Combinatorial Chemistry and Factorial Design

Adapted from Wolkenberg, Scott E.; Su, Andrew I. *J. Chem. Educ.* 2001 *78,* 784-785

The object of this lab is to determine which of nine compounds acts as an antibiotic by killing *E. coli*, which is a bacterium. This can be done in six experiments using mixtures, rather than in nine experiments using pure substances.

# Factorial design

Traditionally, a series of experiments is designed such that only one variable is changed at a time. However, varying several parameters at a time can be more efficient, though analysis of the results is more difficult. An experiment designed such that several parameters are varied at a time is said to have a “*factorial design*”. This lab will illustrate factorial design by determining which of the nine compounds is the antibiotic by doing only six experiments.

To illustrate how this is possible, assume that only one of the nine compounds one is an antibiotic. Let each experiment answer the question, “Does the bacterium survive when exposed to this material?” The answer can be represented by ↑ for yes and ↓ for no. One result might be ↑↑↑↑↑↓↑↑↑. For experiments on pure compounds only nine results are possible, since only one of the compounds is an antibiotic. For the six experiments on mixtures the result might be ↑↑↑↓↑↓. Since each experiment has two possible outcomes, ↑ or ↓, for six experiments the number of possible results is 26 = 64. Because more results are possible with mixtures than with pure substances, more information can be gained in fewer experiments by doing the experiments on mixtures, rather than on pure substances.

# Combinatorial chemistry

The nine compounds to be screened for antibiotic activity will be synthesized from two types of building blocks: an aldehyde and a hydrazine. These combine to form a “hydrazone”.



Three different aldehydes (each with a different R1) and three different hydrazines (each with a different R2­) will be combined to give nine different products. This is an example of *combinatorial chemistry*, in which different components are combined in many possible combinations. With two R groups, the number of possible products is NR1 × NR2. The members of each group of compounds is called a *chemset*. The structures of chemset **1** and chemset **2** are shown in Figure 2 and

Figure 3, respectively. The product of reacting chemset **1** with chemset **2** is chemset **3** {*1*–*3*,*1*–*3*}, Figure 4



Figure . Synthesis of hydrazones.



Figure . Chemset **1**{1–3}, the aldehydes.



Figure . Chemset **2** {1–3}, the hydrazines.

|  |  |  |
| --- | --- | --- |
|  |  |  |
| 3{*1*,*1*} | 3{*1*,*2*} | 3{*1*,*3*} |
|  |  |  |
| 3{*2*,*1*} | 3{*2*,*2*} | 3{*2*,*3*} |
|  |  |  |
| 3{*3*,*1*} | 3{*3*,*2*} | 3{*3*,*3*} |

Figure . The nine possible products, **3**{*1*–*3*,*1*–*3*}.

The pharmaceutical industry uses the approach of combinatorial chemistry—combining building blocks—to create large libraries of compounds. A recent article (B. A. Morgan, *et. al*, “Design, synthesis and selection of DNA-encoded small-molecule libraries”, *Nat. Chem. Biol.*, August, 2009) describes the synthesis of a library containing 800 million small molecules. These libraries can be used to screen for, e.g., antibiotic activity. Usually, the compounds in these libraries are tested individually, rather than as mixtures. (The process called “high throughput screening” uses automation to screen thousands of compounds per day.)

# Resources

Jiju Antony, “Design of Experiments for Engineers and Scientists”, Butterworth-Heinemann, 2003.

<http://www.itl.nist.gov/div898/handbook/pri/section3/pri3.htm> Experimental design is presented on the NIST web site.

# Identification of an antibiotic by the “cup agar diffusion” method

The mixtures will be screened for antibiotic activity using the cup agar diffusion method. The bottom of Petri dishes (pronounced “pē-trē”) is covered with a layer of warm agar that, when cooled, solidifies. Small holes, or “cups” are bored in the agar using a hollow glass tube. A couple of milliliters of a solution containing *E. coli* is poured into the Petri dish to cover the surface; the excess is poured off. To each of the cups is added about two drops of one of the mixtures. Over the next week that solution will diffuse out from the cup through the agar. The result is that, if the mixture contains an antibiotic, no bacterial colonies will be visible around the cups.

# Hazards associated with *E. Coli*

News reports occasionally describe cases of people getting seriously sick from ingesting the bacteria, *Escherichia coli*, or *E. coli*. Since we are using *E. coli* in this lab, an explanation is in order.

*E. coli* exists as several different strains. Whereas some of them are dangerous, others are fairly safe. In fact, *E. coli* is part of the normal flora of the intestines of humans, cattle, and swine. In other words, *E. coli* is growing in your intestine right now.

Some strains of *E. coli* do cause disease. These strains contain genes not found in the harmless organisms. These genes allow the bacteria to produce toxins and proteins that enable the bacteria to invade cells within the body. Several diseases are associated with these *E. coli* strains. Some *E. coli* strains are responsible for the travelers' diarrhea; others are associated with hemolytic-uremic syndrome, which is sometimes fatal.

The strains of *E. coli* usually used in molecular biology research do not contain the genes that produce disease, so these strains are normally harmless. (The strain we are using is “7A”.) However, even the strains of *E. coli* normally present in humans can cause illness if the bacteria escapes the digestive tract, so laboratory strains might cause an infection if introduced into a cut or into the eye. Therefore, some safety precautions should be taken when handling the organism. In fact, every day, hundreds of scientists and students handle *E. coli* without any noteworthy consequences.

# Handling *E. coli*

Here are some techniques for safely handling *E. coli*.

* When the liquid suspensions of the bacteria are being transferred, keep the nose and mouth away from the transfer areas to avoid inhaling any aerosol that might be created.
* At the end of laboratory sessions, wipe down bench top with a 10% bleach solution. (Soapy water or a disinfectant may be used instead).
* Wash your hands before leaving laboratory.
* Disinfect all bacterial cultures and any material that may have come into contact with the cultures, such as tubes and pipets. This may be done by placing these materials in a 10% bleach solution for 15 min. After that, the material may be disposed of in the garbage or down the drain. (Alternatively, the material may be autoclaved.)

# Chemical Hazards

The chemicals used in this experiment are not toxic in the amounts used. For example, for compound A1, 2-nitrobenzaldehyde, to be toxic to a typical adult, 18,000 mg would have to be consumed orally, whereas only about 60 mg of this compound is used for the entire class. Nevertheless, some of these compounds are skin and eye irritants, so avoid contact with these compounds, and wash your hands before leaving the laboratory.

Student tasks

# Prepare sterile Luria-Bertani agar for solid culture Petri dishes

To make 1 L of LB agar, combine 40 g dehydrated LB agar and 950 mL deionized H2O in a large Erlenmeyer flask with a stir bar. Cover with an upside-down 250 mL beaker to keep bacteria out. Sterilize by boiling the solution for a few minutes on a hot plate.

# Prepare components of the mixtures

Prepare a water bath for a 50 mL conical flask by adding enough water to a 400 mL beaker to cover 24 mL of the water in the conical flask. Heat the bath to a gentle boil in a hood. Prepare a large label with one of the following IDs: "**1**{1}," "**1**{2}," "**1**{3}," "**2**{1}," "**2**{2}," and "**2**{3}". Attach the label in front of the bath to the floor of the hood.

(Each bath goes in a separate hood, so that contamination is reduced.)

For each chemical listed below, add the listed mass to the appropriate conical flask.

|  |  |  |  |
| --- | --- | --- | --- |
| **ID** | **Compound Name** | **Molecular Weight (MW)** | **mass to add (mg)** |
| **1**{1} | 2-nitrobenzaldehyde | 151.12 | 108 |
| **1**{2} | 3-nitrobenzaldehyde | 151.12 | 108 |
| **1**{3} | 5-nitro-2-furaldehyde | 141.09 | 102 |
| **2**{1} | 4-bromophenylhydrazine hydrochloride | 223.52 | 160 |
| **2**{2} | 4-cyanophenylhydrazine hydrochloride | 169.62 | 122 |
| **2**{3} | aminoguanidine bicarbonate | 136.11 | 94 |

To each conical flask, add 24 mL of deionized water, using the marks gradations on the side of the flask. This will make the concentration of the solutions 0.030 M. The concentrations of the solutions need to be correct. If, in weighing out the chemical, too much or too little is added, adjust the volume of water so as to obtain the correct concentration. For instance, if you weighed out 120 mg of **1**{1} instead of 108 mg, add 120/108 \* 12 = 13.3 mL of water, instead of 12 mL.

Most of the chemicals will not dissolve in water at room temperature. Gently cap the flasks and place them in the boiling water bath. After about 3 to 5 min of heating, the solids should dissolve when the tubes are shaken.

Keep the solutions in the boiling water bath until they are used in the experiment. (The solutions may be stored at room temperature until needed.)

# Prepare 1/100 dilutions of "overnight culture"

Prepare 100 mL of Luria-Bertani broth by adding 2.5 g of dehydrated LB Broth to 95 mL deionized H2O. Boil to sterilize, then cool in a water bath, so the heat won’t kill the *E. coli*.

Use the gradations on the sides of the centrifuge tubes to estimate the necessary volumes in the following dilutions.

Label a 15 mL sterile conical test tube "*E. coli* 1/10"

Label 6 15 mL sterile conical test tubes "*E. coli* 1/100"

Pour about 9 mL of the cooled, sterile LB media into each of the seven test tubes.

Pour 1 mL of the E. coli overnight culture into the tube marked E. coli 1/10. Cap this tube and invert it several times to mix.

Pour 1 mL of the E. coli 1/10 dilution into each of the tubes marked E. coli 1/100. Cap the 1/100 dilutions and invert them several times to mix. Keep the 1/100 dilutions at room temperature until they are used in the experiment.

# Procedure

Note: To prevent organisms in the air from growing in the Petri dishes, only uncover the dishes when essential, and quickly cover them again.

 **Label.** The bottom of the Petri dish is the smaller half, which fits inside the top half. Do not separate the top and bottom in this step, so that the interior remains sterile. The bottoms of two petri dishes need to be labeled to indicate which mixture is where, as shown below. Using the provided marker, divide each Petri dish into 3 sections. Label the sections of one dish M1, M2, M3, and the sections of the second dish M4, M5, M6. Also, place your initials on the dishes so you can distinguish them from your classmates.

CMK

M1

M2

M3

CMK

M4

M5

M6

 **Add agar.** Pour about 10 mL of the hot (around 65 °C) sterile LB agar into each of 3 sterile Petri dishes (two of them are labeled). Cover the plates and let them cool for about 45 minutes. During that time the agar will solidify. Meanwhile, do steps 3 through 5.

 **Label tubes for mixtures.** Obtain six 1.5 mL microcentrifuge tubes. Label them M1, M2, M3, M4, M5, and M6.

 **Prepare the mixtures.** Using plastic disposable transfer pipets, prepare the mixtures according to the table, below. To prevent contamination, use a different pipet for each solution. The order of addition may be significant, so ***add the reagents in the order shown*** and ***add the correct number of drops***. For example, to prepare M1, add 5 drops of **2**{1} to tube M1, then add 5 drops of **2**{2} to that tube, then add 5 drops **2**{3}, then 15 drops **1**{1}. Cap and shake to mix each mixture.

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Tube | Add 5 drops of | then 5 drops of | then 5 drops of | then 15 drops of |
| M1 | **2**{1} | **2**{2} | **2**{3} | **1**{1} |
| M2 | **2**{1} | **2**{2} | **2**{3} | **1**{2} |
| M3 | **2**{1} | **2**{2} | **2**{3} | **1**{3} |
| M4 | **1**{1} | **1**{2} | **1**{3} | **2**{1} |
| M5 | **1**{1} | **1**{2} | **1**{3} | **2**{2} |
| M6 | **1**{1} | **1**{2} | **1**{3} | **2**{3} |

Record any observations made during the additions:

M1 M4

M2 M5

M3 M6

 Cap the tubes and shake them for 10 to 15 seconds. Again record any observations of the mixtures:

M1 M4

M2 M5

M3 M6

 **Add bacteria.** Pour about 5 mL of the bacterial culture *E. coli* TG1 1/100 onto one of the agar plates. Cover the plate and gently swirl it to ensure that the liquid culture makes contact with all of the solid agar.

 Pour the excess liquid culture onto the second labeled dish. As always, keep the plates uncovered as little as possible. As before, cover it and swirl it to coat the plate with the liquid culture.

 Pour the excess liquid culture in this dish back into the *E. coli* TG1 1/100 test tube.

 Remove the excess liquid from each labeled plate by tilting the plate on edge and using a Pasteur pipet to suck up the liquid that collects at the bottom edge of the plate. Transfer this excess liquid back into the *E. coli* TG1 1/100 test tube. Dispose of the transfer pipet in the *E. coli* waste container at the front of the lab.

 **Make cups.** Use the unlabeled Petri dish to practice making cups in the agar. Press straight down into the agar with the large end of a glass Pasteur pipet, then lift straight up. This leaves a little disc of agar on the plate. Dislodge the disc from the Petri dish by placing the small end of the pipet into the bottom of the crevice around the disk, and then leveraging the disk out. Though not contaminated, this disk may be placed in the *E. coli* waste container for disposal.

 Use the Pasteur pipet to make cups in the center of each section of the labeled plates. A bottom view of the plate is shown below. Dispose of the glass pasteur pipet in the large beaker containing bleach in a hood at the front of the lab.

CMK

M1

M2

M3

CMK

M4

M5

M6

 **Place the mixtures in the cups.** Using a new transfer pipet for each mixture, transfer 2 drops of each mixture to the corresponding cup in the agar plates. To get the solution into the cup can be tricky. To do the job, (assuming you are right-handed) hold the Pasteur pipet in your right hand. Rest your left fist on the bench top near the plates and use your left index finger to steady the tip of the pipet.

 **Incubate.** Carry the plates to the incubator, being careful not to let the liquid spill out of the cups. The the two agar plates will be incubate at 37 °C for 24 h, at which time the instructor will remove them to the refrigerator. They will be analyzed during the next lab period.

# Data Analysis

1. **Record the results** of the experiment in the table below. For each mixture place a "–" in the table if the mixture inhibited growth, or "" if the mixture that did not inhibit growth.

|  |  |  |
| --- | --- | --- |
| Mixture | Contents | Result |
| M1 | **1**{1}, **2**{1}, **2**{2}, **2**{3} |  |
| M2 | **1**{2}, **2**{1}, **2**{2}, **2**{3} |  |
| M3 | **1**{3}, **2**{1}, **2**{2}, **2**{3} |  |
| M4 | **2**{1}, **1**{1}, **1**{2}, **1**{3} |  |
| M5 | **2**{2}, **1**{1}, **1**{2}, **1**{3} |  |
| M6 | **2**{3}, **1**{1}, **1**{2}, **1**{3} |  |

Each mixture’s components react to produce three compounds. For instance, M1 contains the compounds **3**{1,1}, **3**{1,2}, and **3**{1,3}. Therefore, in the mixtures that show inhibition of growth (antibiotic activity) there are three compounds.

1. **Determine which compounds formed** when one of the chemset **1** compounds combined with one of the chemset **2** compounds. As shown in the table below, when the starting material **1**{1} combines with the starting material **2**{1}, the product **3**{**1,1}** is formed. Fill in the empty cells in the table.

|  |  |  |  |
| --- | --- | --- | --- |
|  | **2**{1} | **2**{2} | **2**{3} |
| **1**{1} | **3**{1,1} | **3**{1,2} |  |
| **1**{2} | **3**{2,1} |  |  |
| **1**{3} |  |  |  |

1. **Determine which components are in each mixture.** The components of the six mixtures are given by the components in one of the three rows or one of the three columns. For example, in the table below, left, the shaded row lists the three components of M1. Likewise, in the table below, right, the shaded column lists the components of M4. (Just copy the results above to the two tables below.)

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
|  |  | M4 | M5 | M6 |
|  |  | **2**{1} | **2**{2} | **2**{3} |
| M1  | **1**{1} | **3**{1,1} | **3**{1,2} |  |
| M2  | **1**{2} | **3**{2,1} |  |  |
| M3  | **1**{3} |  |  |  |

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
|  |  | M4 | M5 | M6 |
|  |  | **2**{1} | **2**{2} | **2**{3} |
| M1  | **1**{1} | **3**{1,1} | **3**{1,2} |  |
| M2  | **1**{2} | **3**{2,1} |  |  |
| M3  | **1**{3} |  |  |  |

1. **Determine which compound is an antibiotic.** If one particular compound acts as an antibiotic, then two mixtures will show antibiotic behavior: The “row” mixture containing that compound, and the “column” mixture containing that compound. In the table below, fill in the empty cells (just as you did previously), and shade the columns and rows corresponding to mixtures that acted as antibiotics. Determine which of the nine compounds is the antibiotic, and circle it in the table.

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
|  |  | M4 | M5 | M6 |
|  |  | **2**{1} | **2**{2} | **2**{3} |
| M1  | **1**{1} | **3**{1,1} | **3**{1,2} |  |
| M2  | **1**{2} | **3**{2,1} |  |  |
| M3  | **1**{3} |  |  |  |

**Questions about factorial design**

1. How many separate reactions would need to be carried out to synthesize each of the chemset **3** compounds?
2. How many tests would need to be carried out to screen each compound individually for antibiotic activity?
3. How many separate reaction mixtures were actually prepared?
4. How many separate antibiotic activity tests were actually carried?
5. Consider a larger system, one with 10 hydrazines and 10 aldehydes. How many possible hydrazones can be made from these starting materials?
6. Using the factorial design method, how many mixtures (and antibiotic screenings) would be necessary to identify which one of the hydrazones is an antibiotic?
7. For today’s lab, the fraction $\frac{experiments with mixtures}{experiments with single components }=\frac{6}{9}=0.67$. What is that fraction for the system with 10 hydrazines and 10 aldehydes?
8. Suppose the results of a factorial design experiment gave the following results. (The shaded area indicates mixtures that tested positive for antibiotic activity.) Can the antibiotic compound be unambiguously determined from this data? (Explain)

|  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
|  |  | M9 | M10 | M11 | M12 | M13 | M14 | M15 | M16 |
|  |  | **2**{1} | **2**{2} | **2**{3} | **2**{4} | **2**{5} | **2**{6} | **2**{7} | **2**{8} |
| M1  | **1**{1} |  |  |  |  |  |  |  |  |
| M2  | **1**{2} |  |  |  |  |  |  |  |  |
| M3  | **1**{3} |  |  |  |  |  |  |  |  |
| M4  | **1**{4} |  |  |  |  |  |  |  |  |
| M5  | **1**{5} |  |  |  |  |  |  |  |  |
| M6  | **1**{6} |  |  |  |  |  |  |  |  |
| M7  | **1**{7} |  |  |  |  |  |  |  |  |
| M8  | **1**{8} |  |  |  |  |  |  |  |  |