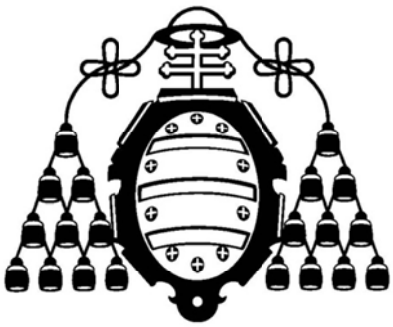
DEPARTAMENTO DE BIOQUÍMICA Y BIOLOGÍA MOLECULAR



UNIVERSIDAD DE OVIEDO

RECOMBINANT DNA TECHNOLOGY- LABORATORY PRACTICE GUIDE

BIOTECNOLOGY DEGREE

NOMBRE Y APELLIDOS:

CURSO Y GRUPO:

Taquilla n º :

Study of the transcription regulatory elements of a gene promoter from *Saccharomyces cerevisiae*

1.- INTRODUCTION

The initiation of mRNA synthesis depends on specific gene promoter elements that are able to respond to physiological changes. There are two essential components involved: the cis-acting DNA elements and the trans-acting protein factors.

In this laboratory practice we will try to identify some regulatory elements that are necessary for the transcriptional regulation of the glucokinase 1 gene (*GLK1*) from *Saccharomyces cerevisiae* .

In yeasts, cis-acting elements, with the exception of the TATA box, can be grouped into two types: positive regulatory elements, also named upstream activating sequences (UAS) and negative regulatory elements or upstream repressing sequences (URS)

One very useful procedure to determine the localization of cis-acting regulatory sequences consists of making gene promoter sequence deletions and constructing plasmids containing the different deletion sequences, each fused to the sequence of a reporter gene encoding for a protein product with an enzymatic activity easy to quantify and not normally present in the host cell.

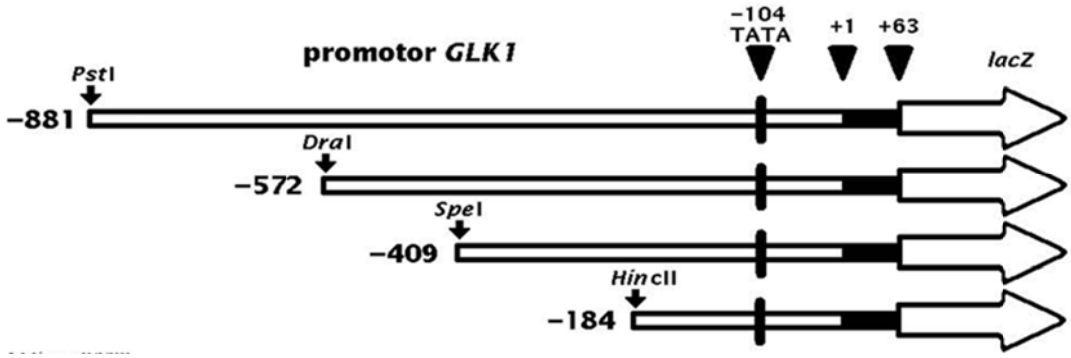


Figure 1: Promoter of gene GLK1

In order to perform the study, the laboratory practice will start with two yeast cell cultures, each containing yeast cells that had been previously transformed by introducing a different recombinant plasmid construct harboring a fragment of the GLK1 promoter sequence fused to the coding sequence of *E. coli* β-galactosidase gene (lacZ). After disruption of the yeast cells and obtention of cell protein extracts from both yeast cell cultures, β-galactosidase assays will provide the means to measure transcriptional activity of the chimera gene present in each of the plasmids introduced into each of the yeast cell cultures.

2.- PROCEDURE AND RESULTS

2.1 β-GALACTOSIDASE ASSAYS:

1. Turn on a heating block and adjust temperature to 30 ^{0}C.

2. Mark two Eppendorf tubes with the numbers 184 for one and 409 for the other and fill them up with corresponding number of yeast cell culture suspension (grown in YPD rich medium at A_{600nm} = 1). Centrifuge for 3 min at high speed in a microcentrifuge, remove the supernatant and **completely** resuspend sediment by adding 300 µl of buffer Z (100 mM phosphate buffer pH 7).

3. Add to each tube an equivalent volume of glass beads and disrupt the yeast cells by vigorous shaking in a vortex at the highest speed. To avoid heat inactivation of the enzyme in the sample, shaking is performed in short, repeated periods of 30 seconds each followed by 30 seconds on ice for a total of 14 periods.

4. Centrifuge each tube at the highest speed for 5 minutes and transfer each supernatant to a clean new tube. Supernatants are the soluble yeast cell protein extracts ready to use for assays.

5. Place 100 µl of supernatant 184 in one Eppendorf tube and 5 µl of supernatant 409 in a separate tube, each previously labeled with the corresponding numbers. Add buffer Z, pH 7 to each tube up to 800 µl. To a third clean Eppendorf tube add only 800 µl of buffer Z, pH 7, which will serve as a blank for the assay.

6. Save the left-over volumes of each extract from step 4 and store them in the refrigerator at 4 ^{0}C until required for the protein assay.

7. Start the β-galactosidase assays by placing the three tubes, prepared as indicated in step 5, in a heating block at 30 ^{0}C and keep them there until the reaction is stopped. The reaction starts by adding 200 µl of the substrate o-nitrophenyl-β-D-galactoside at a concentration of 4 mg/ml. Keep counting the time right from the start until the sample turns yellow. This will generally occur after about 5 minutes incubation but the time needed will depend on the degree of cell disruption obtained in step 3. Stop the reaction at the appropriate time by adding 500 µl of Na_2CO_3 1M.

8. The intensity of the yellow color obtained in the reaction is measured in absorbance units, using a colorimetric spectrophotometer. Since a minimum of 4 ml are needed to measure absorbance, samples have to be diluted by taking 0,5 ml and adding 3,5 ml of Na_2CO_3 1M (to blank sample too).

9. Set the wavelength to 410 nm and adjust to zero the colorimetric spectrophotometer with the sample labeled Blank. Measure the absorbance of each of the two diluted samples.

2.2 RESULTS:

Calculate the enzymatic activity for each sample in units/ml (U/ml). Use the value of the molar extinction coefficient (Ɛ) for the o-nitrophenol: Ɛ= 4500 M^{-1} cm^{-1} in the equation below:

A= Ɛ c l where "l" equals 1 cm and "c" represents the molar concentration

2.3 PROTEIN ASSAY

To achieve accurate values for the transcriptional activity of the gene promoter it is necessary to evaluate the reporter β-galactosidase activity in terms of specific activity in Units/mg protein (U/mg). The reason for this is that the comparison between samples has to be done considering not only the activity of β-galactosidase but also the amount of total protein present in the sample, which is dependent on the degree of cell disruption obtained. Therefore an assay for the amount of protein in the sample is needed.

The protein assay - Lowry method protocol:

1.-Prior to the assay, dilute extracts threefold with MilliQ water.

2.-Label four 12-ml clean plastic tubes with the numbers 409 and 184, two tubes for each number.

3.-Pipet 5 and 10 µl of each diluted extract into each of the corresponding labeled tubes.

4.-Add MilliQ water to each tube up to 1 ml.

5.-Label another five 12-ml clean plastic tubes and pipet the following volumes (in ml) of a 200 µg/ml BSA solution: 0,2, 0,4, 0,6, 0,8 and 1 ml. Adjust the volumes up to 1ml in each tube by adding the following volumes of MilliQ water : 0,8, 0,6, 0,4, 0,2, 0,0 ml, respectively. Additionally, label one tube as blank and pipet 1 ml of milliQ water.

6.-Add 2,5 ml of solution $C_{(*)}$ to each tube (a total of 10 tubes).

7.-Mix and incubate for 10 minutes at room temperature.

8.-Add 0,5 ml of Folin-Ciocalteu reagent to each of the ten tubes. Mix thoroughly and wait 30 minutes.

9.- Measure absorbances at 500 nm.

(*) Solution C : Mix 200 ml of Solution A (2% sodium carbonate / 0.1N NaOH), plus 2 ml of a solution of sodium-potasium tartrate (2%) and 2 ml of $CuSO_4 \cdot 5H_2O$ at 1 %.

2.4 RESULTS:

1.-Plot the standard curve representing the values of absorbance obtained against the respective amounts (mg) of BSA in each tube (tubes 5 to 9).

2.-Calculate β-galactosidase specific activity for each protein extract.

3.-From the values of β-galactosidase specific activity, are you able to make any conclusion about the presence of transcription regulatory elements in the plasmid constructs studied?

2.5 CALCULATIONS:

Sample	Activity (U/ml)	Protein conc. (mg/ml)	Specific activity (U/mg)

2.6 CONCLUSION: