**Modern Biology Inc.**

# INSTRUCTOR MANUAL

## "Synthetic Biology: Using Bacterial Computers to Solve the Pancake Problem"

Questions about this exercise should be directed to:


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**Chemicals and Materials Provided**

**For Bacterial Growth and Transformation:**

-Tube called “Pancake-E. coli” with bacterial cells that contain the (1, -2) construct on a plasmid that carries ampicillin resistance
-Tube called “Hin-Plasmid” with plasmid DNA carrying kanamycin resistance and an expression cassette for production of the Hin recombinase.
-Tube of *E. coli* cells called E. coli not transformed
-Two Vials of $CaCl_2$ with each containing 5 ml
-Two tubes of Nutrient broth for transformation (15 ml each)
-One bottle of 200 ml of Nutrient broth for growth of picked colonies
-Two bottles of Nutrient Agar
-Sterile petri dishes
-Two Tubes with 1.3 ml of 50 mg/mL amplicillin (for Nutrient agar and Nutrient broth)
-Tube with 750 µL of 20 mg/mL kanamycin (for Nutrient agar and Nutrient broth)
-Tube with 250 µL of 40 mg/mL tetracycline (for Nutrient agar)
-1.5 mL sterile microcentrifuge tubes
-Sterile transfer pipets
-Sterile toothpicks

**For Plasmid Isolation:**

-Solution I -Tris Buffer and Ribonuclease A
-Solution II -Sodium Dodecyl Sulphate and Sodium Hydroxide
-Solution III- Guanidium Chloride and Acetic Acid
-Equilibration Buffer- Sodium Hydroxide
-Buffer HB -Guanidinium Chloride and Isopropanol
-*Wash Buffer -Tris Buffer, Sodium Chloride and Ethanol
**Note:** The ethanol must be added to this buffer prior to use.
-Elution Buffer -Tris Buffer
-8 HiBind DNA Columns and Collection Tubes

**Materials that are Needed but Not Provided**

-Micropipettors that can transfer 10 µL and 100 µL
-Ice buckets and ice
-37°C water bath
-37°C dry incubator
-Light box (or overhead projector)
-Handheld UV light and safety glasses (CAUTION: UV light is harmful and safety glasses should always be worn when using it.)
-Microcentrifuge and 1.5 ml tubes
-Vortex mixer (optional)

**Preparation of Solutions**

**-Agar Plates**

You will need the following number and types of plates for these experiments:

8 Amplicillin +Kanamycin Plates
8 Tetracycline Plates
16 Amplicillin Plates

To Prepare the Plates:

1. Melt the two bottles of 400 mL nutrient agar in a boiling water bath or microwave oven with the caps loosened. Add 600 µL of amplicillin to one bottle, mix and pour 16-22 agar plates.
2. Transfer about half ( ~200ml) of the agar from the other bottle into a separate sterile container (Not provided).
3. Add 200 µL of tetracycline to one portion of the nutrient agar,mix and pour 8-10 plates..
4. Add 200 µL of amplicillin and 200 µL of kanamycin to the other portion and pour 8-10 plates.

Plates should be maintained at room temperature for 1-2 days and then placed in the refrigerator. Plates should be used within 2-3 weeks after preparation.

-**Liquid Media**

Add 200 µL of amplicillin and 200 µL of kanamycin to the bottle of 200 mL nutrient broth.

**-Wash Buffer**

Combine the following in a bottle: 16 ml of 94-100% ethyl alcohol (not provided) and 4 ml of Wash Buffer Concentrate.

**Optional Modifications of the Procedure**

1. Once students have successfully exposed the starting construct of (1,-2) to Hin recombinase and isolated plasmids from the resulting clones, they may be interested in repeating the procedure with the isolated plasmids. They could collect interesting data on the distribution of phenotypes obtained by starting with different configurations.

2. Students could grow the Pancake/Hin cotransformations for various periods of time and look for changes in the distribution of resulting phenotypes. The students may expect to find that differences in the time allowed for Hin recombination will change the distribution outcome.

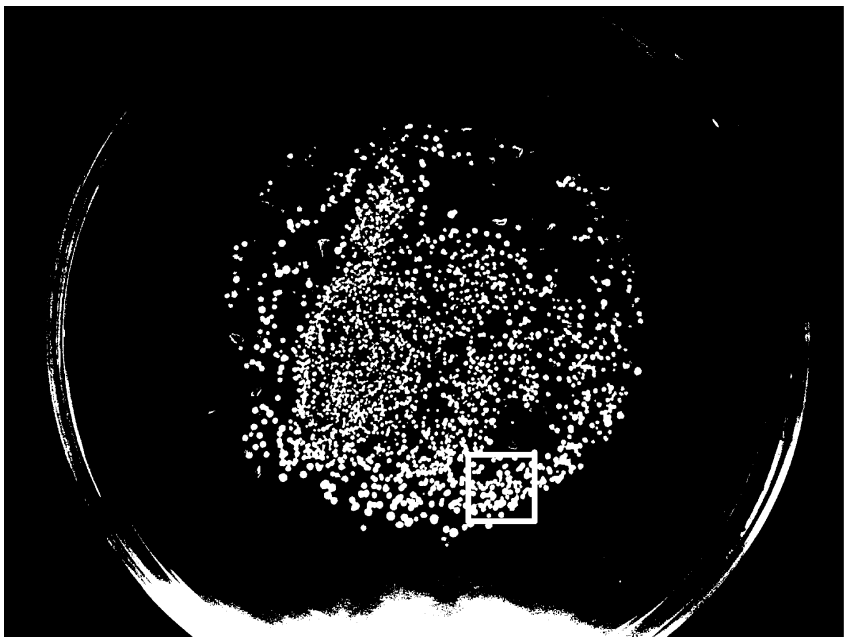
3. Students could verify the pancake configurations by carrying out colony PCRs. Sequences of primers and conditions for PCRs and electrophoresis that could be used for this analysis are described in Haynes, Karmella A., et al. 2008. Engineering bacteria to solve the Burnt Pancake Problem. Journal of Biological Engineering. Vol. 2(8): 1 – 12.

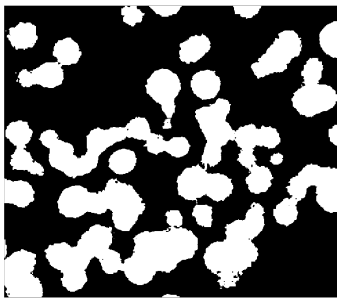
**Experimental Results**

Competent cells with the (1,-2) “Pancake” plasmid carrying ampicillin resistance were transformed with the “Hin” plasmid carrying kanamycin resistance. Below is a nutrient agar + amplicillin (50 µg/mL) + kanamycin (20 µg/mL) plate with the resulting transformant colonies.

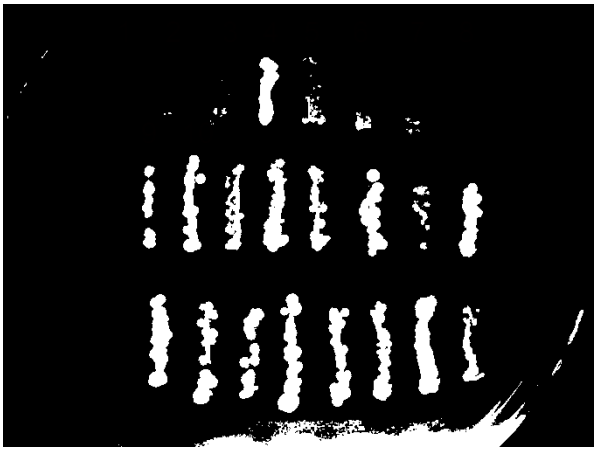
1. Eight colonies from the plate above with picked with a toothpick and transferred to nutrient broth + amplicillin (50 µg/mL) + kanamycin (20 µg/mL) and grown overnight at 37º C with shaking.

2. Plasmids were prepared from the overnight culture and used to transform competent cells without plasmids. Below picture taken on a UV box of a nutrient agar + amplicillin (50 µug/mL) plate with the resulting transformant colonies.





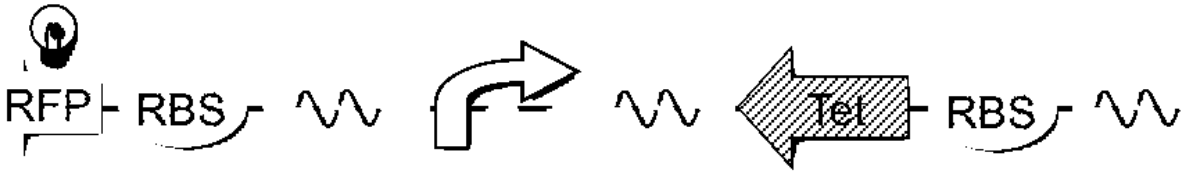
3. Colonies from the plate above were picked with toothpicks. A total of 24 colonies were transferred to a nutrient agar + ampicillin plate, shown below. RFP expression is strong in several of the streaks, weak in others, and not present in others. In addition, some of the streaks appear to have been derived from more than one colony. You can see the red cells without the UV light.



4. The first 10 of the streaks above were transferred to a nutrient agar + tetracycline (40 µg/mL) plate, shown below. Starting at about 1:00 and going clockwise, the clones are the 8 in the top row of the plate shown above and the first 2 in the middle row. Three of the 10 clones show no tetracycline resistance, including 2 that are red above. Clone four is red and tetracycline resistant, so its likely pancake configuration is (-2,-1).

**Data Analysis**

The starting configuration of pancakes is (1,-2), show below.




Below is a chart of the eight possible pancake configurations that result from the action of the Hin recombinase on the starting configuration and their expected phenotypes.

| Configuration | RFP Expression | Tet Expression | Comments |
|---|---|---|---|
| (1,2) | No | Yes | 1 |
| (1,-2) | No | No | 1 |
| (-1,2) | Yes | No | 2 |
| (-1,-2) | Yes | No | |
| (2,1) | No | No | 1 |
| (2,-1) | Yes | No | |
| (-2,1) | No | No | 1 |
| (-2,-1) | Yes | Yes | |

Comment 1 – backwards promotion could produce some RFP expression
Comment 2 – backwards promotion could produce some Tet resistance expression

RFP Expression - As shown in the table, half of the configurations are predicted to result in RFP expression. Of the 24 colonies streaked on the plate above, 9 showed RFP expression. As indicated by Comment 1, the other configurations may be weakly expressing the RFP gene by backwards promotion. Some evidence of this can be seen on the plate (clones 2,3, 5 and 7).

Tet Expression - One fourth of the configurations are expected to result in expression of the tetracycline resistance gene. Of the 10 colonies streaked above, 7 showed tetracycline expression. This is higher than expected and the discrepancy may be explained by backwards promoter activity or readthrough transcription from the plasmid backbone. In addition, random selection of colonies could produce higher than expected frequency by chance alone.

**Answers to Questions**

Questions in Experimental Procedure

Day One Question: What is the expected combination of phenotypes produced by the (1, -2) Pancake clone? Are cells with this plasmid expected to glow red? Are they expected to be resistant to tetracycline?

*Answer: The cells with the (1,-2) Pancake plasmid are not expected to express either the RFP or the Tet resistant genes, so they should not glow red and should not be resistant to tetracycline. These may be faintly red due to backwards promotion.*

Day Two Question: What is the purpose of plating the Pancake cells on media containing two different antibiotics?

*Answer: The media with both ampicillin and kanamycin will select for transformant bacteria that include both the Pancake and Hin plasmids.*

Day Three Question: Based on what you have learned about the Hin/hix recombination system, what do you think will happen to the original (1,-2) pancake configuration during growth in media with ampicillin and kanamycin?

*Answer: During this time, the Hin recombinase will flip the two pancakes into all the eight possible configurations.*

Day Four Question: What will happen to the kanamycin resistance plasmids during the transformation and selection? What will be the effect of the introduction of only one pancake plasmid to each competent bacterial cell?

*Answer: Because there is no kanamycin in the media, there will be no selection for the Hin plasmid. The effect of the introduction of only one Pancake plasmid will be the production of ampiciliian resistant colonies that are bacterial clones.*

Day Five Question: Is it possible for a given cell to express both the red fluorescence and tetracycline resistance phenotypes?

*Answer: Yes, the configuration (-2,-1) has the promoter pointing to the left toward a leftward Tet gene and a leftward RFP gene. This configuration is expected to express both genes. In addition, (-1, 2) could be red and tetracycline resistant.*

Day Six Question: Results from living cells do not always match our theoretical expectations, causing us to reconsider. How would our phenotypic predictions change if the promoter has backwards activity?

*Answer: Several more of the eight possible configurations would be predicted to express either or both of the red fluorescence or tetracycline resistance phenotypes.*

<u>Questions in Mathematical Investigations</u>

**Day One Questions:**

1. To become more familiar with the BPP, experiment with cutout icons for the promoter and the protein coding region. Find and sketch all the possible arrangements of the two biological burnt pancakes.

*Answer: There are eight possible arrangements of the two burnt pancakes. The promoter pancake can be either left-facing or right-facing, as can the RBS-Tet pancake. Furthermore, these two pancakes could be in either order. Thus, there are three independent choices, each with two possibilities. The total number of possibilities is $2^3$.*

2. To make a functional gene, the promoter must precede the protein coding portion (RBS + Reporter), and the promoter and the protein coding portion must face the same direction. How many of the arrangements you found in #1 will make a functional reporter gene?

*Answer: Of the eight, three would express RFP only, one would express Tet only, and one would express both. (See data analysis comments.)*

**Day Two Questions**:

1. In how many ways can 2 unburned pancakes be stacked? List them using permutation notation.

*Answer: Two unburned pancakes can be stacked in two ways: (1,2) and (2,1).*

2. In how many ways can 3 unburned pancakes be stacked? List them.

*Answer: Three unburned pancakes can be stacked in six ways: (1,2,3), (1,3,2), (2,1,3), (2,3,1), (3,1,2) and (3,2,1).*

3. In how many ways can 4 unburned pancakes be stacked?

*Answer: Four unburned pancakes can be stacked in 24 ways.*

4. Find a pattern and give a formula for the number of ways that any number *n* of unburned pancakes can be stacked.

*Answer: For n unburned pancakes, there are n choices for which pancake is on bottom, followed by n-1 choices for the pancake placed in second position, and so on until there is only one pancake left which must be the top pancake. Thus, the number of stacks of n unburned pancakes is n(n-1)(n-2)…(1) = n! ("n factorial").*

5. Suppose the initial starting positions of three objects is (1,2,3). How many of the permutations yield the result in which none of the three objects ends up in the same position that it started? List the permutations that leave no object in its original position. Such permutations are called *derangements*.

*Answer: The only derangements of (1,2,3) are (2,3,1) and (3,1,2).*

6. For four objects and starting position (1,2,3,4), list all of the derangements.
*Answer: The nine derangements of (1,2,3,4) are (4,1,2,3), (3,4,1,2), (2,3,4,1), (2,1,3,4), (4,3,2,1), (3,4,2,1), (2,4,1,3), (3,1,4,2) and (4,3,1,2)*

7. Estimate the number of derangements of five objects.

*Answer: Students will likely guess that "a little more than a third" of the 120 permutations are derangements. The actual number of derangements is the fraction n!/e rounded correctly to the nearest integer where e is approximately 2.718. The number of derangements of five objects is 44.*

**Day Three Questions:**

1. In how many ways can 2 burnt pancakes be stacked? List them using signed permutation notation.

*Answer: There are eight stacks of two burnt pancakes: (1,2), (1,-2), (-1,2), (-1,-2), (2,1), (2, -1), (-2,1), and (-2,-1).*

2. In how many ways can 3 burnt pancakes be stacked?

*Answer: There are 3 factorial (six) ways in which the three pancakes can be ordered and each of the three pancakes can be burned side up or burned side down. Thus, there are* $(6)2^3=48$ *stacks.*

3. In how many ways can 4 burnt pancakes be stacked?

*Answer: There are* $4!\ 2^4 = 384$ *stacks of four burnt pancakes.*

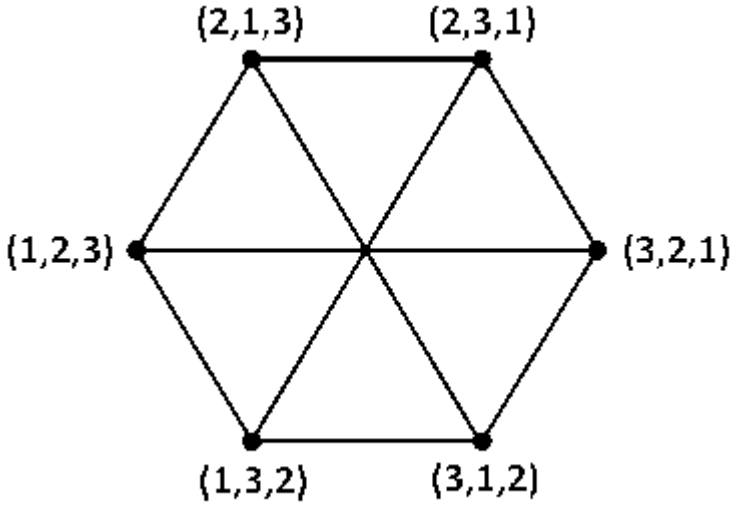
4. Find a pattern and give a formula for the number of ways that any number *n* of burnt pancakes can be stacked.

*Answer: For n pancakes there are n! orderings and each pancake has two orientations. The general formula for the number of stacks of burnt pancakes is* $n!2^n$.

**Day Four Questions:**

1. In the unburned pancake graph below with three pancakes, list all permutations that are adjacent to (2,3,1).

*Answer: The permutation (2,3,1) is adjacent to (3,2,1) by flipping the first two pancakes, (2,1,3) by flipping the last two pancakes, and (1,3,2) by flipping the whole stack.*



2. Above is the pancake graph for three pancakes with the vertices (2,1,3) and (2,3,1) labeled. Label the other vertices with so that the edges correspond to potential flips.

*Answer: Beginning with (2,1,3) and reading clockwise, the vertices could be labeled as (2,3,1), (3,2,1), (3,1,2), (1,3,2), (1,2,3), as shown above. Another possible labeling is (2,3,1), (2,3,1), (3,2,1), (1,2,3), (1,3,2), (3,1,2).*

3. How many permutations are adjacent to both (2,1,3) and (1,2,3)?

*Answer: There are no vertices that are adjacent to both (2,1,3) and (1,2,3) but there is exactly one vertex adjacent to (2,1,3) and (1,3,2), namely (3,1,2).*

4. If we define the *distance* between two permutations as the number of edges in the shortest path that connects one to the other, how many vertices are of distance 2 from (2,1,3) in your unburned pancake graph with three pancakes? List them.

*Answer: At distance 2 from (2,1,3) are (3,2,1) and (1,3,2).*

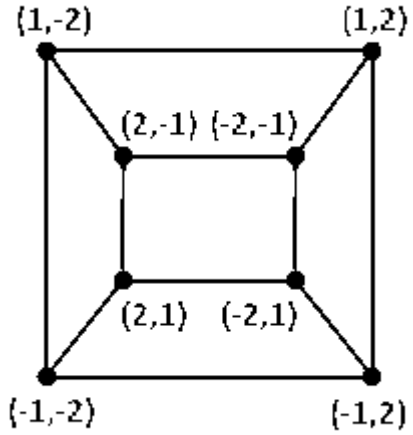
5. List all vertices in the graph that are of distance 3 from (2,1,3).

*Answer: There are no vertices distance 3 from (2,1,3) since you can find some path to any vertex that uses at most two edges.*

**Day Five Questions:**

1. In the burnt pancake graph for two pancakes, list all permutations that are adjacent to (1,-2).

*Answer: The vertices adjacent to (1,-2) can be obtained by reversing the first pancake yielding (-1,-2), reversing the second pancake yielding (1,2), or flipping the stack of two yielding (2,-1).*




2. Above is the burnt pancake graph for two pancakes with the vertex (1,-2) labeled. Label the other vertices with so that the edges correspond to potential flips. (There is more than one possible answer.)

*Answer: There are several possible labelings of the graph. One labeling (shown above) is to begin with (1,-2) and continue clockwise to give (1,2), (-1,2), (-1,-2) and then labeling the inside four vertices clockwise beginning with the upper left as (2,-1), (-2,-1), (-2,1), (2,1).*

3. In the burnt pancake graph with two pancakes, how many permutations are adjacent to both (1,-2) and (-1,2)? adjacent to both (1,-2) and (1,2)?

*Answer: Both (1,2) and (-1,-2) are adjacent to both (1,-2) and (-1,2). No vertex is adjacent to both (1,-2) and (1,2).*

4. How many vertices are of distance 2 from (1,-2) in your burnt pancake graph with two pancakes? List them.

*Answer: The vertices (-1,2), (2,1), and (-2,-1) are all distance 2 from (1,-2).*

5. List all vertices in the graph that are of distance 3 from (1,-2).

*Answer: Only (-2,1) is distance 3 from (1,-2).*

**Day Six Questions:**

1. What fraction of participants finished in 1 move? What fraction did you expect?

*Answer: Empirical results will vary by class, but it is expected that one-third of the participants reach (1,2) in their first step.*

2. What fraction of participants finished in 2 moves? What fraction did you expect?

*Answer: It is not possible to reach (1,2) in two flips.*

3. What fraction of participants finished in exactly 3 moves? What fraction did you expect?

*Answer: To finish in exactly three steps, there are four walks that reach (1,2) for the first time in 3 steps, each with probability 1/27 so the probability of finishing in exactly three rolls of the die is 4/27.*

4. What fraction of participants took more than 10 moves?

*Answer: Observed fractions will vary, but it is not unlikely that a student will go 10 moves or more before finishing the game.*

5. Based on your data, how many moves would you expect it to take to reach the finish?

*Answer: To find an empirical expected value, ask students what they think the number is likely to be. Students will probably suggest finding the mean or median of the observed data values.*

6. Suppose the game did not finish when a participant gets to the (1,2) vertex but continues on with rolls of the dice and moves. What do you think would happen to the distribution of participants?

*Answer: If the game were to continue after reaching (1,2) instead of terminate, one would expect to be on any of the 8 vertices with equal probability; that is 1/8 chance of being at a*

*particular place at a particular time. This is not true at the beginning but should eventually be the case.*

Answers to Study Questions

1. Synthetic biology projects have been conducted by undergraduate college students in the context of the International Genetically Engineered Machines competition, or iGEM, since 2005. Find and describe an example of an iGEM project in each of the categories medicine, energy, and the environment.

*Answer: Performing a search with the term iGEM will lead students to the Wiki pages of all of the teams in each of the years of the competition. Many examples of projects in each of the categories can be found.*

2. What does the term "computer" mean? Describe what you think are the requirements for a device to be called a computer. Is it possible for a bacterial cell or a population of bacteria to function as a computer?

*Answer: This is an opinion question, so there is no correct answer. However, many people describe a computer as a device that takes input, processes it in some way, and reports an output. Some requirements for a computer may be that it performs calculations or analyses, that it has some level of automation, and that computes answers to problems. According to this description, bacterial cells or populations can function as computers.*

3. Describe the purpose of genetic recombination in nature. For what purpose do *Salmonella* bacteria use the Hin/hix recombination system?

*Answer: In a very general sense, genetic recombination serves the purpose of producing new combinations of genes in populations that provide various levels of fitness in changing environments. Salmonella bacteria use the Hin/hix recombination system to produce variation in the expression of antigenic proteins in the changing environment of a host immune system.*

4. Construct a pancake problem of your own that involves five burnt pancakes. Have a friend try to solve the problem. Was your problem relatively hard or easy?

*Answer: The goal here is for students to use as few flips as possible to put the pancakes in the solved order and orientation (1,2,3,4,5). From some starting points it is easy to see that a single flip will do the trick, like (1,-3,-2,4,5). Others, like (-2,-1,3,-5,-4), cannot be solved in one flip but two flips will do it. An interesting challenge is to find the fewest number of flips required to solve the problem from any starting point. Students may decide that the more flips required, the harder the problem. Alternatively, students may discover clever algorithms along the way for getting to the finishing orientation. This should be encouraged; however, the found algorithm may not be the most efficient way to manipulate the stack. As a challenge problem for students, consider the stack (1,4,5,2,3) for which several ways can be devised to reach the final stack in four flips, but it is also possible to reach the final stack in just three; namely, (1,4,5,2,3) → (1,-3,-2,-5,-4) → (1,2,3,-5,-4) → (1,2,3,4,5). Students might choose to define a harder problem as one in which some standard algorithm that that they discover does not yield the most efficient solution. Encourage exploration among interested students.*

5. The pancake problem you investigated involved a promoter and a gene. How could a larger burnt pancake problem be encoded in bacteria?

*Answer: A larger pancake problem could be encoded if additional genetic units were used as pancakes. Gene expression would result in an observable phenotype only if the genetic units were in the correct order and orientation. One method would be to encode a promoter, several parts of a given reporter gene, and a transcription terminator as pancakes. Each of these would be flanked by hix sites, allowing Hin recombinase to produce all of the possible arrangements. The reporter would be expressed only when the pancakes are in the solved configuration.*