

**Semester-long inquiry-based molecular biology laboratory: transcriptional
regulation in yeast**

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Abstract

A single semester molecular biology laboratory has been developed in which students design and execute a project examining transcriptional regulation in *Saccharomyces cerevisiae*. Three weeks of planning are allocated to developing a hypothesis through literature searches and use of bioinformatics. Common experimental plans address a cell process and how three genes that encode for proteins involved in that process are transcriptionally regulated in response to changing environmental conditions. Planning includes designing oligonucleotides to amplify the putative promoter of the three genes of interest. After the PCR, the product is cloned proximal to β -galactosidase in a yeast reporter plasmid. Techniques used include agarose electrophoresis, extraction of DNA from agarose, plasmid purification from bacteria, restriction digestion, ligation, and bacterial transformation. This promoter / reporter plasmid is then transformed into yeast. Transformed yeast are cultured in conditions prescribed in the experimental design, lysed and β -galactosidase activity is measured. The course provides an independent research experience in a group setting. Notebooks are maintained on-line with regular feedback. Projects culminate with the presentation of a poster worth 60% of the grade. Over the last three years, about 65% of students met expectations for experimental design, data acquisition, and analysis.

Keywords: inquiry-based learning; molecular biology laboratory; transcriptional regulation; upper-division undergraduate

Introduction

Among the ten high-impact practices listed by Project Kaleidoscope are collaborative assignments and projects, undergraduate research, and capstone course and projects [1]. Similarly, the BIO2010 report included project-based laboratories in its eight recommendations [2]. The Boyer Commission on Educating Undergraduates recommended making research - based learning the standard in universities [3]. Guided by these recommendations, I have designed a semester-long inquiry-based molecular biology laboratory. It has been taught annually for five years. In groups of three, students use a combination of information collected from the scientific literature, bioinformatics, and creativity to design experiments to investigate transcriptional regulation in the unicellular fungus, *Saccharomyces cerevisiae* (Baker's or Brewer's yeast).

The many advantages provided by *S. cerevisiae* as a model organism also facilitate its use in a teaching laboratory. Complete sequencing of the *S. cerevisiae* genome was achieved 20 years ago [4] and a well-annotated, on-line genome compendium, the Saccharomyces Genome Database (SGD) [5], facilitates bioinformatics analysis. Culture media components are commonly inexpensive and strains readily available from principal investigators or the American Type Culture Collection (ATCC). Plasmid vectors with a range of origins of replication, promoters, and marker genes are available for cloning or other modification using recombinant DNA techniques. Rapid growth, high efficiency transformation, ease of environmental manipulation, and well-established protocols round out the pragmatic benefits of experimenting with *S. cerevisiae*.

Nutrient availability, oxidation, temperature, osmotic environment, concentration of metals, radiation, agonists, and antagonists offer a variety of stimuli that yeast may experience in nature. Each of these has been found to initiate in *S. cerevisiae* a regulatory response with a transcriptional component [6, 7]. Commercial ethanol production byproducts such as acetic acid and furfural also influence transcription [8]. Analysis of the entire yeast transcriptome in response to environmental stimuli has been performed using comprehensive microarrays and RNA sequencing [6]. These data are often available in the public domain linked to publications or annotated genome websites [5]. Accordingly, students can often find a good deal of information on-line about the transcriptional regulation of any gene of interest (GOI).

As in other organisms, transcriptional regulation in yeast is mediated by trans-acting factors (e.g. transcription factors) binding to cis-acting elements (e.g. regulatory elements in promoters) or other trans-acting factors. RNA polymerase is subsequently recruited for or prevented from initiating transcription. Stimuli may initiate signal cascades that influence trans-acting factor abundance, location and / or conformation (Fig.1). While chromatin conformation can influence transcription in yeast, heterochromatin is largely limited to subtelomeric regions and mating loci [9].

Monitoring transcriptional activity in yeast can be performed in several ways. Some are more economical than others. Promoters of a GOI can be cloned into a plasmid lacking cis-acting elements but with a coding sequence for an easily assayable reporter enzyme [10]. Transformation of the plasmid into yeast allows for subsequent integration or episomal maintenance. Exposing parallel cultures of transformed yeast to control or

stimulating conditions and quantifying reporter enzyme activity allows for comparisons of transcriptional activity. Alternatively, transcript abundance, an indirect measure of transcriptional activity, can be measured using quantitative (i.e. real time) polymerase chain reaction (PCR) [11]. This procedure can utilize several chemical detection strategies [12] and often involves a specialized thermal cycler with fluorescence detection. Microarrays / gene chips and RNA-Seq are two more modern technologies that can monitor transcriptome changes in response to stimuli. However, those require instrumentation and funds that may not be readily available.

Course Details and Implementation

Molecular Biology (BIOL/BCHM 474) is a four-credit, combined lecture and laboratory course. Integration between the lecture and laboratory is unstructured and occurs at the topics of DNA replication, transcriptional regulation, methodology, experimental design, and data interpretation. For the laboratory component, the class meets once a week for four hours. Even though only one semester of organic chemistry laboratory and one semester of biochemistry lecture satisfy the prerequisites, the class is typically taken as the third in a series of three advanced biochemistry courses. Each has an associated laboratory. Students are primarily junior and senior biochemistry majors.

Routinely, the same instructor teaches the lecture and two laboratory sections with 15 - 18 students per section. Two undergraduate laboratory preparation staff members prepare and provide the materials listed on weekly “prep sheets”. Prep sheets (in supplemental material) are organized by technique and list equipment, chemicals, disposables, and waste generated. Prep sheets are provided prior to the term along with a weekly schedule of

techniques. That schedule is amended as needed with one week of notice. Laboratory preparation students also routinely facilitate students performing experimental work in between scheduled class meetings if advanced notice is provided. Accordingly, skilled and dedicated laboratory preparation students have greatly aided this course. Since the course is often populated with seniors, preparation students rarely have taken the class previously so they experience some just-in-time learning.

The class meets in a 1,000 ft² laboratory equipped with incubators, centrifuges, water baths, thermal cyclers, freezers, refrigerators, balances, sinks, and two fume hoods. UV-vis spectrophotometers, a cold room, and a gel documentation system are in adjoining rooms. Lap top computers are available for each student. Supplies are purchased with a budget of about \$3500 for the two sections combined. Canvas is our university's learning management system. Electronic resources such as the lab manual and selected manuscripts are provided there. Independent Google sites are generated by each student group for keeping weekly lab notes, data, references, and other supporting documents. These "living" documents are convenient for providing written feedback in a unique color in the body of student work. Using on-line notebooks also guarantees unrestricted access to the data for all group members.

Student Learning Objectives

The course is designed to address four main learning objectives.

- a) *Be able to create a mechanistic hypothesis or question and create a plan to address it experimentally.*
- b) *Become proficient at several molecular biology techniques.*

c) *Adopt a project-oriented, self-motivated, goal-centric approach to a research*

enterprise.

d) *Improve communication skills.*

Laboratory Exercises

The broad aim of the project is to better understand how transcriptional regulation of a gene or genes allows a cell to respond to environmental changes. Information to guide the students in their pursuit of this aim is provided in a 73-page lab manual [13]. Instead of prescribed weekly activities, the manual contains an overview of the project, a suggested sequence of steps, and is a compendium of technique protocols. Each protocol includes a one to two page introduction describing the background, purpose, and mechanisms involved. Students are charged with using the information in the manual to plan and execute their experiments. While providing a suggested sequence of experiments impinges on the inquiry nature of the project, experience indicates that without some provided framework, students are less productive. Pre-laboratory presentations by the instructor routinely last less than 20 minutes and offer just-in-time descriptions of the mechanisms underlying the day's likely techniques. A longer presentation at the first class meeting introduces the mechanisms of PCR and transcriptional regulation.

Stage 1: Planning experiment and designing oligonucleotides (time: three weeks)

After being introduced to the course and reminded of good laboratory practices, students form groups of three. Groups of two are sometimes required. Planning the project begins with students choosing a cell process of interest. Most students find some particular aspect of how life works to be interesting and this approach lends itself to a systems biology approach. Groups then use their own knowledge and/or a search of the

scientific literature to identify three proteins in any organism that are components in that process. Searching the Protein database at the National Center for Biotechnology Information website [14] allows retrieval of the respective amino acid sequences. These may be queries in the blastp function at the SGD to identify yeast homologs. Yeast GOI may also be found using the YeastMine search engine [15] which allows a keyword search of yeast gene descriptions.

After identifying three GOI, students develop ideas about conditions in which those genes may be transcriptionally regulated. Again, the scientific literature and the SGD are suggested resources. Detailed experimental designs are formed including the volume of cells, concentration of media components, and time(s) of exposure. Putative promoter regions of the GOI are then identified in the SGD. This requires determining the genomic (i.e. chromosomal) location of the open reading frames of each GOI using the “Sequence” function at the respective SGD site. This same function allows determination of the genomic location of the gene closest to the GOI’s 5’ end. The sequence that begins with the first nucleotide after the “nearest neighbor” gene and the nucleotide just before the start codon for the GOI is the putative promoter region. Importing this sequence into DNA analysis software allows for the formation of reverse - complement sequences and restriction enzyme site identification. The ApE (A plasmid Editor) program made by M. Wayne Davis [16] is free and provides ample functionality.

Primers for amplifying the putative promoter regions of the respective genes are then designed. This exercise emphasizes the spatial aspects of the anti-parallel DNA double helix and how primers are extended during DNA replication. On-line tutorials and an in-

class exercise with a provided DNA sequence afford auxiliary instruction. Asking students to provide the genomic location of each primer helps detect improper design and allows predicting the length of PCR products. It is uncommon for the sequences between open reading frames to be longer than 1.0 kb. However, if the putative promoter is longer than 2.0 kb, students are encouraged to design primers to the proximal 2.0 kb or less. This avoids the need for PCR conditions specific to “long products.”

Stage 2: PCR and product ligation into the pSF011 reporter plasmid (time: six weeks)

Yeast genomic DNA is isolated and used as the template in a PCR with the designed primers. The products are resolved by agarose electrophoresis (Fig. 2) and extracted if the correct length. In parallel, bacteria harboring the pSF011 reporter plasmid [17], which includes a polylinker next to the β -galactosidase gene, are cultured and the plasmid extracted by alkaline lysis. pSF011 is an integrating plasmid that requires linearization prior to transformation. Integration provides a scientific benefit as it puts the DNA in a chromosomal context. Even so, other yeast reporter plasmids will likely suffice.

Prior to ligation, PCR products and pSF011 undergo modification. Using one strategy, the primers are designed to include unique restriction enzyme sites at the 5' ends. The two chosen enzyme sites are unique in the pSF011 polylinker so that the site in the oligonucleotide near the GOI is closer to β -galactosidase in pSF011. This allows for directional cloning. Alternatively, no sites are engineered into the primers. The PCR products are treated with T4 DNA polymerase and T4 DNA kinase to create blunt, phosphorylated termini. pSF011 is digested with EcoRV and dephosphorylated with calf

intestinal phosphatase to create blunt, dephosphorylated termini. Students have used both strategies with similar success.

Ligation and transformation into competent *E. coli* (generated by students if time permits) generates bacterial colonies that may contain the desired pSF011 / promoter plasmid. Culturing of the bacteria in liquid Luria-Bertani broth with ampicillin, plasmid isolation, and analytical restriction enzyme digestion ideally identifies one or more plasmids of intended construction.

Students who successfully construct the intended plasmid are heartily congratulated. There are many potential pitfalls. Unproductive PCR reactions are often due to improperly (i.e. wrong orientation) designed oligonucleotides. Otherwise, about 70% of the oligo pairs yield products of the expected size. Obtaining low to absent yields when extracting DNA from agarose gels is another common problem. Optimizing this protocol or identifying another for use by novice experimenters is a future goal. Time constraints sometimes require shortening digestion times which may also decrease the success rate. Submitting positive clones for sequencing at a core facility may allow for students to examine sequence output and look for PCR induced sequence artifacts. However, time has never allowed for this option.

If groups do not successfully generate the plasmids by the end of week nine, then there is little chance that the project can be completed prior to the end of the term. Two contingency plans have been used. Students have used the literature to find papers that describe the construction of promoter / reporter plasmids. Kindly contacting the corresponding authors to request the plasmids routinely resulted in letters arriving within

two weeks. This “clone by phone” approach allowed the groups to move forward with the caveat that the hypothesis and growth conditions required amendment. Groups from later years have benefitted from these plasmids being readily available. Similarly, clones generated in previous semesters may be used. This requires careful sample placement during the flurry of semester-end activity. Alternatively, groups can use their original hypothesis and use quantitative PCR to monitor transcriptional regulation in response to the conditions originally proposed. This “plan B” is described later.

Stage 3: Transformation of yeast, culturing under stimulating conditions, and monitoring transcription. (four weeks)

Transforming the pSF011 / promoter constructs and empty pSF011 (control) into yeast is preceded by plasmid digestion with NcoI to linearize the plasmid. This divides the *URA3* locus in the plasmid and allows integration into the *ura3* (i.e. mutated *URA3*, found in many lab strains of *S. cerevisiae*) locus by recombination. Transformants are selected by autotrophy on synthetic complete (SC) media lacking uracil. In parallel to transforming wild-type yeast, transforming a strain with a targeted gene deletion offers an experimental variable. Such deletion strains may lack a transcription factor or receptor hypothesized to be necessary for the transcriptional regulation under study. These strains may be obtained from the corresponding authors of studies describing the strains or from the ATCC.

Successfully transformed yeast can then be used to study transcriptional regulation in response to the proposed stimuli. If nutrients, or the lack of them, are the independent variables, then commercially available synthetic media are necessary. Synthetic media contain four components: a drop out media (DOB or CSM; a mixture of amino acids and

bases), yeast nitrogen base (YNB; a mixture of cofactors and coenzymes), a nitrogen source (e.g. ammonium sulfate) and a carbon source (e.g. glucose). Media which lack one or more components of DOB or YNB allow the experimenter to completely control the concentration of that nutrient. Time of exposure and concentration of the component(s) are two variables often studied. Considering appropriate negative controls to address possible confounding effects (e.g. osmolarity) is suggested.

After the treatments, cells can be frozen so to allow simultaneous assay of samples collected over time or at a later class period. Lysis and colorimetric analysis of β -galactosidase activity uses straightforward protocols. The number of cells harvested, as measured by Abs_{660} , serves as the normalizing value. Protein quantification assays allow for more rigorous normalization if time permits.

Contingency Plan “B”: quantitative PCR (time: four weeks)

Due to the challenges that novice experimenters face handling and manipulating DNA, only about 40% of the groups construct the promoter / reporter plasmids by the end of the ninth week. One contingency experimental plan is to use quantitative PCR with the original experimental plan for the GOI and stimulating conditions. Yeast are grown in the stimulating conditions and RNA is extracted using a “hot phenol” method. Following quantification and DNase I treatment, the RNAs are reverse transcribed to first strand cDNA using commercially available kits. Quantitative PCR using serial dilutions of the cDNA is performed using newly designed primers complementary to the 3' end of the GOI open reading frame. Mixtures of polymerase, buffer, deoxyribonucleotides, and SYBR green dye are purchased and the manufacturer' recommended cycles are used. Expression

levels are calculated using the $\Delta\Delta C_q$ method [18]. Students have had good success generating interpretable data with these assays (Fig. 3). This experiment can also be used independently as a module or a smaller scale independent project.

Reflection and Future Modifications

This is an ambitious project for students without molecular biology research experience. Accordingly, about 5 - 10% of groups successfully generate β -galactosidase data for their GOI. While this rate seems strikingly low, the students appear to become more comfortable with experimental logistics over time. Contingency plans provide a safety net. Anecdotally, many students become engaged in their projects and commit time and effort to them.

Future plans include testing different protocols for extracting DNA from agarose and creating compatible termini for PCR products and plasmids. This may increase the students' success rate. Including peer evaluation of the posters prior to public presentation may enhance student consideration of poster formatting. Projecting posters onto a screen instead of printing them may allow for more drafts and reduce costs.

Scaling up the laboratory to accommodate more than two sections of students would provide a logistical challenge. Each group of three students comprises a research entity that likely benefits from customized input and feedback. However, if graduate students were available to act as teaching assistants, their contributions may allow expanding the student population.

Evaluation of Student Performance

As indicated in the syllabus in supplemental materials, groups are evaluated based on the completeness of their on-line weekly lab notebook entries (20%), experimental design (8%), effort (12%), and the poster (60%). Notebooks are regularly graded with feedback topics ranging from suggested information to calculations. Posters are evaluated based on hypothesis and background (13%), experimental design and methods (13%), results (40%), conclusion (6.5%), answering of questions at the oral presentation (6.5%), and poster layout (20%). Detailed feedback of the posters is provided to each group as a Word document.

Student Learning Outcomes

Since the posters were the culmination of the students' work, learning outcomes were mainly monitored by compiling poster evaluations. The performance of 30 groups from 2014 - 2016 were analyzed. Constructing and communicating a detailed and comprehensive background section and articulating a mechanistic hypothesis reflect successful achievement of learning outcome (a) described above. 63% of the student groups met or exceeded these expectations. Groups not meeting expectations commonly provided insufficient description of the cell process under study. Another common shortcoming was not describing a proposed mechanism that underlies the hypothesis. Proficiently conducting molecular biology experiments, learning goal (b), and adopting a goal-centric approach, learning outcome (c), was measured in the "results" criteria of the posters. Cloning at least one putative promoter region into pSF011 or generating interpretable quantitative PCR data with appropriate controls was the expectation. 67% of groups met or exceeded the expectation. Groups not meeting expectations commonly hit logistical challenges with the initial PCR and then were not expedient after shifting to

quantitative PCR. Communication was assessed in the “poster layout” section. Including strategic text to provide flow through the Results section, proper labeling of data (e.g. providing informative lane labels for gels and axes labels for graphs), and logical placement of panels was the expectation. 80% of students met or exceeded that expectation. Providing content without consideration of connection between the neighboring panels was a common problem.

Conclusion

In line with the philosophy that student engagement promotes learning, an inquiry-based molecular biology laboratory has been developed and taught for five years. While students have freedom to choose the cell process and growth conditions that may induce transcriptional regulation of relevant genes, the general experimental format (i.e. recombinant DNA construction of promoter / reporter plasmids) and protocols are provided. Of the types of inquiry proposed by Buck et al. [19], this approach best resembles “open inquiry”.

Few molecular biology laboratories with this degree of inquiry have been described in the literature. Perturbation of HeLa (i.e. human fibroblast cells) and broad monitoring of the cells’ proteome by SDS-polyacrylamide electrophoresis was done with a single, class-chosen environmental stimulus [20]. Transfer RNA conformation analysis is part of a course that includes a four-week, student planned experiment component [21]. PCR and subcloning of bacteriophage genomic DNA fragments is part of a semester-long, molecular virology project [22]. In biochemistry laboratories, examples of full-semester projects include the overexpression of guanylate monokinase, production of monoclonal antibodies, and ELISA analysis [23]. Development of a functional assay for a protein of interest in the

second semester of a two-semester biochemistry laboratory course utilizes techniques learned in the first semester [24]. Similarly, in a single semester course, ten weeks of guided instruction lead into a three-week project studying *H. pylori* urease [25].

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Supporting Information

The laboratory syllabus and a compendium of laboratory preparation sheets are available as supporting information. The laboratory manual is available at:

<http://www-personal.umd.umich.edu/~poelkers/OelkersMolbiolmanualUMD2016.pdf>

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Accepted Article

Figure Legends

Fig. 1. Schematic of promoter reporter plasmid activation. The binding of a ligand to a cell surface receptor (1) is one of the ways in which yeast may sense environmental changes. Subsequent signaling (2) may induce a transcription factor to relocate to the nucleus (3) and bind to cis-acting elements. Binding proximal to the β -galactosidase (β -gal) reporter gene may influence its transcription. Changes in transcription rate may be detected by the change in rate at which the corresponding transcript is translocated (5) to the cytosol and translated (6). Measurement of β -gal activity is thus an indirect measurement of transcriptional regulation.

Fig. 2. GelRed stained agarose gel of PCR products. Two different lengths of the putative promoters of (A) *GSH1* or two different genes, (B) *DTR1* and *GAS4*, are shown.

Fig. 3. Real-time PCR analyzing the effect of nitrogen availability on *GRE3* expression. Wild type yeast were cultured in synthetic complete media with either the full complement (100%) of ammonium sulfate or the indicated percentage for 1, 2, and 3 hours. After culturing, RNA was isolated, cDNA was synthesized using oligo dT primers, and real time PCR performed using a SYBR green master mix (BioRad) and primers complimentary to *GRE3* or *ACT1*. Fold expression was calculated using the $\Delta\Delta C_t$ method. For each time point, the expression of *GRE3* in 100% nitrogen was set to 1.0. n=1.

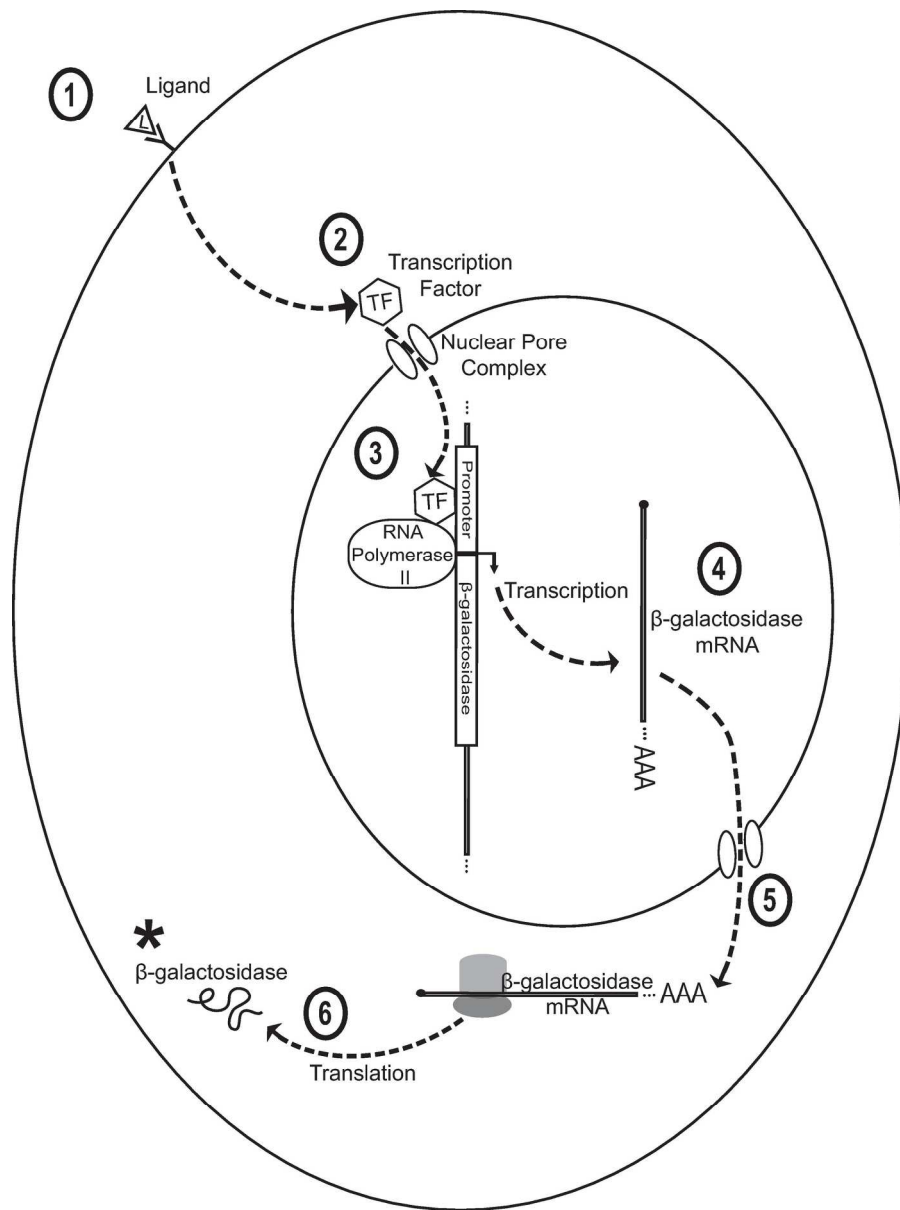


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Fig. 1

175x234mm (300 x 300 DPI)

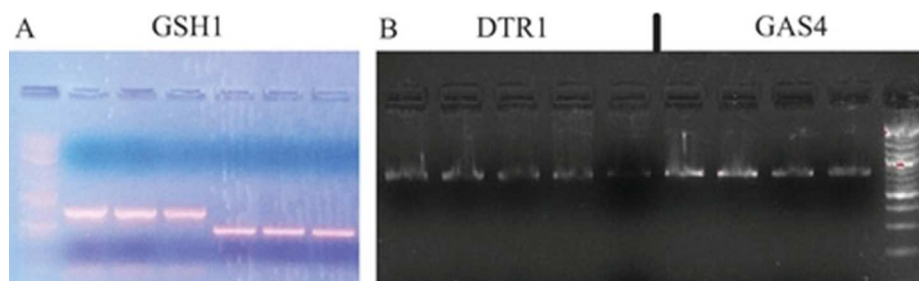


Fig. 2. GelRed stained agarose gel of PCR products. Two different lengths of the putative promoters of (A) GSH1 or two different genes, (B) DTR1 and GAS4, are shown.

Fig. 2

38x11mm (300 x 300 DPI)

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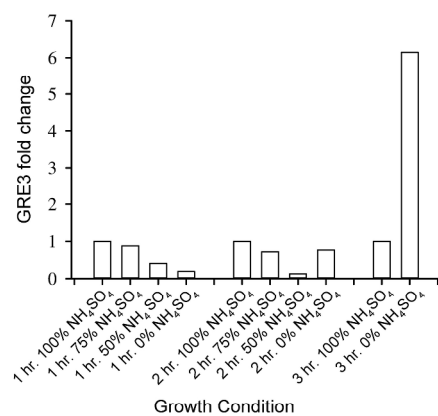


Fig. 3. Real-time PCR analyzing the effect of nitrogen availability on GRE3 expression. Wild type yeast were cultured in synthetic complete media with either the full complement (100%) of ammonium sulfate or the indicated percentage for 1, 2, and 3 hours. After culturing, RNA was isolated, cDNA was synthesized using oligo dT primers, and real time PCR performed using a SYBR green master mix (BioRad) and primers complimentary to GRE3 or ACT1. Fold expression was calculated using the $\Delta\Delta C_t$ method. For each time point, the expression of GRE3 in 100% nitrogen was set to 1.0. $n=1$.

Fig. 3

278x359mm (300 x 300 DPI)