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A Yeast Reporter Assay to Analyze the Effect of Dietary Supplements on Gene Expression

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Regulation of gene expression is an essential topic introduced in undergraduate level genetics courses. However, understanding how environmental factors can directly lead to changes in gene expression can be challenging. In this inquiry-based laboratory exercise, a yeast *LacZ* reporter assay is used to demonstrate how environmental cues can regulate gene expression. The assay utilizes *Saccharomyces cerevisiae* that has been transformed to carry a plasmid with yeast variants of the AP-1 response elements in the promoter upstream of the *LacZ* gene. The expression of *LacZ* in these strains is induced by oxidative stress to activate the AP-1 transcription factors. Students then assess if dietary supplements, such as caffeine, modify or counteract these effects. This lab can be easily modified to test a range of variables, including the type, concentrations, and source of dietary supplement as well as varying effects from different yeast AP-1 homologs. Although developed for an introductory genetics course, the lab can be easily adapted for an upper level genetics course to provide additional opportunities for open-ended research and literature review.

Keywords: genetics, inquiry-based learning, gene regulation

Introduction

Oxidative stress is known to affect cell signaling pathways that ultimately alter gene expression. Dietary supplements often claim to counteract oxidative stress and therefore may potentially affect transcriptional regulation of genes induced by oxidative stress. In this lab exercise, students will learn how one dietary supplement, caffeine, can regulate gene expression during oxidative stress using a yeast *LacZ* reporter assay. The exercise uses the yeast *Saccharomyces cerevisiae* as a model organism. The assay focuses on transcriptional activation mediated by yeast homologs of the AP-1 transcription factor, Yap1 and Gcn4, under oxidative stress and in the presence of caffeine.

To perform the experiment, the reporter gene was introduced to *S. cerevisiae* via plasmid transformation using a standard lithium acetate transformation. The plasmid was obtained from Dr. Yoshiharu Inoue and originally contained response elements that specifically bind only the Yap1 transcription factor (Maeta *et al*., 2004). Activation of the response elements leads to expression of the *LacZ* gene and production of β-galactosidase. The plasmid was mutated so that the response element would specifically bind Gcn4. Thus, two versions of the plasmid are available; (1) a Yap1 Reporter and a (2) Gcn4 Reporter. Strains containing either plasmid are available upon request.

Overview of Lab Exercise

The lab exercise is written to be completed in two, 3-hour lab periods in an introductory genetics level lab course. The exercise is performed in groups of 2-4 and can be used in large or small class sizes. The lab handout as written uses the Yap1 reporter; however, it can easily be modified to use the Gcn4 reporter. The experiment as written is performed by a group of four students, with one pair in the group completing Experiment A and the other completing in experiment B. This will allow students to see the effects of oxidative stress alone and in the presence and absence of the supplement. Comparing the results between Experiment A and B may also reveal dose-dependent effects of the supplement. Furthermore, comparing the data across multiple groups may reveal differences in effects due to the caffeine course. In the first lab session, students induce oxidative stress in the yeast in the presence and absence of caffeine. The experiment is done in triplicate. At the end of the first lab period, students collect cell samples and measure cell density via spectrophotometry. The samples are then frozen until the next lab period. In the second lab period, students lyse the cells and perform standard *LacZ* assays to measure expression levels. At the end of the exercise, students perform statistical tests and graph their results. An Excel template for graphing and performing statistical tests is available upon request.

Student Learning Outcomes

Students learn the basic concept of how transcription factors regulate transcription. This experiment also teaches students to connect cell signaling with gene transcription. The experiment is easy to perform but requires students to be careful and consistent in their pipetting to ensure good reproducibility between the triplicates and conditions. They analyze their data using Excel and perform basic statistical analysis. The lab write-up directs students to think through the experimental design, to develop hypotheses and predictions, and then to compare their results with these.

Student Outline

Learning Outcomes

By the end of this lab, students will be able to:

- Explain how some transcription factors interact with promoters to regulate gene expression.
- Describe in general how cell signaling is integrated with gene regulation.
- Relate the similarities in cell signaling involving the AP-1 transcription factor between humans and *Saccharomyces cerevisiae*.
- Develop hypotheses and predictions for how caffeine may affect AP-1 dependent transcription.
- Perform a *LacZ* reporter assay to test the hypotheses.

Cell Signaling and Transcriptional Regulation of Gene Expression

Transcriptional regulation of gene expression is mediated by **transcription factors**. These proteins either enhance or decrease the affinity of RNA polymerase to bind to the promoter of a gene and therefore activate or repress gene expression, respectively. Often these transcription factors must bind to regulatory sequences called **response elements** at or near the promoter to exert their effects (Fig.1). There are numerous families of transcription factors, many of which are downstream effectors for cell signaling pathways.

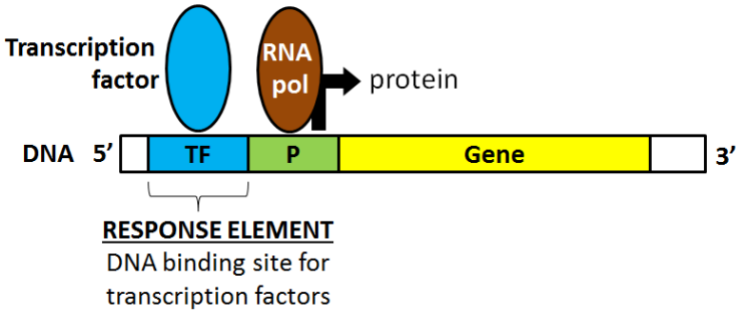

Figure 1. Regulation of transcription by transcription factors.

Cell signaling pathways control and coordinate the cell's responses to its external and internal environment. Cell signaling then is central to not only homeostasis but how a cell responds to environmental cues, such as a mitogen that promotes growth or a potentially damaging molecule that can lead to cellular stress. As part of these responses, many cell signaling pathways alter the activity of transcription factors to promote or enhance transcription (Fig. 2).

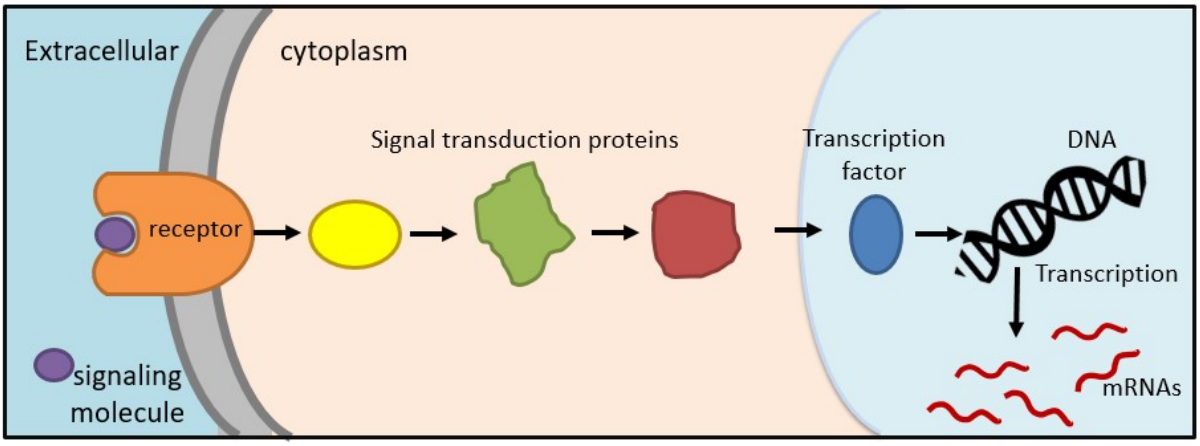

Figure 2. Cell signaling pathways can regulate transcription factors to control gene expression.

In this lab, we will be examining transcriptional regulation by the Activator Protein-1 (**AP-1) transcription factor**. AP-1 is conserved in all eukaryotes and has a central role in many cellular responses. It is a basic leucine zipper (bZIP) transcription factor, which is active only when it dimerizes with another AP-1 transcription factor (Shaulian and Karin, 2002).

Multiple signaling pathways found in all eukaryotic cells, including the Ras/MAPK, TOR, and AMPK pathway, converge on the AP-1 transcription factor. The activation of AP-1 induces the expression of a number of genes involved in growth, development, stress, and nutrient supply (Shaulian and Karin, 2002). AP-1 is a dimer and in mammalian cells, AP-1 monomers are from the Jun and Fos family of proteins. Mutations to Jun or Fos have been shown to promote cancer. Yeast have several genes homologous to AP-1, two of which are *YAP1* and *GCN4* (Moye-Rowley *et al*., 1989). These transcription factors have been shown to induce gene expression in response to various environmental and physiological stresses. Although they are homologous, their responses to these stresses can vary. For example, Yap1 is known to induce gene expression early in response to oxidative stress, while Gcn4 has been suggested to be important for cellular responses after prolonged oxidative stress (Maeta *et al*., 2004; Mascarenhas *et al*., 2008). **In this lab, we will examine the role of Yap1 in regulating gene expression during oxidative stress.**

Analyzing Gene Regulation using a *LacZ* Reporter Assay

To understand how internal and external cues affect gene regulation, scientists often to turn simple model organisms and systems in which they can manipulate the promoter and/or transcription binding site sequences. The *LacZ* reporter assay has been used extensively to examine gene regulation. In this system, a plasmid carries the reporter gene *LacZ*, which encodes the enzyme β-galactosidase (Maeta *et al*., 2004). This gene is expressed only when RNA polymerase and specific transcription factors binds to the promotor region and transcription binding sites, which are found upstream of the gene. For this lab, we will analyze how AP-1 regulates gene expression in the yeast *Saccharomyces cerevisiae* (Fig. 3). The assay utilizes yeast strains that have been transformed with a plasmid that contains AP-1 response elements from Yap1 incorporated upstream of the *LacZ* gene (Maeta *et al*., 2004).

For this plasmid, AP-1 will bind to its response element in the promoter and activate transcription of the *LacZ* gene when cells are subjected to oxidative stress. In our system, we will induce oxidative stress by incubating the yeast with hydrogen peroxide (H_2O_2). Upon activation, β-galactosidase will be produced.

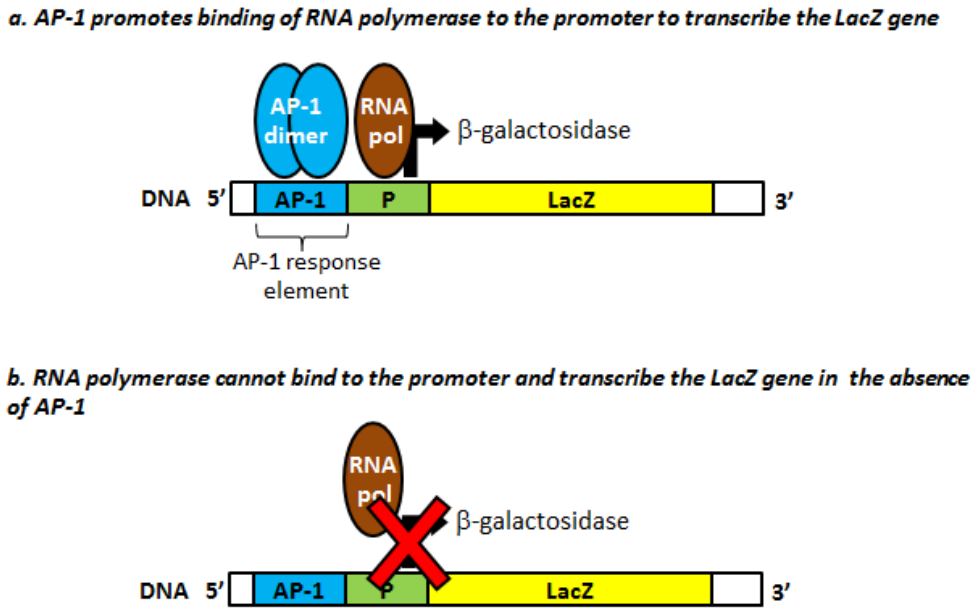

Figure 3. AP-1 Regulation of transcription.

β-galactosidase can convert a clear chemical ONPG to a yellow metabolite, o-nitrophenol (Fig. 4). This compound absorbs at 420nm. Thus the concentration of this compound, and in turn the amount of *LacZ* expression, can directly be quantified through spectrophotometry.

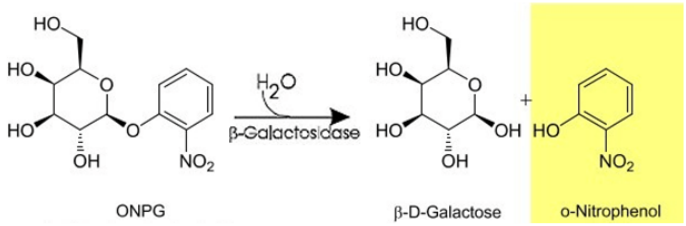

Figure 4. Reaction catalyzed by β-galactosidase.

The quantification of the products formed by the reaction above is calculated in "Miller Units" (Rupp, 2002). A Miller Unit then is a standardized amount of β-Gal activity and is calculated using the equation below. For this equation, the volume of culture assayed in measured in mL and time is measured in minutes.

$$Units\ of\ beta\ Gal\ (Miller\ Unit) = \frac{A_{420}}{A_{600}}\ x\ volume\ assayed\ x\ time$$

The Role of Caffeine on Oxidative Stress

The accumulation of reactive oxygen species, termed **oxidative stress**, has been implicated in aging and the progression of many diseases, including cancer, diabetes, and Alzheimer's disease. Each year, Americans spend tens of billions of dollars on dietary supplements, such as vitamins, herbal products, and minerals (Garcia-Cazarin *et al*., 2014). Studies have suggested that caffeine has anti-oxidant properties and therefore can reduce oxidative damage or stress (Devasagayam *et al*., 1996; Yu *et al*., 2017). This would in turn likely affect the expression of genes regulated by AP-1, which is activated in response to oxidative stress (Shaulian and Karin, 2002).

In this lab, we will determine the effect of caffeine on transcriptional activation mediated by one of the AP-1 transcription factors in the yeast *Saccharomyces cerevisiae*. We will use a *LacZ* reporter assay to measure transcriptional activation by Yap1 in the presence and absence of caffeine during oxidative stress. In addition, each group will be given a different source or form of caffeine to test.

Experimental Questions

1. How does caffeine affect AP-1 regulated transcription during oxidative stress?

2. Do different forms of caffeine have varying effects on AP-1 regulated transcription during oxidative stress?

Materials

- Spectrophotometer with 1-mL cuvettes
- Cultures of yeast transformed with Yap1 reporter plasmid, grown to log phase
- 50mM H_2O_2
- 100mM stocks of caffeine from various sources (examples: pure caffeine, guarana, gingko biloba)
- Chloroform
- 0.1% SDS mix
- Z-buffer (60mM Na_2HPO_4, 40mM NaH_2PO_4, 10mM KCl, 1 mM $MgSO_4$; pH 7)
- 4 mg/mL ONPG in Z Buffer, made fresh
- 1M Na_2CO_3

Safety Considerations

You will be working with live yeast cultures and chloroform, a known toxin and potential carcinogen. Therefore make sure to wear eye protection and gloves. If you spill any yeast cultures or chloroform on yourself, wash the affected area immediately and let your instructor know.

Week 1- Cell Treatment with H_2O_2 and Dietary Supplement

You will work in groups of four to perform the experiment and will be given actively growing yeast transformed with the reporter plasmid. Each condition will be tested in triplicate. One pair in the group will perform Experiment A and the other pair will perform Experiment B. The data will then be aggregated and analyzed together.

Table 1. Experimental setup.

Experiment A	No caffeine	2mM caffeine
No H_2O_2	Samples 1, 2, 3	Samples 4, 5, 6
+ 0.5 mM H_2O_2	Samples 7, 8, 9	Samples 10, 11, 12

Experiment B	No caffeine	10mM caffeine
No H_2O_2	Samples 13, 14, 15	Samples 16, 17, 18
+ 0.5mM H_2O_2	Samples 19, 20, 21	Samples 22, 23, 24

1. Label your sterile Eppendorf tubes with your initials, experiment letter, and number them 1-12 or 13-24.

2. Swirl the yeast culture and then promptly add 0.5 mL of yeast to each tube.

3. Add the caffeine or an equal amount of water (no caffeine) to the tubes (Table 2).
    a. Experiment A
        i. For tubes 1-3 and 7-9, add 10 µL of water.
        ii. For tubes 4-6 and 10-12, add 10 µL of the caffeine stock.
    b. Experiment B
        i. For tubes 13-15 and 19-21, add 50 µL of water.
        ii. For tubes 16-18 and 22-24, add 50 µL of caffeine stock.

4. Vortex the tubes to mix.

5. To the tubes add H_2O_2 to a final concentration of 0.5 mM or water (Table 2).
    a. Experiment A
        i. For tubes 1-6, add 5.1 µL of water.
        ii. For tubes 7-12, add 5.1 µL of the 50mM H_2O_2 stock.
    b. Experiment B
        i. For tubes 13-18, add 5.5 µL of water.
        ii. For tubes 19-24, add 5.5 µL of the 50 mM H_2O_2 stock.

Table 2. Summary of volumes added for water, caffeine, and H_2O_2 for Steps 3 & 5.

		Experiment A Samples				**Experiment B Samples**			
		1-3	**4-6**	**7-9**	**10-12**	**13-15**	**16-18**	**19-21**	**22-24**
Step 3	**water**	10 µL		10 µL		50 µL		50 µL	
	caffeine		10 µL		10 µL		50 µL		50 µL
Step 5	**water**	5.1 µL	5.1 µL			5.5 µL	5.5 µL		
	H_2O_2			5.1 µL	5.1 µL			5.5 µL	5.5 µL

6. Vortex the tubes to mix.

7. Incubate the tubes in the 30°C water bath for 1.5 hours.

8. During the last half hour of the incubation:
    a. Turn on the spectrophotometer and set the wavelength to 600nm.
    b. Set up the blanks, one for each condition according to Table 2. The blanks will contain everything in the sample except instead of using the yeast culture you will use sterile yeast media. Thus, Experiment A and B will each have four blanks.

9. Read the Absorbance (OD_{600}) of each sample. This will tell us if the samples have the same density of cells. We will use this reading to normalize, or adjust, our measurements for differences in cell density.
    a. Blank the spectrophotometer with the appropriate blank. Pipet the blank back into the tube. Do not discard the blank until you have taken all your readings.
    b. Vortex the tube and pipet the sample into the cuvette. Record the reading in the table at the end of the handout.
    c. **DO NOT DISCARD THE SAMPLE! Pipet the sample back into its tube.**
    d. Rinse the tube out with distilled water.
    e. Repeat for each sample.

    Note: You do not need to blank the spectrophotometer in between samples of the same condition, only before taking measurement of a different condition.

10. Spin down the cells at 6000 rpm for 1 minute.

11. Remove the growth media by pipetting; leave the pellet of cells. It is important that you remove as much of the liquid above the pellet as possible.

12. Freeze the pellet until the next class period.

Week 2- Standard ONPG Spectrophotometric Assay (GLOVES AND EYE PROTECTION MUST BE WORN!)

1. Defrost the cell pellets.

2. Resuspend each cell pellet in 800 μL of Z buffer, vortex to mix thoroughly.

3. Lyse the cells by adding 40 μL of 0.1% SDS followed by 60 μL of chloroform.

4. Vortex for 10 seconds.

5. Incubate samples at 30°C for 5 minutes.

6. Add 200 μL ONPG/Z buffer to each sample. Vortex briefly and record starting time. This step must be done quickly.

7. Incubate at 30°C for about 30 minutes, until yellow color appears in most samples.

8. Stop the reaction by adding 400 μL 1M Na_2CO_3. Record stopping time.

9. Centrifuge for 10 minutes at maximum speed.

10. Remove 1 mL of the supernatant and place into new clean tube. Do not pipet up any of the cell debris in the chloroform. Discard the tubes with chloroform in the appropriate waste container.

11. Measure absorbance at 420nm.
    a. Blank the spec with a solution of 800 μL Z buffer + 200 μL ONPG/Z buffer + 400 μL 1M Na_2CO_3.

b. Vortex the tube and pipet sample into the cuvette. Record the reading in the table at the end of the handout.
c. **DO NOT DISCARD THE SAMPLE! Pipet the sample back into its tube.**
d. Rinse the tube out with distilled water.
e. Repeat for each sample.

12. For each sample calculate the number of units of β-Gal produced as a result of AP-1 activation. Volume of culture assayed in mL and time assayed in minutes.

$$Units\ of\ beta\ Gal\ (Miller\ Unit) = \frac{A_{420}}{A_{600}}\ x\ volume\ assayed\ x\ time$$

13. Average the data for each condition and present the results in the form of bar graphs. Use Excel to construct the bar graph and to put error bars that indicate standard deviation.

14. Use the Student's t-test to compare the following for each reporter.
    a. No caffeine to 2mM caffeine
    b. No caffeine to 10mM caffeine

15. Answer the following questions.
    a. Why do we include H_20_2 in the experiment?

    b. What is/are the independent variables? What is the dependent variable?

    c. What does the dependent variable(s) tell you?

    d. State one possible hypothesis for each of questions addressed.
        - How does oxidative stress affect AP-1 regulated transcription?
        - How does a low concentration of caffeine affect AP-1 regulated transcription during oxidative stress?
        - How does a low concentration of caffeine affect AP-1 regulated transcription during oxidative stress?

    e. Predictions: Based on your hypothesis in 4, predict what you would expect the outcome of each condition would be, i.e. the level of expression for each condition. Use (-) to indicate no expression; and (+), (++), (+++), and (++++) to indicate varying levels of expression.

Experiment A	No caffeine	2mM caffeine
No H_20_2		
+ 0.5mM H_2O_2		

Experiment B	No caffeine	10mM caffeine
No H_20_2		
+ 0.5mM H_2O_2		

    f. Do you think different forms of caffeine have varying effects on AP-1 regulated transcription during oxidative stress? Develop a hypothesis and predictions based on your thoughts.

16. Write a paragraph discussing your results for +/- caffeine and low vs. high concentration of caffeine. How do your results compare to your predictions?

17. Now compare your results to the results obtained for the other sources of caffeine. Discuss in a paragraph, how the results were similar and different and which sources most affected AP-1 dependent transcription.

18. Discuss the potential effects caffeine may have on the effects of oxidative stress in humans.

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Materials

Equipment and Supplies

The following general equipment should be present in the lab for the experiment: a shaker for growing yeast, microcentrifuge, a freezer, and a water bath or dry incubator set to 30°C. If using a water bath, make sure there are sufficient floaters to hold the tubes. Each group should also have the following: a spectrophotometer with 1-mL cuvettes, vortexer, set of pipettors (P10/P20, P200, P1000) and tips, and a microcentrifuge tube rack.

Sources of Caffeine

Caffeine can be obtained from a variety of sources, including commercially prepared forms from Sigma. Caffeine can also be obtained from dietary supplement pills found from health food stores. Dietary supplements containing "natural" sources of caffeine, such as guarana and gingko biloba, can also be tested. When using caffeine sources in pill form, the pills should be crushed with a mortar and pestle, then mixed with the appropriate amount of water for the desired molarity of caffeine, vortexed vigorously and allowed to incubate for several hours before use. Because these pills often contain fillers, it will not completely dissolve and only the liquid portion should be used.

Yeast Strains

The experiment uses the diploid yeast strain BY4743, transformed with either the reporter plasmid that contains the AP-1 response elements from Yap1 (YCp50-Yap1-CYC1-LacZ) or Gcn4 (YCp-50-Gcn4-CYC1-LacZ). Both are available by contacting the author. To maintain the plasmid, the yeast must be grown in synthetic dextrose media with all amino acids but uracil (SD-ura). To make 1 L of the media, add 6.7 g yeast nitrogen base without amino acids (Fisher DF-0919-15-3) and 1.92 g of yeast synthetic dropout medium supplement without uracil (Sigma Y1501) with 960 mL of water. Autoclave to sterilize then add 40 mL of filter sterilized 50% (w/v) glucose before use. Once grown, the yeast strain can be frozen as a glycerol stock and stored at -80°C.

Yeast Growth Instructions and Optical Density Reading

Prior to the experiment the yeast strains should be grown to mid-logarithmic phase (OD_{600} ~0.5-0.7). This can be done by inoculating the yeast into 4 mL SD-Ura the night before, then transferring the culture to a flask containing 100 mL SD-Ura. The culture should be grown 4-6 hours and the OD_{600} checked before use. When reading the OD_{600} of the yeast, it is important that each culture sample is mixed thoroughly just before the reading. Yeast tend to settle quickly and even waiting a few minutes will prevent an accurate reading.

Solutions and Buffers

For a section of 24 students working in six groups of four (12 pairs total), the following solutions and buffers should be available or made.

For Day 1:

- 1 mL of 50mM H_2O_2 (5 μL of 30% H_2O_2 in 995 μL of water) per group or pair
- 100mM caffeine made in water per group
- 1 mL of sterile water per pair

For Day 2:

- 200 mL Z Buffer (60mM Na_2HPO_4, 40mM NaH_2PO_4, 10mM KCl, 1mM $MgSO_4$; pH 7) with 50mM DTT (dithiothreitol) freshly added; store at 4°C
- 100 mL 1M Na_2CO_3; store at room temperature
- 50 mL 4 mg/mL ONPG (ortho-Nitrophenyl-β-galactoside) in Z-buffer (NOTE: the ONPG should be freshly added)
- 15 mL chloroform*
- 10 mL 0.1% (w/v) SDS (Sodium Dodecyl Sulfate)

*Note: Chloroform is used to better separate the particulate cellular material from the liquid volume. It can be omitted; however care should be taken to ensure that no particulate material is introduced into the cuvette as this will affect the spectrophotometry readings.

The solutions should be distributed in the following amounts to each pair. Note: The volumes listed contain ~50% more solution than is required for the experiment.

Z buffer: 15 mL
1M Na_2CO_3: 7.5 mL
ONPG in Z buffer: 4 mL
Chloroform: 1 mL
0.1% SDS: 0.75 mL

Consumables

The experiment requires the use of microcentrifuge tubes and pipet tips. Although not strictly required because of the short duration of the experiment, it is advisable that all plastic ware is autoclaved to prevent contamination.

Notes for the Instructor

Prior to starting this lab, students should be familiar with the central dogma and have a basic understanding of cell biology. Students should also be familiar with and adept at pipetting as pipetting errors are a significant source of experimental variation. Depending on the skill level of the students, the results may be aggregated across the class.

The lab uses Excel's basic graphing and statistical analysis features. Instructors can use this lab to reinforce

these skills to students but in my experience, there is a great amount of variation in the students' level of skill with graphing and statistical analysis software. Thus, I provide my students with an excel template (available upon request) to perform this part of the lab.

Example of Results

Figure 5 provides sample results obtained by students who tested two different forms of caffeine, one from Sigma and the other in the form of guarana from a health food store, in the presence and absence of oxidative stress. Neither affected AP-1 dependent transcription at low concentration but at higher concentrations, both reduced AP-1 dependent transcription, although this was not statistically significant for guarana due to experimental error.

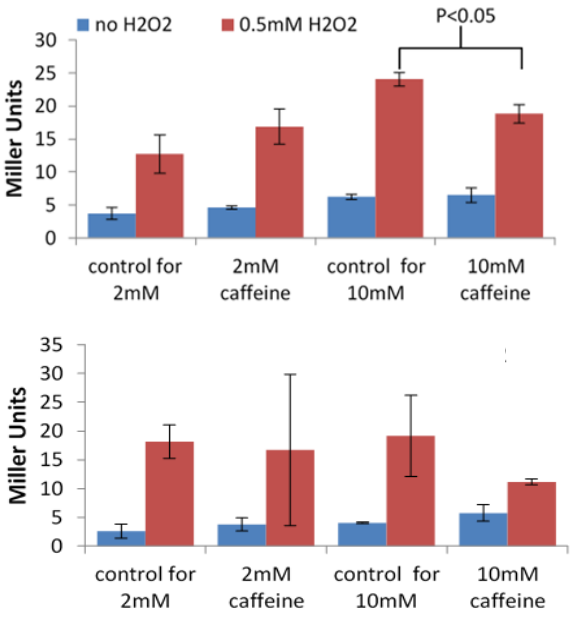

Figure 5. Student examples of the reporter assay using the Yap1 reporter. Students tested two different sources of caffeine, a commercially available form (Sigma Aldrich Cat # C0750; top) and a guarana supplement (bottom) obtained from a health food store, at 2mM and 10mM in the presence and absence of 0.5mM H_2O_2.

Variations of the Experiment

The experiment can be modified to provide more open-ended research questions. I have had students test different sources of caffeine (Sigma vs. generic health food store brand vs. guarana) within a section and then compare their results to see if there are differences in efficacy. Other dietary supplements can also be tested for their effects on AP-1 dependent transcription. Students can perform a primary literature search to identify a dietary supplement and appropriate concentrations to test. In addition, differences between the Yap1 and Gcn4 reporter plasmids can be tested as they have been reported to have different effects on transcription in response to oxidative stress.

The experiment can also provide a potential avenue for students to conduct open-ended research. Using the basic experimental design described, students can investigate the efficacy of different dietary supplements and/or differential effects of single supplement on variants of AP-1.

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About the Authors

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APPENDIX A:
Lab Datasheet on - β–Gal Reporter Assay

Sample	A_{600}	A_{420}	Time (min)	Miller Units	Average Miller units per condition	Standard deviation
1						
2						
3						
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