stopped labeling reaction (125 µL) to the top of the column and collect the liquid displaced from the column in the first tube. Add 125 µL of TE to the top of the column and collect the displaced liquid in the second tube. Repeat the process for the remaining tubes.

e. Measure the amount of radioactivity in each fraction by Cerenkow counting. There should be two peaks: The first one (starting about fraction 5 or 6) contains the labeled fragment DNA; the second contains the unincorporated nucleotides. Pool the probe peak fractions (usually about four fractions). In total this should give rise to $>10^6$ counts/min; a good high-specific activity labeled probe will give rise to ~10^7 c.p.m.

9. Decant the prehybridization buffer from the filter and replace with fresh hybridization buffer, in a minimum volume necessary to cover the filter.
10. Denature the probe by heating at 100°C for 7 min (*see* Note 2). Add the denatured probe directly to the hybridization buffer and ensure that it is mixed in thoroughly. Hybridize at 65°C for 18 h, using an agitation system that provides continuous, even distribution of the probe solution over the surface of the blot filter. Once this is complete, the filter should be washed six times, each at 65°C for 30 min using the washing solution, which should also be kept at 65°C.
11. After hybridization and washing, the filter is blotted dry between 3MM Whatman (Maidstone, UK) filter paper. It is then sandwiched between two pieces of cling film and adhesive labels affixed around the periphery of the filter. Radioactive ink markings are then made on the labels using a toothpick and once dry, further cling film is used to cover these markings. The filter is then exposed to X-ray film.

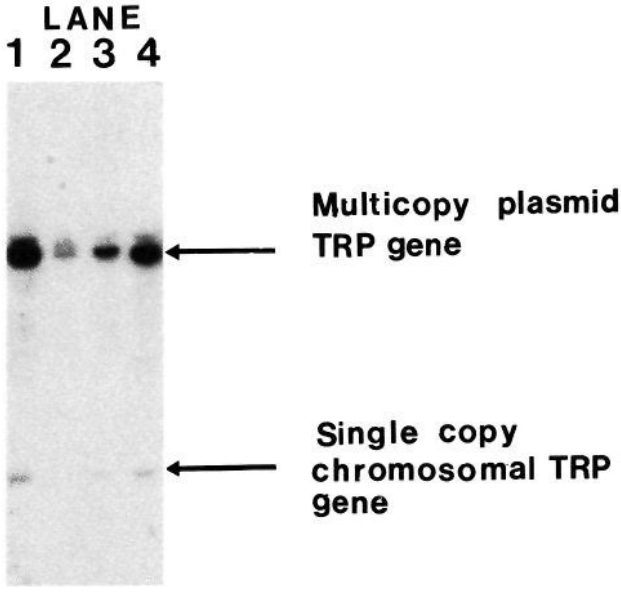

Fig. 3. An autoradiograph showing the bands obtained from a filter containing restricted genomic DNA samples probed with a *TRP* probe. Lanes 1 and 4 are overexposed, but can be employed to facilitate the excision of the single copy band from the filter. The exposure of lane 2 is that required to remove the plasmid *TRP* band.

12. On developing the film, two bands should be visible (*see* Fig. 3 and Note 5). It is usually arranged so that the upper band (larger fragment) represents the marker present on the plasmid, whereas the lower band is the single copy chromosomal band. The X-ray film image is then aligned over the cling film covered filter, using the radioactive ink markings. The edges of the band areas on the X-ray film are pierced with a syringe needle through to the filter, which enables the areas of the filter containing the two bands to be cut out, using the holes as a guide. The cling film is peeled away from both sides of each filter fragment, which are subsequently placed into a separate scintillation vial. To each vial, add 2.5 mL of nonaqueous scintillation fluid and count in a scintillation counter (*see* Notes 6–9).
13. The ratio between the counts for the upper and lower band areas can be calculated and this is the average plasmid copy number for cells in the population (*see* Notes 10 and 11). If the average copy number per plasmid-containing cell is required, then the average copy number must be adjusted to take into account the percentage of plasmid-free segregant cells in the population (*see* Note 12).

4. Notes

1. Nylon filters (such as Hybond-N [Amersham]) are recommended for the Southern blotting of the DNA, since the nylon membrane is more robust than nitrocellulose and is therefore easier to handle.
2. The 100°C bath can be boiling water, a heated polyethylene glycol bath, or dry-block heater. It is important that the caps of microfuge tubes placed at 100°C are pierced with a needle-hole prior to heating, as otherwise internal pressure will cause the cap to fly off in an explosive manner.
3. Although the probe may be used directly following the labeling reaction, this is inadvisable, as it usually results in a high background owing to unincorporated nucleotides.
4 If a trapped air bubble forms in the G50 column, fill the column with TE buffer, insert a Pasteur pipet (attached to a filling device) and gently swirl to suspend the GSO particles above the bubble; then suck the air bubble into the Pasteur pipet. The column material will settle again and surplus buffer drain away.
5 If additional bands are observed on the autoradiograph, this is usually indicative of partial restriction if 2-μm-based vectors are used Alternatively, it could be a result of an integration, particularly if an *ARS* based vector has been used
6. It is recommended that scintillation vials are treated with an antistatic device or that static is removed by some other means, prior to scintillation counting. Static can interfere markedly with the counting process and cause abnormally high counts, which is a common source of error. It is also advisable to count the vials several times, to be sure that static interference has been eliminated.
7. A clean radioactivity-free background is crucial, as background radioactivity can interfere with the counts obtained and hence the accuracy of the determination Most affected will be the single-copy chromosomal band counts since these are the weakest. If the background is not clean, it may be possible to subtract the background radioactivity counts from the band counts. To estimate background, excise an appropriate piece of filter of similar area to the band, scintillation count, and subtract the scintillation counts obtained from the fragment band counts
8. To ensure that the whole band area has been excised from the filter, the filter can be re-exposed to X-ray film. If band areas can still be observed, the data obtained clearly will not be correct.
9. To aid the excision of the filter bands, a number of autoradiograph exposures generally are required. Generally, longer exposures will be required for the single copy chromosomal band and shorter exposures for the multicopy plasmid fragment (Fig. 3)

10 Scintillation counting has been shown to be accurate for copy-number determinations of up to at least 80 plasmids per cell.

11. Densitometer scans of autoradiographs are not so easy to employ accurately for estimating plasmid copy numbers, as the signal from the multicopy plasmid fragment is usually manyfold greater than that of the single chromosomal fragment. Consequently when the single-copy band is visible, the plasmid band is overexposed on the autoradiograph, so that its density range has gone beyond the reactive range.

12. To determine the percentage of plasmid-containing cells, a microbiological plate assay can be performed. Ideally, this should be performed with cells used to make the total DNA preparation, or failing that, cells grown under identical conditions. The cells are plated out onto nonselective agar medium, to give a number of plates containing a maximum of 300 colonies per plate The plates are subsequently replica-plated onto selective agar medium and the number of colonies growing, represent plasmid-containing cells. These are scored and the percentage calculated.

13 The recent development of phosphoimager technology now enables the amount of radiolabeled probe bound to restriction fragment bands on filter blots to be quantified directly. If available, this is easier than the scintillation counting method described.

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