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PROFESSOR: Good morning, good morning.

So last time, we talked about the most remarkable biochemical purification procedure ever invented-- cloning. You remember the issue with biochemistry. You're going to grind up a cell, you're going to take the contents and run it over different kinds of separation columns or centrifuge it or things, in order to separate some things from other things. The problem with purifying a gene that way, away from all the rest of DNA in a cell, is that the gene has exactly the same biochemical properties as all the other genes. How in the world are you going to do it?

The solution was devilishly simple and devilishly complex at the same time. All you have to do to purify something is dilute it. If I take any substance an I add enough water, it's very dilute.

And in any little drop in any little test tube there will only be one DNA molecule. It's now purified. I could do that for every molecule there is.

The problem is, it's not very useful unless I have a way to take that molecule and make extra copies of it. But if I could do that, dilution is purification. The trick we use initially for making more copies is we ask E. coli to do it for us.

That, as usual, is the solution to most issues. Find something in life that already does it. And so, just to remember what we did, we took total DNA. Maybe it was yeast DNA, maybe it was human DNA, maybe it was zebra DNA. And we cut it up with some restriction enzyme. Our restriction enzyme we used was EcoR1 And it therefore cut at the EcoR1 sites, G, A, A, T, T, C. We could have used a different restriction enzyme.

We then added it to a vector. The vector was cut open at an EcoR1 site. The vector had an origin of replication, ORI, and the vector had a resistance marker, some resistance gene that made a protein that could break down some antibiotic found in nature. We combine these two pieces.

Vector now gets its insert. We call this vector, we call this insert. We attach them together using what?

AUDIENCE: Ligase.

PROFESSOR: Ligase, the enzyme that ligates DNA. We then take this, we transform it into a bacterium. The scale is obviously off, right? This DNA plasmid here is tiny compared to this bacteria. But if I draw it to scale, it won't be very helpful.

So we then transform it in here. We do that in a test tube. We treat the bacteria a little roughly so it likes to slurp up the DNA. We then plate it on a plate.

And those bacteria that have acquired our plasmid containing the antibiotic resistance gene are able to grow on this plate that has antibiotic on it. And we call this thing a library. So that's it. We can make a library.

And we have in effect, then, what I said. We've deluded the individual molecules out, and each one went into its own bacteria, and each one got replicated by that bacteria. Those plasmids are replicated by the bacteria. In fact, we choose plasmids that are called multicopy plasmids, where there's not just one copy but the cell might make 50 copies of it. We grow up a whole colony of it and there you go.

We talked about some of the issues with it. Where do we get the restriction enzymes, the ligases, the vectors, et cetera? It's in the catalog, right? We used to purify them ourselves, but they're all in the catalog.

So any questions about this? We had some questions. I have a question about this. How come when we add ligase the vector itself just doesn't close up into a circle without an insert in it? It might. What would happen then?

AUDIENCE: You'd just have your original vector.

PROFESSOR: You'd just have your original vector. And what happens when I transform into the bacteria?

AUDIENCE: It'll still survive.

PROFESSOR: It'll still survive. So will the unimolecular closure of that circle be more common than the bimolecular interaction between a vector and an insert?

AUDIENCE: Probably.

PROFESSOR: Probably, because the two ends of the circle are pretty close to each other. So what do I do? Yeah?

AUDIENCE: Because [INAUDIBLE] are the same. [INAUDIBLE] same restriction enzymes because if they're two different enzymes so they don't match.

PROFESSOR: Ooh. That's cute trick. Two different restriction enzymes so that they couldn't reclose. But then my fragments better have those two different sites, too, and only be able to clone those fragments that have the two different ones. But that could work. So you want a trick for making sure it doesn't reclose.

Any other tricks? But the problem is, I won't get all the fragments, only the ones that have, say, an Eco at a Bam site. But that's--

So I bring this up not because it's particularly important, but to tell you the kind of engineering that really does have to go on in molecular biology. What happens is when you have your DNA put in here, we have a sugar-phosphate backbone in both cases. And if we look up close, one of these sides has the phosphate, the other has a hydroxyl. Phosphate, hydroxyl, phosphate, hydroxyl, right? And ligase comes in and joins that.

So this guy has a phosphate on this strand, and this guy has a phosphate on that strand, hydroxyl there, hydroxyl there. What would happen if I got rid of the phosphates on the vector? Could it reclose?

AUDIENCE: No.

PROFESSOR: No. So if there were an enzyme that removed phosphates, I could treat my vector first with the phosphate-removing enzyme. And now it couldn't possibly reclose on itself. And is there such an enzyme? And what's it called?

Phosphatase, because it takes phosphates so easy. Phosphatase, it takes off the phosphates. And then it can't reclose anymore. And where do we get phosphatase? It's in the catalog. Exactly.

So now what happens is that the vector only has an OH here. What happens to ligase? When I put an insert in here, ligase can make a covalent join on this strand. But it can't actually make a covalent join on the other strand.

But does it matter? No, because I've closed up my circle on that strand, and I close up the circle there on the other strand, and I just throw it into E. coli. And you know what happens when it goes into E. coli? It's got a nick, obviously, on that strand. It hasn't closed up.

But what does E. coli do when it sees that DNA? Must be damaged DNA. I'll fix it. So E. coli actually does the last little trick of closing that up for you with its own enzymes for repairing its own DNA.

I bring this up not because it's crucial that you should worry about it, but because I want to know that there's a whole layer of these interesting engineering tricks that get developed. Every one of them exploits enzymes that we know. Every one of them deals with questions like, will my vector reclose on itself, how do I avoid that? And there's a vast cooking book of protocols in molecular biology. And we constantly are just cribbing from things life does to make our protocols more and more efficient.

So I bring it up more because it's kind of a cool thing that all that goes on, and because it helps you remember that these phosphates are very important to joining things up. That's a digression. Now, let's go to the topic.

How do we actually read the library? How do we read our library? How do we use the library, read from the library?

Well let's say we're going to try to find the arginine gene. We talked about the gene for arginine in yeast. So I'd like to clone the gene for ARG1.

We found mutants before that were unable to grow without supplemental arginine. They somehow had a defect in producing their own origin. It's a mutant. I want to find the gene, please. How do I do it?

We've got to think about what's our insert DNA. What are our vectors? What insert DNA should we start with, zebra? No. Human? No.

How about yeast? We're trying to clone a gene from yeast, right? So let's start with yeast.

OK. So we're going to start with yeast DNA. We're going to cut up yeast DNA. We're going to attach it to our vector, we're going to transform it into E. coli, E. coli will grow up on our plates.

And one of these guys, I happen to know it's that one there, contains the ARG1 gene. The problem is I happen to know it, but you don't. How are you going to find out where the ARG1 gene is? Any takers? Yeah?

AUDIENCE: It could be like when you put the gene in make it flourescent.

PROFESSOR: A fluorescent tag? So I should just attach the fluorescent tag to the ARG1 gene?

AUDIENCE: Yes.

PROFESSOR: How do I do that? All the DNA looks the same in the tube. How do I know where to attach the fluorescent tag?

AUDIENCE: Maybe you could size it [INAUDIBLE].

PROFESSOR: There's a lot of pieces of DNA there. And my eyes are not that good.

AUDIENCE: Separate it?

PROFESSOR: Separate it. But will I know which one is ARG1? See, I don't actually know anything about ARG1.

All I know is I made a mutant. The mutant is unable to grow without arginine. I

haven't got a clue what that gene is. I don't know what it encodes, I don't know how big it is, I don't know nothing. All I know is that whatever it is, it's a gene which when mutated prevents you from growing without arginine.

AUDIENCE: Could you plate all of your colonies onto a-- could you put [INAUDIBLE]?

PROFESSOR: Minimal medium. What if I plate on minimal medium? Now what? What are you hoping for?

AUDIENCE: The one that has the ARG1 gene will not grow.

PROFESSOR: The one that has the ARG1 gene won't be able to grow-- oh wait, yeah. But something like that. Let's work it through. We've got my idea here. What are we going to do with it?

AUDIENCE: [INAUDIBLE]

PROFESSOR: I've got a working ARG1 Mutate ARG1 afterwards?

AUDIENCE: [INAUDIBLE]

PROFESSOR: OK. How will that work? I'm open for-- got an idea here?

AUDIENCE: If you have your different colonies [INAUDIBLE], you could have a secondary plate them all over to one of middle medium. The ones that die are the ones that already [INAUDIBLE]--

PROFESSOR: So guys, I have a concern. I'm just transferring this into E. coli. E.coli grows just fine without arginine. I mean, I'm going to take this yeast DNA. I'm going to put it in E. coli.

E. coli was kind enough to grow it for me. But frankly, E. coli doesn't need this ARG1 gene. E. coli can grow without arginine. I can plate this with and without arginine, E. coli grows just fine.

But you're on to something. You're onto the idea that somehow, the only thing we know about ARG1 is that the functional, wild-type copy of that gene confers an

ability to grow without arginine. And who does it confer it on? And what kind of yeast? Haploid mutant yeast.

Ah. So suppose I put a working copy of ARG1, a good copy, a wild-type copy, into a mutant yeast. Now what would happen to that mutant yeast? What would happen? The mutant yeast before, could it grow without arginine? No.

If I put in a working copy of the ARG1 gene, what will happen? It grows. Now let's design a scheme.

Do I want to use E. coli at all? No. What do I want to use? I want to use a yeast. So let's get rid of E. coli and let's instead use yeast.

And which yeast should we use, wild-type or mutant? Mutant yeast. What mutation? ARG1 mutant yeast. ARG1 minus yeast.

Now, if I plate ARG1 minus yeast on minimal medium, what happens? It doesn't grow. It dies.

What DNA should I be putting in? Yeast DNA. Mutant or wild type? Why wild type? Because it'll have a working copy of ARG1.

So I want yeast, wild type. Now what happens? One of these guys, and only one of these guys here, this one, has a ARG1 gene. That's ARG1.

When it goes in, that plasmid has the ARG1 plus gene, whereas other plasmids don't. That cell that inherits that gene there, that gets that gene, is not green. I just drew it green for you. But it has the ARG1 gene.

And when I plate this on minimal medium, what's distinctive about it? It grows. That's how you can clone the ARG1 gene. You clone it by the only thing you know about it. Namely, it confers a function. This is called cloning by function.

Or, what did we do when we crossed two mutants together to see if things were in different genes? It was a test of complementation. Really what we're asking is, is there a plasmid that complements the defect?

In effect what's happening is in this cell, right here, we have a yeast cell. And the yeast cell has a defect in its ARG1. But the plasmid has a working ARG1.

So for that one gene, this cell could be thought of maybe a little bit like a diploid, just at that one gene. And we've done a complementation, just a teeny little complementation for one gene. And we could call this cloning complementation. It's essentially cloning by function.

Any questions? Yes?

AUDIENCE: [INAUDIBLE] --they all have functioning [INAUDIBLE]?

PROFESSOR: Yep.

AUDIENCE: So why does that then have a function?

PROFESSOR: Oh, oh. You see, the yeast genome has about 4,000 different genes. I chop it up with my EcoR1. Some plasmids get ARG1 but most of them get leucine 2 or [INAUDIBLE] or other things. And each yeast cell in my library only picks up a plasmid with one chunk of DNA, one gene.

So it turns out that the yeast cells in my library, each one has one of thousands of alternative possibilities. And it's just the guy who gets ARG1 who grows.

AUDIENCE: But you're saying that the yeast [INAUDIBLE] plasmid. All code for--

PROFESSOR: That's right.

AUDIENCE: But I don't get why you would end up with [INAUDIBLE].

PROFESSOR: What do I--?

AUDIENCE: Why do you end up with a strain that has ARG1 in it?

PROFESSOR: Oh. ARG1 is the ARG1 working copy. In the yeast, I'm talking about this yeast here has the working copy of ARG1, ARG1 plus. So this guy has an ARG1 plus. It also has lots of other genes.

Each of these plasmids gets one gene. Some of them get an ARG plus. Some of them happen to get a leucine gene or some other gene that's irrelevant. And the plasmids that contain the working copy of the gene, they, when they go into the cell, give the cell the ability to grow. So that's why.

All right. So that's how we get a gene by function.