Genetic Techniques for Biological Research
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ISBNs: 0-471-89921-6 (Hardback); 0-470-84662-3 (Electronic)

2 Techniques in Yeast Cell and Molecular Biology

The techniques used to study *Saccharomyces* are not unique to *Saccharomyces* but have been adapted where possible to the special needs of this small eukaryote. Those methods that are most commonly used by researchers are described in this chapter and are presented in enough detail to allow one to understand the basics and read the literature. Additional reading material and techniques manuals are listed at the end of the chapter for those needing more information.

CELL FRACTIONATION

The study of subcellular components often requires purifying a large amount of a particular component. One might be interested in isolating and purifying for further analysis the components of a complex unit like a ribosome, proteasome, or spliceosome. Or one might want to measure the metabolic activity of organelles such as the mitochondrion or peroxisome. Cell fractionation methods have been developed that allow the researcher to separate relatively pure samples of subcellular components in reasonably active states. Lloyd & Cartledge (1991) and Zinser & Daum (1995) review methods of isolation of yeast subcellular organelles. Additional methods for specific organelles are listed in Walker (1998).

PREPARATION OF THE CELL EXTRACT

The first step is to rupture the cells and release the contents. A variety of methods are available for *Saccharomyces* that are similar to those used for other types of cell with variations to accommodate the rigid cell wall and the high level of protease activity found in *Saccharomyces*. Cells are grown under the appropriate culture conditions, harvested by centrifugation or filtration, and resuspended in a buffered salt solution containing protease inhibitors. A number of mechanical, chemical, or enzymatic methods are available for breaking open the cells and releasing the contents. A French press is used for large-sized samples (several grams of cells). This device forces cells through a small hole under pressure. Cells can also be ruptured by aggressive agitation of a cell suspension with glass beads. This can be done on small samples simply by vortexing the cell–glass bead suspension or by shaking the suspension in any of a variety of devices designed specifically for this purpose. Alternately, the *Saccharomyces* cell wall may be stripped using glusulase or zymolyase, enzymes that attack the structural components of the cell wall, after which the cells are burst by altering the osmolarity of the cell suspension.

DIFFERENTIAL-VELOCITY CENTRIFUGATION

Differential-velocity centrifugation separates subcellular components based on size/shape and density. The theory is that the larger more dense components will pack at the bottom of a centrifuge tube faster and at lower speeds then smaller less dense ones. Initially, the total cell extract is centrifuged at a low speed for a short time to remove unbroken cells. In the next steps, the speed and time of centrifugation are progressively increased removing some components (packing them at the bottom of the tube as a precipitate) and leaving others in the supernatant at each step. The final supernatant, after the step-wise removal of nuclei, mitochondria, vacuoles, peroxisomes, plasma membrane and vesicles, endoplasmic reticulum, and ribosomes, is called the soluble fraction and contains soluble proteins and other small molecule components of the cytosol such as tRNAs.

EQUILIBRIUM DENSITY GRADIENT CENTRIFUGATION

Equilibrium density gradient centrifugation separates subcellular components based only on their density. For this method, one must first prepare a density gradient in a centrifuge tube. A nonionic molecule like sucrose, glycerol, or Ludox is used to vary the density of the buffer solution. The concentration of the molecule is varied, and therefore the density of the solution, and the concentration, is greatest at the bottom of the centrifuge tube and decreases slowly towards the top of the tube. Special devices are available for making these gradients. A step gradient can also be prepared. Here a series of solutions of different concentration (30%, 25%, 20%, etc.) are layered on top of one another with the step with the highest concentration at the bottom.

The cell extract is layered at the top of the gradient and the tube is subjected to centrifugation at high speed for several hours. During this time the different subcellular components move down the tube until they reach the position in the density gradient that corresponds to the density of the component and will remain in this position indefinitely. In a step gradient, the organelle will position itself between two steps. Using a fraction collector, individual small samples are gently removed from the tube starting at the top or bottom in a manner that does not disturb the gradient. The samples are then analyzed by Western analysis, electron microscopy (EM), or biochemical assay to identify the subcellular location of a particular protein. The purified fractions also can be used for other biochemical studies.

Figure 2.1 illustrates the results from a typical equilibrium density gradient separation experiment. Western analysis (see below) was used to identify the fractions containing the protein of interest, Gap1p (the general amino acid permease) in this experiment, and biochemical assays of marker enzymes from the different subcellular compartments were carried out to identify the location of the compartment in the gradient.

The purity of the subcellular fractions is often at issue. Samples obtained by differential-velocity centrifugation are generally not considered to be a highly homogeneous purified product. No matter which method is used it is essential to test the purity. The samples can be observed by EM to determine the presence of contaminating components. Marker enzymes or proteins characteristic of a particular

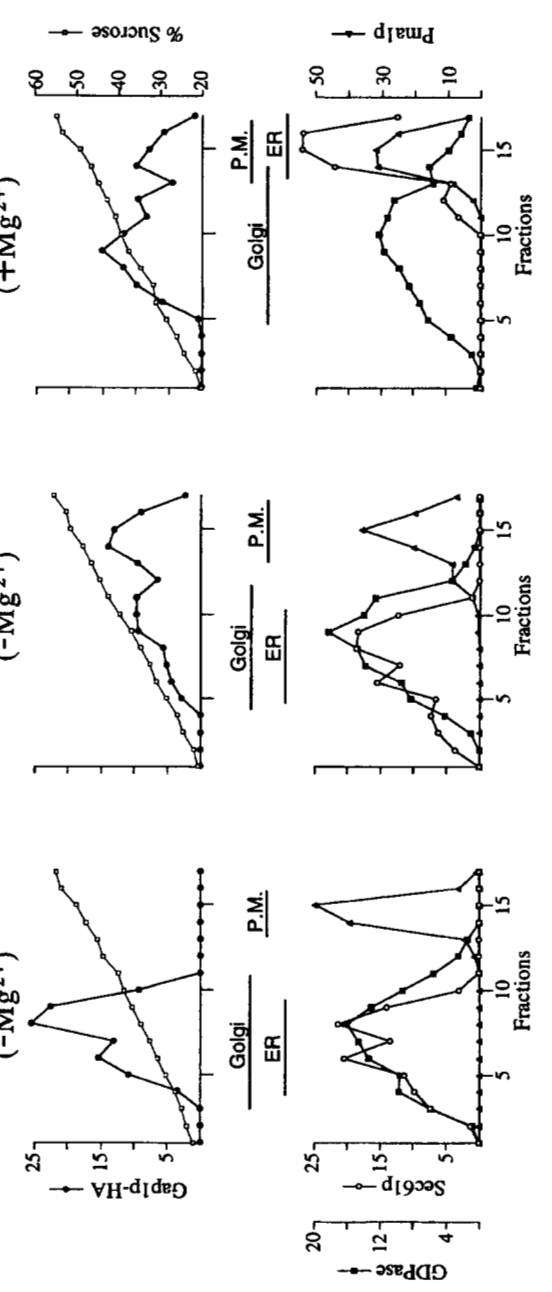

Figure 2.1 Equilibrium density gradient analysis. Shown are the results of an equilibrium density gradient analysis carried out to determine the subcellular localization of Gap1p, the general amino acid permease, under different culture conditions (glutamate or glutamate transferred to urea). (Taken from Roberg *et al.*, 1997.) The density gradient is 20%–60% sucrose with and without Mg^{2+}. The upper row of panels shows, for each fraction, the sucrose concentration (open circles) and the relative levels of Gap1p-HA (hemagglutinin-tagged Gap1 protein) as determined by Western analysis. The lower row of panels shows the relative levels of Pma1p (plasma membrane marker) and Sec61p (endoplasmic reticulum marker) as determined by Western analysis and the activity of GDPase (Golgi marker) as determined by enzymatic assay. The horizontal bars above the lower set of panels indicates the position in the gradient of each membrane type. Reproduced from *The Journal of Cell Biology*, 1997, by copyright permission of The Rockefeller University Press

subcellular component can be assayed (either biochemically or by Western analysis) to assess the purity of a sample. Examples of such marker enzymes can be found in Kreutzfeldt & Witt (1991), Griffin (1994), and are listed in Walker (1998).

MICROSCOPY TECHNIQUES

In standard light (bright-field) microscropy, a beam of light from a source (usually placed below the specimen) is focused onto a specimen, passes through the specimen, is focused by a second series of lenses, and is then observed by eye or photographed. Samples are usually fixed to denature the proteins in the specimen, sectioned into thin slices (if needed), attached to a solid substrate (the slide), and stained using any of a series of chemicals that specifically react with cellular components such as DNA or protein. It is hoped that these treatments do not significantly alter the subcellular structures or their organization. Because of the small cell size of *Saccharomyces*, bright-field microscropy is very uninformative and researchers have developed other methods for visualizing subcellular structures.

FLUORESCENCE MICROSCROPY, IMMUNOFLUORESCENCE, AND GFP

Fluorescence microscropy allows the researcher to localize a specific protein to its subcellular site by providing a mechanism for a fluorescent dye to specifically bind to a particular protein or subcellular component. A fluorescent molecule is one that becomes activated by absorbing light of one wavelength (the excitation wavelength) and then returns to the resting state by emitting light at a longer wavelength (fluorescence wavelength) still in the visual range. The fluorescent molecule is visualized in the specimen using a fluorescence microscope that is designed to shine light of the excitation wavelength on the specimen (usually from above) and to allow one to observe the emitted light (again from above). The emitted light is passed through filters that block all except light of the fluorescent wavelength before it is viewed or photographed.

The most commonly used fluorescent dyes are rhodamine (which emits light in the red range) and fluorescein (which emits light in the green range). In immunofluorescence the dye is covalently conjugated to an antibody specific to the protein of interest. Cells are fixed and made permeable to the antibody. The sample is then treated with the fluorescent dye-conjugated antibody and the antibody then binds to its target antigen/protein. The position of the antibody is visualized using a fluorescence microscope. Microscopes can be fitted with several different sets of filter pairs thereby allowing one to observe, in a single cell, the location of two or even three antibodies each conjugated to a different dye and emitting light of a different fluorescent wavelength. In this way one can compare the localization of two or more different proteins within a single cell. For further information, the *Handbook of Fluorescent Probes and Research Chemicals* from Molecular Probes, a supplier of such reagents, is an excellent resource (http://www.probes.com).

A number of variations on the immunofluorescence theme have been developed for use in *Saccharomyces* and other cells. The fluorescent dye can be conjugated to a

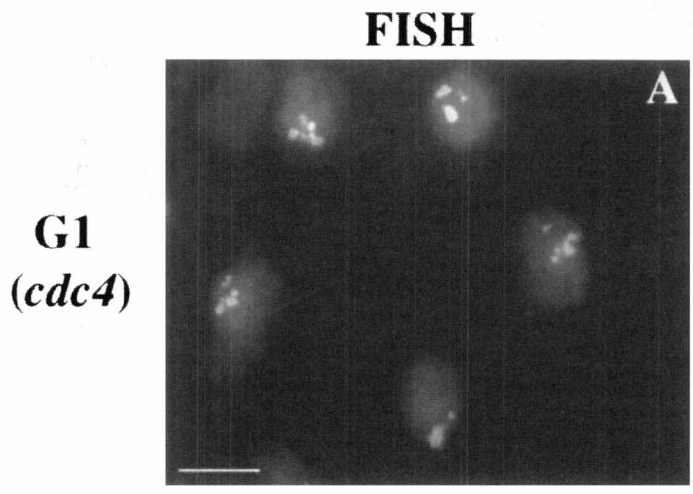

Figure 2.2 Fluorescence *in situ* hybridization—FISH. Shown is the result of FISH analysis of centromere localization in the nuclei of diploid cells arrested in G1 of the cell cycle (using a temperature sensitive mutation of *cdc4*). Chromosomal DNA was stained with propidium iodide (grey) to show the outlines of the nucleus. Oligonucleotide probes were made from sequences tightly linked to *CEN1*, *CEN4*, and *CEN16* and thus should hybridize to six chromosomal sites in these diploid cells. Each probe is tagged with an antigenic compound called digoxigenin and the location of the tagged oligonucleotide is visualized by immunofluorescence using dye-conjugated antibodies. The photograph shows that the centromeres are clustered and, using immunofluorescence to localize the position of the microtubule attachment to the nuclear envelope (data not shown), the authors demonstrate that the clustering is in the region closest to the spindle pole body. Taken from Guacci *et al.* (1997). Reproduced by permission of the American Society for Cell Biology

molecule other than an antibody that specifically interacts with a particular cellular component. For example, phalloidin is a small cyclic peptide derived from the death cap fungus *Amanita phalloides*. It specifically binds to polymerized actin microfilaments. Rhodamine-conjugated phalloidin will enter permeablized cells and bind to the actin cytoskeleton thereby allowing this complex meshwork to be visualized by fluorescence microscropy (see Chapter 3, Figure 3.9). Fluorescence *in situ* hybridization, or FISH, uses nucleic acid hybridization to bind the fluorescent dye to specific DNA sequences in chromosomes. The fluorescent dye is conjugated to a DNA oligonucleotide probe, introduced into cells, and allowed to hybridize to the complementary site(s) on the chromosomes. Examination by fluorescence microscropy allows these positions to be visualized. FISH has been used to demonstrate the location of chromosomal telomeres in interphase *Saccharomyces* nuclei and to study chromosome separation during cell division (Figure 2.2). Finally, Walker (1998) lists several cytofluorescent dyes for yeast microscropy that interact with specific cellular molecules. One example is calcofluor white that binds to the chitin found at the site of bud scars. Another is DAPI (4,6-diamidino-2-phenylindole) that binds specifically to DNA and can be used to visualize the nucleus and even mitochondrial DNA (Figure 2.3).

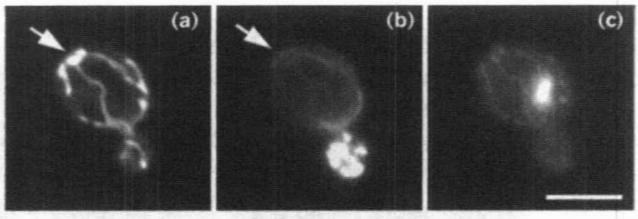

Figure 2.3 Visualization of mitochondria by different methods. Cells were grown to the midlog phase on a rich medium with galactose as the carbon source. Panel (a) shows the mitochondria stained by immunofluorescence. In panel (b) Alexa-phalloidin was used to detect polymerized actin. Panel (c) shows cells stained with the DNA binding dye DAPI. The large bright spot in the mother cell in panel (c) is the nucleus and the less brightly staining spots are the mitochondria, indicating that several copies of mtDNA can be found. Note that the mitochondria accumulate in the mother cell distal to the site of bud emergence and appear to lie along the length of the actin cables. Taken from Yang *et al.*, 1999. Reproduced with permission from Elsevier Science

Perhaps the most powerful advance in fluorescence microscropy came with the development of GFP, by Martin Chalfie and coworkers (Chalfie *et al.*, 1994). GFP is responsible for the bioluminescence exhibited by the jellyfish *Aequorea victoria*. What makes GFP such a valuable tool for the study of cell biology in the age of recombinant DNA technology? GFP fluorescence occurs *in vivo* in the living cell simply by shining light of the correct excitation wavelength on the cells. Since there is no fixation or staining necessary, it is believed that a more accurate view of the *in vivo* situation is obtained. The amino acid sequence and structure of GFP is solely responsible for its fluorescent activity, and no exogenously added cofactors or exogenously produced modifications are required. In fact, the GFP chromophore is synthesized autocatalytically by a series of intramolecular reactions involving the side-chains of several amino acid residues in the GFP sequence. Therefore, whether expressed in the native organism *Aequorea victoria* or heterologously in the cells of any other species, GFP undergoes this autocatalytic reaction to produce the chromophore and emits light at its characteristic fluorescent wavelength. GFP is nontoxic and thus can be expressed in all cell types. Most importantly, GFP fusions are usually functional and results obtained with these fusions are therefore biologically relevant.

To use GFP to study the localization of any protein, one constructs an in-frame fusion of the GFP ORF to that encoding the protein of interest. Plasmid vectors containing the full ORF of the GFP gene are available (Niedenthal *et al.*, 1996). These contain multiple cloning sites positioned so as to allow the gene of interest to be inserted either upstream or downstream of the GFP. For use in *Saccharomyces*, a high-level constitutive promoter, such as the *ADH1* promoter, is usually included. Using PCR-based methods, the sequence of the GFP ORF can be inserted at any position in any gene of interest. The GFP-fusion construction is then introduced into cells and transformants will produce a GFP-fusion protein whose subcellular localization can be visualized by fluorescence microscropy. In *Saccharomyces* time-lapse photography of cells expressing a GFP-fusion protein has been used to

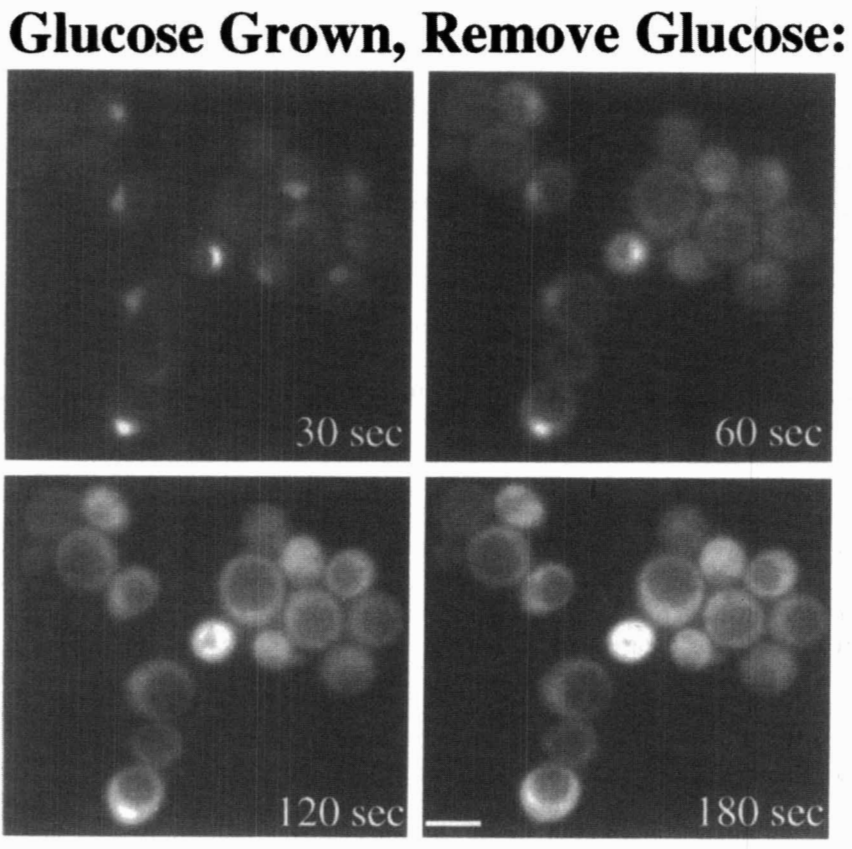

Figure 2.4 Green fluorescent protein fusions for visualization of living cells. The photo shows a time-course of Mig1 repressor exit from the nucleus. These cells are expressing a fully functional Mig1p–GFP fusion protein. Following growth on glucose, the cells are harvested and placed in medium lacking glucose. The first panel shows the cells 30 seconds after glucose removal. Mig1p–GFP is seen localized to a discrete subcellular site that, by DAPI staining, is shown to be the nucleus (data not shown). Very little fluorescence is observed in the cytoplasm. The same cells are photographed every 30 seconds for 1.5 minutes during which time the fluorescence can be seen to leave the nucleus and accumulate in the cytoplasm. The large poorly fluorescent region seen in the cytoplasm is the vacuole (based on Nomarski optics). Taken from De Vit *et al.* (1997). Reproduced by permission of John Wiley & Sons Publishers

demonstrate changes in subcellular location of the fusion protein resulting from changes in growth conditions, such as carbon source or temperature. An example of this type of analysis is shown in Figure 2.4, which demonstrates the time-course of Mig1p GFP-fusion protein exit from the nucleus after cells are shifted from a glucose-containing medium to a medium lacking glucose. To control for the possibility that fusion to GFP inactivates some or all of the functions of the protein under study, it is essential that the GFP-fusion construction be tested for its ability to complement the mutant phenotypes of a null allele of the gene of interest.

The introduction of a limited number of amino acid changes in the wild-type GFP gene has allowed for improvements in the fluorescence characteristics of GFP, such as increased emission of light or spectral resolution, and has even produced mutant products that absorb and emit light at slightly different wavelength ranges (for example, blue fluorescent protein or BFP). The coexpression of GFP- and BFP-fusion genes allows the researcher to compare the subcellular localization of these two proteins *in vivo*. Several different variants are now available.

CONFOCAL SCANNING MICROSCROPY

Most of the high-resolution subcellular localization studies done using fluorescence microscropy would not have been possible without the development of an improved imaging technique called confocal scanning microscropy. Fluorescence microscropy is generally done using whole cells because the embedding media used for sectioning often is fluorescent and obscures the fluorescence derived from the sample. Since eukaryotic cells, even *Saccharomyces*, have a thickness, the fluorescence one observes is coming not only from the molecules in the plane of focus but also from molecules above and below. The greater the thickness of the sample the greater the problem one will have in resolving specific structures.

The confocal microscope uses a laser light source to produce the excitation light beam that can be focused into a narrow focal plane allowing only a thin optical section of the sample to be illuminated. The laser beam set at a specific excitation wavelength rapidly scans the sample. The position and intensity of the emitted light is recorded and the information stored for computer analysis. The results of these scans are combined to generate a composite digital image of the fluorescence from a sample. Because only information from a narrow focal plane is used this method produces a high-resolution map of the subcellular position of the fluorescent molecule.

Other forms of image analysis can also be carried out. The amount of fluorescence can be quantified by computer analysis of the digital image and used as a measure of gene expression. Depending on the laser source and the filter sets available for the particular microscope, one can create images of the fluorescence produced by different antibody–dye conjugates. These images, when superimposed by the computer software system, can very accurately demonstrate whether the two antibodies are colocalized in the cell. Confocal imaging is also used to create a three-dimensional image by a method referred to as optical sectioning. The microscope stage is moved vertically in small steps thereby moving the focal plane through the cell. At each step an image is generated and these are then combined to create a single three-dimensional image that can be rotated in space on the computer screen.

NOMARSKI INTERFERENCE MICROSCOPY

Nomarski interference microscopy (sometimes called DIC) can be used to visualize live unstained cells or tissue samples. It makes use of the differences in thickness and refractive index of different parts of the cell and gives a three-dimension-like image. Light moves more slowly through material with a higher refractive index. Nomarski imaging requires a microscope equipped with special polarizing lenses and prism. A

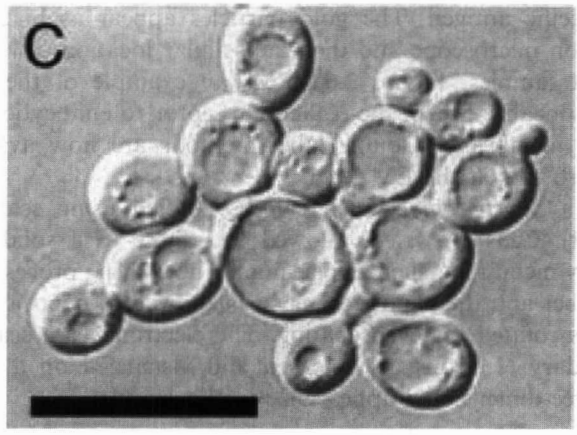

Figure 2.5 Nomarski optics view of *S. cerevisiae*. Nomarski optics can be used to visualize live or fixed cells. The vacuole is by far the most predominant organelle of *S. cerevisiae* and is very clearly observable by this method. The multiple punctate structures in the cytoplasm surrounding the vacuole are vesicles of various types. Taken from Lang *et al.* (2000). Reproduced by permission of the American Society for Microbiology

plane-polarized light beam is split into two and allowed to pass through the sample at nearly adjacent sites after which the two beams are rejoined. If the two sites differ in refractive index, then the beams of light will be out of phase when they exit the sample and when joined will interfere with each other thereby reducing the intensity of the light beam. If there is no difference between the two sites, then the intensity of light will be high. If the difference is substantial, then the beams will completely interfere and a dark region will result. When observed by Nomarski optics, the most prominent organelle in *Saccharomyces* is the vacuole (Figure 2.5).

ELECTRON MICROSCROPY

The electron microscope uses an electron beam instead of a light beam to visualize the cell and its components. The transmission electron microscope passes the electron beam through the sample and to do so the sample must be sliced into very thin sections using special tools and an embedding material. In order to see cellular structures the sample must be stained with an electron dense agent such as osmium. The electron beam is focused with magnetic lenses and this is projected onto a viewing screen or photographed.

The major problem with EM analysis is sample preparation. EM is not often used for *Saccharomyces* but it has proved to be very useful for some studies, particularly on the cytoskeleton. The development of antibody localization techniques for EM work has encouraged more researchers to attempt this difficult method of analysis. The method is referred to as immuno-gold localization because it uses antibodies that are bound to gold particles via *Staphylococcus aureus* Protein A. The surface of the gold particle (about 4 nm in diameter) is covered with Protein A, which also binds very tightly to the constant region of IgG antibodies. The EM sectioned sample is treated with the antibody–gold complex under conditions that allow

binding to the specific antigen. The gold particles appear as black dots in the transmission electron microscope and their subcellular location can be determined in osmium stained samples. Figure 2.6 shows an example of the immuno-gold labeling method to localize Ste2p, the α-factor receptor to endocytic vesicles forming at the plasma membrane. Double label methods are now available for the colocalization of proteins at the EM level.

The surface of a *Saccharomyces* cell can be studied using scanning electron microscropy (SEM). Cells are fixed, allowed to adhere to a solid support, and coated with a heavy metal film, such as platinum. The coating process is carried out in a vacuum chamber and the vaporized metal is allowed to deposit on the sample while the sample is rotated for even coating. The electron beam scans the sample and excited secondary electrons are released and visualized on a monitor. This method gives a three-dimensional appearance to the sample.

FLOW CYTOMETRY

Cells can be sorted into different classes using a fluorescence activated cell sorter (FACS). For this, cells are selectively labeled with a fluorescent dye and sorted into classes based on the extent of labeling. As described above, fluorescence labeling can be done using an antibody-conjugated fluorescent dye or a fluorescent dye such as DAPI that binds a specific cellular component. For example, an antibody specific to a particular cell surface protein is conjugated to a fluorescent dye. This will bind to the surface of cells that express the surface protein and these cells will be fluorescent. Cells lacking this surface protein will not be fluorescent. The extent of the fluorescence can also be quantified and the cells sorted based on the amount of fluorescence.

In the FACS, cells treated with the fluorescent dye label flow past a laser beam and will or will not fluoresce based on the level of bound dye. The cells are then dispersed into droplets containing no more than one cell. Droplets containing a fluorescent cell are made negatively charged and the charge is used to separate the droplets into categories. The number of cells in a category can be quantified and those cells falling into a specific category can be separated from the remainder of the cells and used for further analysis by other methods. DNA staining with a fluorescent dye is used to distinguish cells in G1 of the cell cycle containing the haploid amount of DNA (1 C amount of DNA) from cells in G2 (2 C) from cells in the S phase (1–2 C). Figure 2.7 shows such an analysis. Cells containing the temperature-sensitive mutation *mcm2-1* arrest at the nonpermissive temperature with 2 C DNA content while wild-type cells continue to proceed through the cell cycle (evidenced by a distribution of cells with 1 C or 2 C DNA content or amounts in between the two). Also, Figure 2.7 shows that normal cell cycling is restored in the *mcm2-1 dbf4-6* double mutant strain. This is an example of suppression (see Chapter 8).

PROTEIN EXTRACTION AND PURIFICATION

Standard analytical methods for protein purification and characterization are routinely used in the study of *Saccharomyces* proteins. It is important to have a

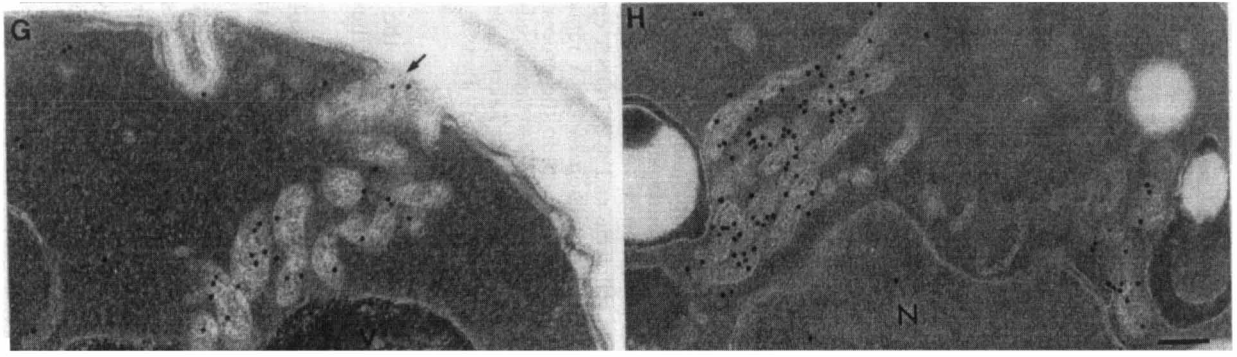

Figure 2.6 Immuno-gold localization. α-Factor receptor Ste2p is localized to the plasma membrane of **a** mating type cells. In response to the binding of **a**-factor, Ste2p is internalized in the form of vesicles by a process called endocytosis. Immunogold labeling methods are used here to visualize the subcellular location of Ste2p during this internalization process. The cells are fixed, sectioned, stained with osmium to visualize subcellular structures, and treated with anti-Ste2p antibody followed by the gold-labeled secondary antibody. One can see a series of black spheres (the electron dense gold beads) concentrated over a tubular–vesicular membrane compartment that appears to span the region from the plasma membrane to the vacuole (labeled V). Taken from Mulholland *et al.* (1999). Reproduced by permission of the American Society for Cell Biology

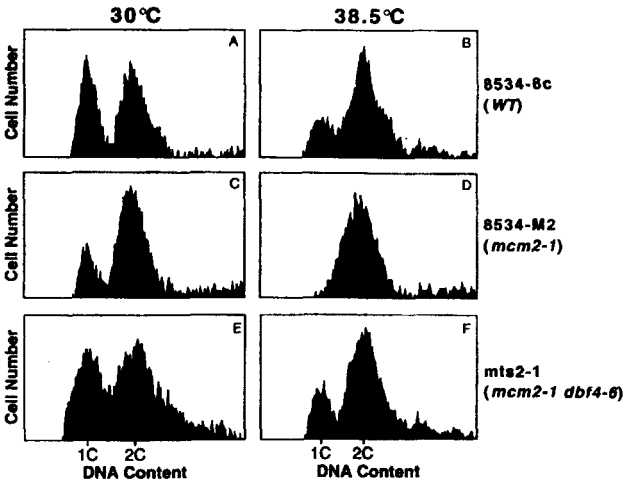

Figure 2.7 FACS analysis of DNA content. Strains carrying the temperature-sensitive mutation *mcm2*-1, encoding a protein required for the initiation of DNA replication at chromosomal origins of replication, and the mutation *dbf4*-6, a component of a regulatory kinase, and the wild-type strain are analyzed for DNA content by FACS analysis. Cells were grown at the permissive temperature and exposed to the nonpermissive temperature for at least one cell cycle. The DNA was stained with propidium iodide and the cells subjected to FACS analysis to determine the number of cells containing different amounts of DNA. The results are presented graphically, as seen in the figure. A 1C DNA content is the amount found in a haploid cell during G1 of the cell cycle and a 2C DNA content is the amount found in a haploid cell in G2 following the completion of DNA replication. Cells having DNA levels between 1C and 2C are in the S phase and in the process of replicating their DNA. As can be seen, the effect of the *mcm2*-1 mutation is to arrest cells in G2 but the *dbf4-6* mutation suppresses (bypasses) the *mcm2-1* alteration. Taken from Lei *et al.* (1997). Reproduced by permission of Cold Spring Harbor Laboratory Press

basic understanding of these methods and be familiar with the terminology since one will undoubtedly encounter many of these while reading the literature whether on *Saccharomyces* or other systems.

Saccharomyces can be cultured in liquid in large volume sufficient to provide milligram quantities of many proteins that then can be purified by standard methods such as gel filtration, affinity chromatography, and ion exchange chromatography. A thorough description of the variety of protein purification techniques available to the researcher is beyond the scope of this book. Instead the reader is referred to the several reference texts on protein purification, particularly Marshak *et al.* (1996), Ausubel *et al.* (2001), Spector *et al.* (1998a,b,c) and certain volumes of *Methods in Enzymology* edited by Abelson and Simons and others.

In *Saccharomyces* these protein purification techniques may be used in conjunction with overexpression vectors that place the ORF of any cloned gene under the control of high-level constitutive promoters in multicopy plasmids (see the discussion of expression vectors in Chapter 1). In this way, one can achieve sufficiently abundant levels of expression to allow the purification of almost any desired protein. Overexpression of a particular protein makes possible its purification but

may not be appropriate for the *in vivo* analysis of function. Artifacts such as mislocalization to unusual subcellular compartments may occur. The protein may have toxic effects when produced in abundance. For example, overproduction of an integral membrane protein may have serious consequences for the transit of other proteins through the secretory pathway. In fact, overproduction of any protein that relies on a saturable process for its synthesis and localization may lead to unforeseen effects. Examples of saturable processes are nuclear import via import proteins and the nuclear pore complex or protein modification and processing events. Additionally, many proteins are components of larger functional complexes or interact in a highly regulated manner with several different competing regulatory components. Overproduction of one member of a complex may impact the level of expression of the other components of the complex or may alter their interactions so as to cause aberrant regulatory patterns. Caution must be used in the interpretation of any result obtained by the overproduction of a single protein.

WESTERN ANALYSIS

Western analysis is very powerful technique for protein characterization and expression studies. The method is used for analysis to evaluate the amount of a particular protein for which protein-specific antibodies are available. Because of the antibody specificity, proteins can be identified even when present in a complex mixture such as a cell extract.

A special section of Ausubel *et al.* (2001) is devoted solely to *Saccharomyces* and describes in detail several extraction methods for protein studies. Many other methods are described in the literature. A major difference in these methods is whether the conditions of the extraction buffer are denaturing or nondenaturing. Nondenaturing conditions are similar to *in vivo* conditions and are designed to maintain the integrity of subcellular structures and protein complexes. One would use this type of condition if experimental interest were in identifying proteins found in association with one another. For example, is a particular protease found in a proteasome? Denaturing conditions, 5% sodium dodecyl sulfate or 8 M urea included in the extraction buffer, disrupt all protein–protein interactions. Denaturing conditions would be used if one simply wanted to compare the amount of a particular protein in a sample.

The proteins of a sample under analysis are size-separated by SDS polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred from this gel to a sheet of nitrocellulose (membrane) by electrophoresis. The proteins in the sample are now fixed in place in this two-dimensional membrane and represent the position to which the protein migrated in the gel. The position of a protein on this membrane is visualized using an antibody specific to that protein, referred to as the primary antibody or probe. The membrane is incubated in a solution containing the primary antibody giving sufficient time for the antibody to bind to the protein that is itself bound to the membrane. A secondary antibody specific to the constant region of the primary antibody is used as a probe to localize the position of the primary antibody on the membrane. This secondary antibody is also conjugated to horseradish peroxidase, biotin, or some other compound whose presence can be assayed easily.

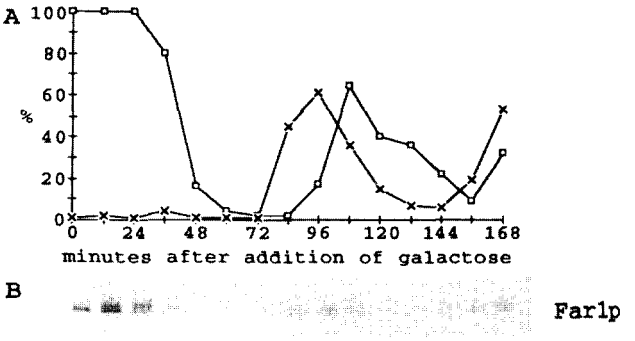

Figure 2.8 Western analysis. This figure follows the relative level of the cell-cycle-regulated protein Far1p during the course of about two cell divisions. Cells were synchronized in G1 (by depletion of an essential cyclin). The addition of galactose allows the cells to initiate a new round of cell division by restoring the expression of the cyclin. Panel (A) follows cell division by monitoring the level of unbudded cells (□) and binucleate cells (×), both determined by microscopic observation. Protein extracts were prepared from the cells in panel (A) and subjected to Western analysis using anti-Far1p antibody. The results of the Western analysis are shown in panel (B) and indicate that Far1 protein accumulates in G1 prior to Start. Far1p runs as a close doublet of bands suggesting that the protein is found in a phosphorylated form. Taken from McKinney *et al.* (1993). Reproduced by permission of Cold Spring Habor Laboratory Press

Typically, the detection assay produces a colored, luminescent, or fluorescent precipitate on the surface of the membrane. This is then photographed, detected by X-ray film, or scanned by some form of densitometer for a permanent record and quantification where possible. Figure 2.8 illustrates the results of a Western analysis of Far1p during a cell division cycle (McKinney *et al.*, 1993). The reader should note that often in addition to the level of the protein of interest the level of another protein, one considered to be relatively constant in amount during the cell cycle such as actin, is also determined using the same membrane. This protein serves as a control demonstrating that equal amounts of extract were loaded into each lane of the SDS-PAGE gel.

The different assay methods vary in their ability to be evaluated quantitatively. Moreover, determination of amounts is always relative to a standard sample run (for example the control condition) in the same gel. Some methods, such as chemiluminescence, are linear over only a single log while others, such as those using a fluorescent secondary antibody, can be linear over several logs. Primary antibodies, secondary antibodies, and detection kits are commercially available and a wide variety of products for all aspects of Western analysis can be obtained from several suppliers.

For detailed information on Western analysis Ausubel *et al.* (2001) is an excellent starting point and provides updated procedures, theoretical discussions, and additional references. Others references listed below are also valuable resources. In addition, there is an abundant technical literature available from the commercial suppliers (Molecular Probes for example) evaluating the capabilities of the various

products on the market as well as technical information from instrumentation manufacturers like Molecular Dynamics that manufactures densitometer, phosphorimager, and fluorimeter equipment.

EPITOPE TAGGING AND IMMUNODETECTION OF EPITOPE-TAGGED PROTEINS

An **epitope** is a structural feature of a molecule that is specifically recognizable by an antibody and to which the variable region of the antibody binds. Short amino acid sequences, branched-chain carbohydrate groups, or even the phosphorylation site on a peptide can serve as an epitope. Most large molecules like proteins contain several epitopes and, when such an antigenic molecule is injected into an animal, it will induce the production of several different antibodies to many or all of these epitopes in that individual animal. Serum isolated from this individual will contain a mixture of several secreted IgG antibody proteins each produced by a different line of antibody-producing B lymphocytes and each capable of recognizing a different epitope of the antigen. This type of antibody is referred to as a **polyclonal antibody** because several different clones of antigen-producing B lymphocytes produce the several serum antibodies.

The advent of **monoclonal antibody** technology solved the problems of antibody heterogeneity and made possible the method of epitope tagging for protein detection. Monoclonal antibodies are produced by mouse lymphocytes. An antigen having multiple epitopes is injected into the mouse and B lymphocyte differentiation is allowed to initiate. Multiple B lymphocyte clones each producing a different antibody specific to a particular epitope of the antigen will begin to undergo this differentiation process. The immunized mouse is sacrificed, the B-lymphocyte-containing spleen removed, and the developing B lymphocytes fused with an 'immortal' line of B lymphocyte tumor cells. Normally, B lymphocytes are able to undergo only a very limited number of divisions in culture, but these tumor cell fusions divide indefinitely. Individual hybrid cell clones are cultured separately and allowed to produce their unique antibody. This immortalized antibody-producing B lymphocyte cell line is called a **hybridoma**. The antibody product of each hybridoma line is then tested against the antigen, and fragments of the antigen, and the specific epitope recognized by the antibody determined. The antibody product is referred to as a monoclonal antibody because it is produced by a single hybridoma clone of B lymphocytes.

A variety of epitope-specific monoclonal antibodies are commercially available but not all are equally useful for different protein analysis techniques. Some are excellent as the primary antibody in Western analysis while others are poor for this application but work extremely well in immunoprecipitation. One should be guided by the methods used by published journal articles and by the recommendations of colleagues and commercial suppliers. Commercial suppliers provide technical assistance for customers and detailed information can be found in the product descriptions of most catalogues. Since all monoclonal antibodies are murine antibodies, all contain the murine constant region. For techniques such as Western analysis that often use a monoclonal antibody as the primary antibody, the secondary antibody

must be produced in an animal other than the mouse and directed against the mouse IgG constant region.

The most common type of epitope used by molecular biologists is the peptide epitope. This is because one can easily attach such an epitope to any protein of interest using recombinant DNA technology. This is referred to as **epitope tagging**. For this one simply needs to construct an in-frame fusion of the ORF of the gene encoding the protein of interest to an oligonucleotide encoding the epitope sequence. Most often, the epitope is placed at the N-terminal or C-terminal end of the ORF. It is essential to test the epitope-tagged allele for function to ensure that the presence of the epitope does not interfere with the functional activity, subcellular localization, or stability of the protein. In *Saccharomyces* this is done by determining whether the tagged allele is capable of complementing all of the mutant phenotypes of the null allele.

Vectors specific for constructing these in-frame epitope fusions can be obtained commercially or from colleagues. Many are described in the literature. These typically contain the epitope sequence located immediately upstream or downstream of a multiple cloning site (MCS). The ORF of interest is simply amplified using appropriate PCR primers and inserted into the MCS thereby placing the epitope at the N-terminus or C-terminus, respectively, of the encoded fusion protein. The resulting protein is said to be epitope tagged. Expression of the epitope-tagged gene product in these constructions is usually from a promoter such as the *ADH1* promoter.

One need not use these vectors, particularly if one wants to use the native promoter. Because the epitope sequence is frequently quite short, one can synthesize oligonucleotide primers containing the sequence and use these for PCR amplification of the ORF of interest. This product can then be inserted into any vector containing any desired promoter sequence (see the discussion of expression vectors in Chapter 1). The sequence of the epitope also may be inserted into any desired site in the ORF of a gene using *in vitro* mutagenesis techniques or by PCR-based methods. This is important if the N-terminal or C-terminal versions of the epitope-tagged protein are not functional and another insertion site for the epitope must be found.

Longtine *et al.* (1998) constructed a series of modules for use as PCR templates for the creation of tagged fusion genes at genomic sites by one-step gene replacement. The modules allow for C-terminal fusion of GFP, three copies of the HA epitope, 13 copies of the Myc epitope, or GST or for N-terminal fusion of GFP, three copies of the HA epitope, or GST to the gene of interest. The N-terminal fusions replace the native promoter with that of *GAL1*. Each module contains the protein tag and a selectable marker. This is amplified using primers containing 40 bp of genomic sequence at the 5' end and sequence complementary to the module template and the 3' end. The amplified product is then transformed into the host strain and *in vivo* recombination between the amplified DNA fragment and the genomic site creates the desired tagged fusion gene.

The researcher has a choice of any of several peptide epitopes. The most common are listed below. Monoclonal antibodies specific to each is commercially available. For some, the researcher has a choice of different monoclonal antibodies based on their performance in a particular technique such as Western analysis or immunoprecipitation or even whether the epitope is at the N-terminal or C-terminal end of the protein or in the middle.

HEMAGGLUTININ (HA) EPITOPE

The HA epitope is a nonapeptide (YPYDVPDYA) derived from the influenza virus hemagglutinin protein. Anti-HA antibody is quite specific and cross reaction to other yeast proteins is not seen. The HA-tag can be used for Western analysis, immunocytochemistry, and immunoprecipitation (see below). Often, the HA epitope sequence is repeated up to three times or more to improve antibody binding and make this tag more useful for immunoprecipitation.

FLAG EPITOPE

The FLAG epitope (DYKDDDDK) is recognized by three commercially available monoclonal antibodies, M1, M2, and M5. M1 and M5 require that the epitope be placed at the N-terminus of the tagged protein and M1 will even bind to a shorter version of the FLAG sequence. M2 is able to recognize the epitope at all locations in the protein. Moreover, M2 can be used for both Western analysis and immunoprecipitation.

Myc EPITOPE

The Myc epitope (EQKLISEEDL) is derived from the human Myc protein, the product of the *myc* oncogene. A number of different monoclonal antibodies are available from commercial sources. Some are suitable for Western analysis and immunoprecipitation while others are more suitable for immunocytochemistry.

IMMUNOPRECIPITATION AND RELATED TECHNIQUES

These methods are used to physically separate a particular protein from a cell extract. They rely on a high-affinity, sequence-specific interaction between the protein and another molecule capable of specifically binding to that protein, such as an antibody. To achieve physical separation, the molecule providing the recognition specificity is bound to an inert substrate that can be separated physically from the binding reaction mix. Once purified by one of these methods, the protein can be characterized further or its level of expression determined, often by Western analysis. Moreover, these methods can be scaled up for large samples and can thus be used for protein purification.

IMMUNOPRECIPITATION

Immunoprecipitation uses protein- or epitope-specific antibodies for protein recognition. The complex is then removed from the mixture because it is bound to small beads, usually composed of Sepharose (a form of starch), that are themselves bound to protein A, protein G, or a mixture of both. Protein A is a product of the bacterium *Staphylococcus aureus* and binds to immunoglobulin constant region with very high affinity. Protein G is also a product from a *Streptococcus* bacterial species and also binds to IgG constant region. Proteins A and G differ slightly in their

affinity for the IgG constant region of the different species commonly used to produce antibodies, like horse, mouse, or goat. Thus, researchers often use Sepharose beads bound with a mixture of proteins A and G in order to achieve high-affinity binding to a broad range of antibodies that they might be using in different experiments.

An antibody specific to the protein of interest is added to the cell extract (prepared under the appropriate conditions) and allowed to bind. Protein A/G-bound Sepharose beads are added and incubated to allow binding of the antibody–protein complex to the beads. Centrifugation is used to pellet the beads with the bound protein. The sample is then analyzed by Western analysis. If a protein-specific antibody is not easily available but the gene encoding the protein is, then researchers often choose to epitope-tag the protein of interest using the methods described above.

Immunoprecipitation is most often used to identify other proteins that may be found complexed with the protein of interest. For this, the cell extract must be prepared under nondenaturing conditions. Any proteins that interact with the protein of interest should **coimmunoprecipitate** (so-called co-IP). For example, if one's genetic analysis indicates that proteins X and Y form a complex, then protein Y should co-IP with protein X when anti-protein X antibody is used. Western analysis of the pelleted sample should detect protein X and, if the two proteins co-IP, should also be able to detect protein Y when anti-protein Y antibody is used as the probe.

METAL CHELATE AFFINITY PURIFICATION

Many natural proteins have metal binding sites for ions such as Ni^{2+} and Zn^{2+}. Metal chelate affinity purification makes use of this to purify proteins. If the protein of interest does not contain a metal binding site, then one can be added by epitope-tagging. By far the most common metal chelate tag is a series of six histidine residues that specifically binds to Ni^{2+} ions and is referred to as a **His-tag**. The His-tag is short enough to be inserted by PCR-based methods, but a variety of vectors are available commercially for the construction of His-tagged alleles of the protein of interest.

Metal chelate affinity purification uses resin beads to which a metal chelating group has been bound. Ni^{2+} ions are then bound to this chelating group in such a way as to allow the Ni^{2+} ion also to be available for binding by the metal binding group of the protein, the His-tag of the protein of interest. The Ni^{2+}-bound resin beads can be used either as a slurry or packed in an affinity column. First, the His-tagged protein is allowed to bind to the Ni^{2+}-bound resin by incubating the resin with cell extract. After washing off all the unbound protein, the His-tagged protein is released from the beads using an excess of imidazole (an analogue of histidine).

Proteins purified in this manner from cell extracts are frequently contaminated with other cellular proteins that normally contain Ni^{2+} binding sites. Snf1 kinase of *Saccharomyces* is an example. Therefore, to obtain a strictly pure product one will have to carry out a second purification step. This may not be necessary for many of the characterization methods to be undertaken in follow-up studies, particularly

Western analysis that can detect the protein of interest alone by the use of specific antibodies.

GST-TAGGED AND MalB-TAGGED PROTEINS

Other affinity purification methods are available and can also be used like immunoprecipitation for studies of protein complexes or for protein purification. Two of the most common affinity purification methods use GST and MalB protein fusions. GST is glutatione S-transferase, is a product of *Schistosoma japonicum* and binds glutathione with high affinity. GST fusions to the protein of interest can be made by standard epitope-tagging methods and many fusion vectors are commercially available to simplify these constructions. Cell extract containing the GST-tagged fusion protein is passed over a resin-bound glutathione affinity column and immobilized. The GST portion is cleaved from the protein of interest with enterokinase or thrombin both of which act on the sequence at the fusion junction site. This releases purified protein from the resin along with any associated proteins.

MalB protein is an *E. coli* product involved in maltose transport. It is localized to the periplasmic space and is used as a maltose carrier. Its high-affinity binding to maltose and amylose is the basis of this purification method. Using methods such as those described above, one constructs an in-frame fusion of the protein of interest to MalB. Vectors for these constructions are commercially available. The MalB portion will provide specific binding to a column-bound amylose and the MalB fusion protein will be retained by the column. The fusion protein is released from the amylose column by excess maltose. The purified fusion protein is then cleaved at the junction site with a site-specific protease and the protein of interest further purified by passage over an amylose column to remove the free MalB protein and any uncleaved residue.

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