

# The effect of an SV40 nuclear localizing signal in *Saccharomyces cerevisiae*

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In eukaryotes, it is necessary to possess the ability to target proteins to various intra-cellular compartments. Among these compartments is the cellular blueprint storage container known as the nucleus. Specific targeting of proteins to the nucleus is accomplished using a lysine rich, eight amino acid oligopeptide known as an SV40 nuclear localizing signal (NLS). In this experiment, an NLS sequence was attached to the green fluorescence protein (GFP) gene, which originated from the deep ocean jellyfish species, *Aequorea victoria*. Poly-linkers *SalI* and *KpnI* were added to the ends using the polymerase chain reaction (PCR) which also amplified the NLS-GFP DNA sequence. The resulting fragment was sub-cloned into the *Saccharomyces cerevisiae* vector pRS426. The vector was transformed into *S. cerevisiae* using electroporation. The organisms were then allowed to grow and express the GFP gene. Fluorescence microscopy was used to look for nuclear localization. This experiment was repeated omitting the NLS from the cloning vector, which produced no nuclear localization. This procedure was designed to be completed in four 3-hour sessions so that it could be used as a teaching tool. Cloning, PCR, plasmid mini-prep, fluorescence microscopy, microorganism culturing, restriction enzyme digests, ligation reactions, and electroporation are demonstrated in this experiment. This procedure will be made available to instructors at the University of Wisconsin La Crosse as well as other institutions via the world wide web.

## INTRODUCTION

Proteins are the machinery that perform most of the work in cells. However, in order for proteins to act, they must come in contact with the appropriate substrates. In prokaryotes, the proteins have access to most of the cellular components. This is because prokaryotes have no internal membrane-bound compartments. Eukaryotes, on the other hand, are compartmentalized, which creates the need for proteins to enter and leave these compartments as necessary. This is accomplished by means of protein sorting. One specific type of protein sorting is nuclear localization, which is the targeting of proteins into the information storage center of the cell, the nucleus.

In this experiment, *Saccharomyces cerevisiae*, a eukaryotic microbe was chosen as the model system. *S. cerevisiae* or baker's yeast was chosen because of its similarity to higher eukaryotes from a genetic standpoint and because it was relatively easy and inexpensive to use. *S. cerevisiae* is a microscopic, single celled organism, which contains chromosomes composed of DNA which are stored in a membrane bound nucleus. It also carries out protein synthesis in a similar fashion to higher eukaryotes by undergoing post-transcriptional and post-translational modification of RNA and proteins respectively. This makes *S. cerevisiae* an ideal model for use in studying nuclear localization.

Additionally, *S. cerevisiae* is a commonly used organism, which made it easy to find information on culturing and genetic techniques. Furthermore, it was an inexpensive and easy to use alternative to mammalian or insect tissues cultures, which are difficult to maintain and expensive.

There are several mechanisms for sorting proteins in cells. Nuclear localization uses short protein fragments attached to the ends of the targeted protein called signal sequences. When cells need to target proteins into the nucleus, a nuclear localization signal (NLS) is attached to the end of the protein and the cell will transport it into the nucleus. Some common proteins that need to be nuclear localized are DNA replication enzymes, RNA polymerase, and DNA repair enzymes.

To study nuclear localization, a method needed to be derived which would allow visualization of the nuclear localization. The method chosen was fluorescence, which used a fluorescent protein called Green Fluorescence Protein (GFP) which is natively found in the deep sea jellyfish *Aequorea victoria*. GFP fluoresces green (515nm) when exposed to blue light (400nm). To demonstrate nuclear localization, an NLS was attached to GFP and the resulting cells were viewed under fluorescence microscopy.

In addition to studying nuclear localization, the experiment has tremendous value as a teaching tool. By completing the experiment in a teaching lab, many of the fundamental principles of molecular biology and microbiology are introduced. Ultimately, this experiment will be converted into a laboratory procedure and potentially used in teaching laboratories.

## MATERIALS AND METHODS

Two cloning vectors were used in this experiment, pRS426 (pRS) and pPD80.08 (pPD). The pRS was a *S. cerevisiae* vector that contained a yeast promoter with a multiple cloning site and the gene for uracil production (used in a selection), which the experimental cells lacked. In addition to a yeast promoter, pRS also contained a promoter for *Escherichia coli* and the gene for resistance to the antibiotic, ampicillin. The pPD contained the GFP gene along with the NLS. The pPD was a cloning vector for *C. elegans* and was only used as a source for the GFP and NLS. It did contain a promoter for *E. coli* and the ampicillin resistance gene so that it could be copied in bacteria. The plasmid pPD80.08 containing GFP and the NLS was transformed into *E. coli* using electroporation at 1600V for 0.5 msec, plated onto LB agar + ampicillin plates and incubated for 24 hours at 37°C. The plasmid was then recovered using a Qiagen mini-prep kit.

The polymerase chain reaction (PCR) was performed on the pPD using the primers GFPJP.1 (GGCCGTCGACATGACTGCTCCAAAG) and GFPJP.2 (GCCGGGTACCTTTGTATAGTTCATC). The resulting PCR product and the yeast plasmid pRS426 were then digested with *KpnI* for two hours at 37°C. The PCR product and pRS were digested with *SalI* for two hours at 37°C. The DNA was then extracted by ethanol precipitation and re-suspended in 10 µl TE.

A ligation reaction was performed by mixing the cut pRS and the cut PCR fragment at a ratio of 3 to 1 and also at 6 to 1. DNA ligase and ligase buffer were added and the reactions were incubated at 13°C overnight. The ligated plasmid was ran on a 0.8% agarose gel at 100V for 45 minutes, stained with ethidium bromide and photographed (Figure 1).

The newly ligated plasmid (pRS426-GFP-NLS) was transformed into *S. cerevisiae* using electroporation at 2500V for 0.5msec, was plated onto SCD-Ura- + streptomycin plates and incubated for two days at 30°C.

An overnight culture of *S. cerevisiae* pRS426-GFP-NLS (from the plates above) was incubated at 30°C for 14 hours. A wet mount was prepared and viewed under a fluorescence microscope with a FITC filter in place to view the fluorescent GFP. Photographs were taken at varying exposure times (6-10 min). The fluorescence filter was removed and several pictures were taken under phase contrast to view the entire cell.

The above procedure was repeated, using the PCR primers, GFPJP.3 (GGCCGTC-GACATGAGTAAAGGAG) and GFPJP.2. The resulting PCR product did not contain the NLS.

## RESULTS

Figure 1 demonstrated PCR fragments of approximately 700 base-pairs. It also revealed pRS to be approximately XX kb. In the ligation mixtures, a 4.2 kb band was expected, but not observed.

Visualization of *S. cerevisiae* pRS426-GFP (no NLS) produced generalized fluorescence throughout the cell (Figure 2A). Visualization of *S. cerevisiae* pRS426-GFP-NLS demonstrated some general fluorescence but a bright concentration of fluorescence in a small area, assumed to be the nucleus (Figure 2B).

FIGURE 1. Agarose gel (0.8%) ran at 100 V for 45 minutes. Lane 1 was blank, lanes 2 and 8 contained BRL 1kb ladder, lane 3 contained the NLS PCR product, lane 4 contained the non-NLS PCR product, lane 5 contained pRS426, lane 6 contained pRS426-GFP (no NLS), lane 7 contained pRS42-GFP-NLS.

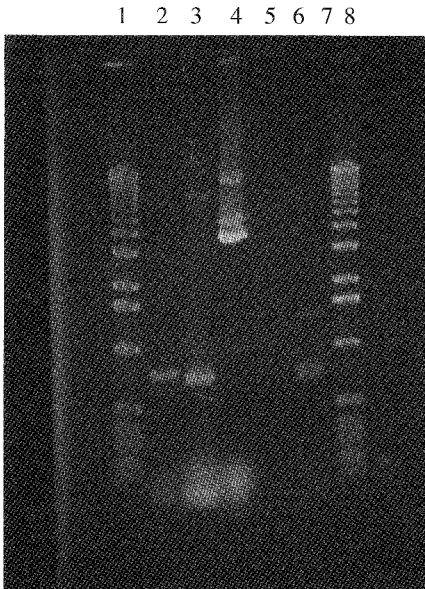
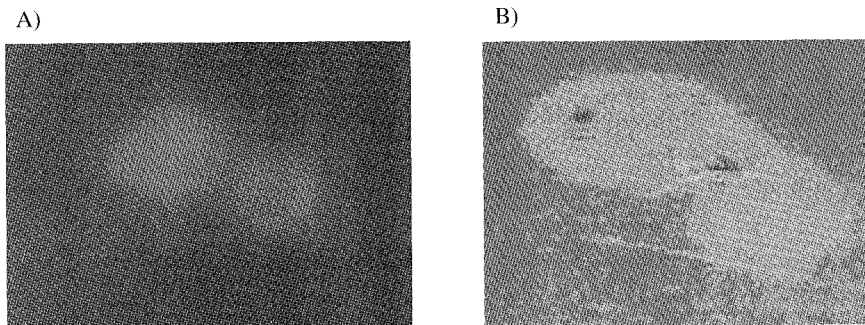


FIGURE 2. Fluorescence micrographs of *S. cerevisiae* using a FITC filter at 1000X. A) Generalized fluorescence of budding yeast with no nuclear localization. B) Nuclear localized fluorescence of budding yeast (the higher intensity fluorescence originally white has been darkened for contrast.)



## DISCUSSION

The agarose gel demonstrated that a successful ligation had not occurred in both the NLS and non-NLS experimental groups. When the various bands were compared to the size standards, the pRS (vector) was roughly 3.5 kilobases, the PCR product (insert) was 0.7 kilobases. The size estimates were consistent with the expected values for each of the pieces of DNA. It should be noted that even though the ligated plasmids were not observed on the agarose gel, the expected fluorescence was observed. This suggested that there possibly wasn't enough ligated plasmid to produce a band on the gel.

While the gels did not demonstrate a ligation had occurred, the photographic data supported that the presence of an NLS on GFP did cause a higher concentration of fluorescence to accumulate in the nucleus of yeast. This was verified when the absence of an NLS produced generalized/non-localized fluorescence.

As stated earlier, the procedure was not only designed to demonstrate protein sorting in eukaryotic cells, it was also designed as a teaching tool. The experiment demonstrated fundamental procedures and principles in both molecular biology and microbiology. The project used several techniques, including; cloning, sub-cloning, PCR, plasmid mini-prep, fluorescence microscopy, microorganism culturing, restriction enzyme digests, ligation reactions, transformation, protein fusion and electroporation.

Along with many successes, there were a few problems that arose. The first and foremost was the potential auto-fluorescence of *S. cerevisiae*. When the yeast were suspended in TE buffer to view under fluorescence microscopy, a generalized fluorescence was encountered, however nuclear localization was still visible. When the organisms were dried onto the slide, the auto-fluorescence was eliminated or greatly reduced. In future experimentation, the organisms should be suspended in a wide variety of media to discover if one will not demonstrate non-GFP fluorescence.

Along with auto-fluorescence, the exposure times for photographing were too long. In the future an exposure time of less than three minutes should be used to reduce the background fluorescence and washing out effect.

An additional problem arose concerning the growth of pure culture following elec-

troportion. Ampicillin was added to the media, but several ampicillin resistant Gram positive rods still grew. In the future, media with a significantly lower pH (3.0-3.2) should be utilized to eliminate the contaminant organisms and allow for a pure culture recovery following electroporation.

Future directions for the project are to write and publish a laboratory procedure that is geared for completion in three or four three-hour sessions. This procedure could be utilized in the molecular biology teaching laboratory (Bio 436) as a major unit. In addition to utilizing the procedure at UW-La Crosse, it will be published on the world wide web, via the UW-La Crosse web page under the molecular biology pages.

## REFERENCES

- Ausubel, F. M., et. al. (1992). In Short Protocols in Molecular Biology 2nd ed. (Brent, R.). Greene Publishing Associates and John Wiley and sons. New York, NY
- Cooper, S. T. (1996). Biology 436 Laboratory Manual. University of Wisconsin-La Crosse. La Crosse, WI.
- Lodish, H., et. al. (1995). In Molecular Cell Biology. p 840-844. W. H. Freeman and Company, New York, NY.
- Winfrey M., M. Rott, and A. Wortman. (1997). Unraveling DNA: Molecular Biology for the Laboratory. Prentice Hall, Upper Saddle River, NJ.