ELIZABETH NOLAN: Where we left off yesterday was beginning to discuss methods for unnatural amino acid incorporation into proteins using the ribosome. And the methodology that was introduced and where we need to continue today is the Schultz method of using the native ribosome to play some tricks and get unnatural amino acids into proteins.

So we'll work through this further to show how the rest of the machinery was generated and then we'll consider some of the limitations and some of that came up in questions last time. And then we'll close with a discussion of one strategy that's a different strategy that uses actually an orthogonal ribosome, which is really, really neat here.

So where we left off last time in terms of the Schultz Method was that we needed a unique codon for the unnatural amino acid, right? And a stop codon was reassigned. So TAG or Amber stop. And the other thing that we need that we'll discuss now involves the requirement of an orthogonal tRNA and aminoacyl-tRNA synthetase pair that can be used in this method.

So the question is, where does this come from? So where do we get a tRNA and an aaRS that can be used for this unnatural amino acid of interest. And one way to think about this in terms of a search is to think about different tRNAs and aaRS from different organisms. And so what's found if tRNAs are compared between bacteria, eukaryotes, [INAUDIBLE], there's evolutionary divergence.

And so can that evolutionary divergence be taken advantage of? And effectively, is it possible to find some tRNA and its aminoacyl-tRNA synthetase from one organism that's orthogonal to the corresponding tRNA and aminoacyl-tRNA synthetase in the organism of interest. So effectively, if we want to use E. coli, we want to find a pair from another organism that's completely independent of the endogenous E. coli machinery.

So what does this mean? A lot of trial and error was done to identify a pair from another organism. And where they ended up finding one is from a methanogen. So methanococcus jannaschii here. OK, and this initial pair was for tyrosine.

And so, there's some features of this pair that are noteworthy to bring up. So first if we think about the aminoacyl-tRNA synthetase here. This one has an unusual feature. So when we discuss these aaRS, remember we discussed the mechanism and we also discussed what happens if a wrong amino acid is selected. And we learned that they have editing function and that there's editing domains.

What also came up in those discussions is that we need to take every one of these enzymes as a case-by-case basis. And as it turns out, this particular enzyme does not have an editing domain. So then the thing to think about is, why would that be useful from the standpoint of incorporating an unnatural amino acid? So what's the benefit there?

So what does this editing domain do?

AUDIENCE: It's one less thing to have to fix if you're assuming that the editing domain would recognize and hydrolyze an unnatural amino acid that you put in even if you got the binding site to recognize it, or the first binding site to recognize it.

ELIZABETH NOLAN: Exactly right. There's no deacylation happening, so no hydrolosis. So it's just more likely this unnatural amino acid can be a successful substrate and there's less engineering that has to be done in terms of modifying the enzyme here. So, another point in terms of-- they found that it does not acylate E. coli tRNA. OK, and that's important for trying to use this in E. coli here.

What's the potential problem? So is this going to be specific for the unnatural amino acid of interest? No way, right? Unlikely at least, and depending on what type of a natural amino acid you're thinking about, it may definitely be a no way. So there's some experimental work to do to make this specific for the unnatural amino acid of interest, which means there has to be some mutagenesis and selection, which we're not going to talk about in detail here.

So what about the tRNA? So we need to think about the tRNA structure right and how tRNAs interact with aaRS, right? And recall, we had in an earlier lecture one example of a crystal structure of this complex. And we saw there's many positions where they interact.

What was known in this system here is that they figured-- OK, and also keep in mind, just backing up a minute, this tRNA, as we know, based on this nomenclature has an anticodon for tyrosine. So that's going to have to be mutated to be the anticodon on the Amber stop in order to use in this method. Right?

So this is going to have to be mutated to give us tRNA(CUA) where this is indicating the anticodon here. So that mutation, we don't want that mutation to disrupt the interaction between the tRNA and aminoacyl-tRNA synthetase, right? And it turns out there were minimal interactions in that area for the native system. So the thinking was that these mutations could be tolerated here.

So with that said, what is the potential problem with this tRNA? So if we want to put this tRNA into E. coli, it can't be recognized by any of the E. coli aaRS. So all of these recognition issues come up.

So, here again with another example, where there needed to be some mutagenesis and selection to prevent interactions between this tRNA from the methanogen and the aaRS of E. coli. And effectively what they did is to pick 11 positions on the tRNA, which I'll just chart out.

OK, so here's our tRNA. Here's our CUA anticodon here. And effectively these ends are positions where they randomized and did mutagenesis here. So they identified these 11 positions. OK? And these 11 positions do not interact with the aaRS here of the pair.

So the idea is to maintain this interaction, but prevent any interaction of this tRNA with E. coli machinery here. So effectively, they used a method called directed evolution to do selection. And what might happen out of that, imagine you have some large pool of mutant tRNA, what might happen?

So here OK, so the end result is that the tRNA might be non-functional. Right? So the mutation was not helpful. OK? It might be non-orthogonal, meaning that it's recognized by the endogenous E. coli machinery or it may be orthogonal here. OK, so recognized only OK? And so this is what needs to be selected for here.

And so assays need to be done that allows these to be differentiated here. So the end result is an orthogonal pair. But the point is, you can't just take this pair from the other organism. It needs to be further modified. So where does that put us in terms of the cartoon we saw yesterday without some of these details?

So here we have the tRNA that has this amber anticodon. So that's our orthogonal tRNA. We have an unnatural amino acid that's able to get into the organism of interest. And we have the orthogonal tRNA synthetase. So these give us this aminoacyl-tRNA with the unnatural amino acid.

And then that can be incorporated into the A site of the ribosome. Right so this is a case where we have a plasma DNA. Here's the gene of interest in red. And somewhere in that gene, a stop codon has been placed to allow for incorporation of this unnatural amino acid somewhere within the polypeptide chain as shown here.

So if we think about the scope of this methodology, where does this take us? So, it's quite broad. This type of work has been applied beyond E. coli, so in yeast and mammalian cells. At present, there is many, many different unnatural amino acids that can be incorporated and it's used by many labs.

So that's something to keep in mind. If you're developing a new method, you'd really like other folks in other labs to be able to use your method. There's a lot of troubleshooting to do experimentally to get it up and running. And Joanne's a wonderful person to talk about that if you're curious for details.

Just some amino acid scope, and you know what maybe we could do. So these are some earlier examples of unnatural amino acids that can be incorporated. And what are some of the neat things? If we look just here for example, there's an azide. Why might we want an azide?

AUDIENCE: Click chemistry.

ELIZABETH NOLAN: Yeah, click chemistry, right. Some chemistry that could be done after protein expression or maybe in a cell. Here we have a benzophenone. So they're useful for cross linking experiments and we'll likely talk about benzophenone cross linking in recitation five in detail. We see some sugars here.

This is the damsel group. That's a fluorophore. So there's many possibilities here. Just looking at these molecules, what's something similar about all of them? We think about them compared to a native amino acid.

AUDIENCE: I was just that they're small.

ELIZABETH NOLAN: OK, they're quite small.

AUDIENCE: It's a kind of modified tyrosine. It will have some sort of benzo group that's modified.

ELIZABETH So they're sort of phenylalinine or tyrosine like, right? And does that make sense from the

NOLAN: standpoint of using this machinery initially? Yes, and you can imagine looking for other pairs to put in other types of unnatural amino acids. So that's reflective there.

Just as some further examples, So this is another example of using an unnatural amino acid that can be useful for click chemistry. And I picked this in part, for one, this unnatural amino acid looks very different than the ones we saw on the prior slide. But there's aminoacyl-tRNA synthetase and the tRNA for this alkyne.

And so you can imagine expressing a protein with this at a specific location. And then after the fact, clicking on a molecule like this fluorophore here. So just thinking about this process, why maybe was this put on later rather than in the cell?

AUDIENCE: Do you mean clicking it on or synthesizing-- or putting that whole thing on?

ELIZABETH NOLAN: Yeah, as you can imagine someone could have thought, rather than clicking this on after the fact, why not just use this whole moiety here as the unnatural amino acid? So this fluorophore.

AUDIENCE: It would be hard to find a synthetase to accommodate that fluorophore.

ELIZABETH NOLAN: It might be hard to find a synthetase.

AUDIENCE: Might just be too much [INAUDIBLE]. It might not fit physically within the ribosome machinery.

ELIZABETH NOLAN: That could be.

AUDIENCE: Are you asking why we would not put it in?

ELIZABETH NOLAN: Yeah, I'm just asking you to think about this, right? So you know, what needs to be thought about, right? So here, there's still a chemical step after this unnatural amino acid was put in. And in this case, why might that be? Maybe it's a permeability issue. We don't know if that molecule readily taken up by the organism.

Is it a size issue, that it's hard to get machinery to accommodate this type of molecule here.

AUDIENCE: Is it folding?

ELIZABETH NOLAN: Folding of--

AUDIENCE: If you had it, is the question like, you put it on the floor, which is after like it's been processed--

ELIZABETH NOLAN: Yeah, maybe it messed up.

AUDIENCE: If it's a floppy thing, it might interfere with folding, or folding might interfere with its, like--

ELIZABETH NOLAN: Right, so can the the polypeptide breach its native confirmation with this perturbation. Just to think about. And here are just some examples of unnatural amino acids that can be used for fluorine NMR as was mentioned last time.

OK. So this is all really exciting, but what is the limitation? And there is a major limitation of this methodology as it was first described. So the major limitation is that the efficiency is low. OK?

And if we consider wanting to incorporate one unnatural amino acid into a polypeptide, so there is one amber stop codon put in, what was found is that about 20% to 30% efficiency for incorporation of one unnatural amino acid. OK? And then this value plummeted to less than 1% for incorporation of two unnatural amino acids. So imagine there's two amber stop codons put within the gene.

So why is this? This is because what's observed is that only a small amount of the protein or polypeptide synthesized reaches completion. And so, how can we think about this? Imagine here, I'm just going to draw some polypeptide chain going from end to C terminus.

Let's imagine this is 20 kilodaltons in size. And maybe this unnatural amino acid is being placed right in the middle. OK? So we want to put an unnatural amino acid here. OK? So, imagine you make your plasma DNA to do this.

You have the tRNA and aaRS and the unnatural amino acid, and you do your expression, and then you take a look by SDS page, so gel electrophoresis, what you see? So imagine here we have 20, 10, five, so kilodaltons here. Right? So we have some molecular weight markers let's just say here.

If you do this, say for the native sequence. So you haven't put in the stop codon. Imagine there's your protein. If we have the unnatural amino acid, what do we see? Something like this. So what does this tell you?

First of all, why do you look at the native one? Effectively, you want some positive control

because if you can't express your polypeptide with the native sequence, you're not going to want to go try to stick in an unnatural amino acid, right? There's a problem. So that's your positive control.

So we see in this make believe gel, there's one band at 20 kilodaltons, which is the size of that. If that ever happens to you, you've had an instant gratification protein trap. So, what about this lane with the unnatural amino acid? What do we see and what does this data tell us? Lindsey.

AUDIENCE: It's like early truncation.

ELIZABETH NOLAN: Yeah, something happened. So early truncation, and why are you saying that? We see two bands. There's one band with the expected migration to about 20 kilodaltons. And then there's the second band that's coming up around 10 kilodaltons.

And based on what I sketched out here, that unnatural amino acid is roughly around the 10 kilodalton mark. OK? What about the relative intensity of these bands? What do we see more of?

AUDIENCE: The truncated one.

ELIZABETH NOLAN: We see more of the truncated form. So what's going on? we? Need to think about our ribosome. And there's some polypeptide being made. And then what's coming here?

We either have our tRNA with the unnatural amino acid or the release factor, right? So there's going to be competition for binding in the A site between the tRNA and the release factor. And so this is getting back to, I believe, Max's question from last time about using the stop codon, right? There's fundamentally a problem here.

So, yeah.

AUDIENCE: How does the release time test different for different stop codons?

ELIZABETH NOLAN: Yes, so we discussed that I think in lecture four. So there's a release factor one and release factor two, and there's three different stop codons. So they both recognize one of the same and two different. And in this case release factor one recognizes the amber stop codon here.

So we're not worrying about release factor two competing with this stop codon because it doesn't recognize this stop codon here. Right? So if release factor one goes in, we get

premature termination. And that results in truncated protein.

So is this a problem? And how much of a problem is it?

AUDIENCE: So you're saying that the release factors comes in because it's recognizing the codon that's trying to-- or that originally was a stop codon?

ELIZABETH NOLAN: Yeah, because the codon is still a stop codon.

AUDIENCE: So in the wild type, it wasn't that we replaced-- sorry. So we replaced it with a stop. But the stop wasn't there originally. And so that's why you get the full 20 length, right?

ELIZABETH NOLAN: Yes. So you have--

AUDIENCE: Yeah,

ELIZABETH NOLAN: OK, continue.

AUDIENCE: There was no stop before. Now there's a stop, but it's not supposed to act like a stop, right?

ELIZABETH NOLAN: Right.

AUDIENCE: So here it is acting like a stop kind of?

ELIZABETH NOLAN: It depends what enters the A-site. So a stop codon is a stop codon. But the idea is that this tRNA has been tweaked to allow a tRNA to recognize the stop. But there's going to be competition because you have the tRNA that's going to deliver the unnatural amino acid. But you also have release factor around.

So this release factor one is in the endogenous pool. So the question is, which one gets there and does the job? Right? And so what that gel is telling you is that there's a mixture. Right? Sometimes the tRNA will get there and translation continues until you get to the desired stop where you want translation to stop, in terms of stopping.

Or if the release factor gets there, you get termination. So you get some truncated protein.

AUDIENCE: How do you know, though, that you've got in the end-- that you actually got the unnatural amino acid in the 20 [INAUDIBLE] and not just the original? Is that fluorescing?

ELIZABETH NOLAN: No. I mean, just imagine we're just looking at protein here-- I mean, where this came from.

AUDIENCE: So it would look the same?

ELIZABETH NOLAN: If you had a fluorescent amino acid, you'd see something-- no. Because if you didn't have the unnatural amino acid there, what else could be there?

AUDIENCE: Just like the native.

ELIZABETH NOLAN: But what native amino acid can be incorporated if there is a stop?

AUDIENCE: Oh, because you also put in the mRNA.

ELIZABETH NOLAN: Yeah. Right. So there has to be a stop. Now, that's also backtracking why you need to make sure everything's orthogonal. Because you don't want one of the endogenous amino aminoacyl-tRNA synthetases to put some endogenous amino acid on this tRNA. OK? So either full length with the unnatural amino acid or truncated because RF1 came along here.

Right? So in terms of how much of a problem this is, in some respects, it depends on what you need and what you want to do. If you're over expressing protein and you can deal with this mixture and get enough full length, maybe that's OK. If you're doing an experiment in cells, you have to ask, what is the consequence of also having some truncated protein around?

What does that mean for the cell? What does that mean for your measurement there for that? So how can we get around this problem of RF1? So effectively, we want to diminish RF1 mediated chain termination. What are some possibilities? Is that feasible?

So we could do that and we could get a better yield. That would be great for protein overexpression. If we could minimize truncated phenotypes, that would be great for an experiment in cells. You don't need to worry about what this truncated protein might do.

So what are possibilities? So can we knock down or knock out our RF1?

AUDIENCE: [INAUDIBLE]

ELIZABETH NOLAN: So this is a wonderful little story. I'll just tell a little bit about, we're not going to go into huge detail. But for quite some time, it was thought that RF1 was essential in E. coli. So a lot of experiments were done with E. coli K12 and even if you go look on a website about all the genes in E. coli K12, it will tell you RF1 is essential. But then in 2012, a paper came out in ACS Chemical Biology, where they were doing some work in a different strain of E. coli.

So there's many different E. coli's. And K12 is a laboratory workhorse. And there's also strains, E. coli B. And they're also laboratory workhorses. So maybe many of you have used BL 21DE3 cells for protein expression.

So this lab was working in E. coli B strain, and found that RF1 could be knocked out; that it's not essential. So then the question is, what's going on? And as it turns out, the essentiality of RF1 in E. coli turned out to be due to an issue with RF2. And in the K12 release factor 2 has a single point mutation that makes it less able to stop at certain stop codons. So when you had both of those together, it was deleterious.

So RF1 can be knocked out. Would you want to do that?

AUDIENCE: So, RF1 can be knocked out without RF2 or RF3, I don't remember.

ELIZABETH NOLAN: Yeah, there are three release factors. RF3 is a GTPase. It's a little different.

AUDIENCE: There's redundant kind of behavior.

ELIZABETH NOLAN: There's some redundancy. And I mean, something too just to ask is, if you can knock it out and the cell is viable, viability is different than normal healthy cell. So those E. coli B, without RF1 will grow, but are they growing and replicating as well as the wild type? No. No. But is it good enough?

And I think again, it comes down to asking what is it that you want to do? So maybe if you're over expressing protein and you're going to purify that, it's not such a big deal. But again, if you're looking at some cellular process, you're going to need to think about what's happening if RF1 can't terminate translation for, you know, its repertoire of proteins and genes there.

There will be some consequence of that perturbation just to keep in mind. But there's certainly work going on with that now that it was found not to be essential.

So in vitro translation, just something to think about. If you're going to work in a test tube, could you just do this outside of the cell? And then, the possibility we're going to discuss in closing is this one of a new ribosome, which I think is pretty cool.

So, is it possible to have an orthogonal ribosome here to get around this problem? So effectively, can we make a new ribosome that only translates the message encoded in a plasmid that has the gene of interest where you want the unnatural amino acid to go?

And so thinking about this in cartoon form, imagine we have E. Coli or some organism, and there's the native ribosome, and this native ribosome translates all of the native wild type mRNAs and gives synthesis of the proteome. But then imagine we can put in an orthogonal ribosome into this organism.

And this orthogonal ribosome only recognizes an orthogonal mRNA, which means it only translates off of this orthogonal mRNA and only gives you synthesis of the protein you want with the unnatural amino acid. So how to think about doing this? Need to think back about the initiation process, and that mRNAs have a ribosome binding site.

So effectively, it's necessary to engineer an mRNA that contains a ribosome binding site that will not direct translation by the endogenous ribosome, so some new ribosome binding site. OK? And then this orthogonal ribosome needs to be engineered such that it's specifically binding to the orthogonal mRNA.

And it doesn't bind to the wild type mRNAs there. So no translation of the cellular message because this ribosome binding site and orthogonal ribosome are a match. OK? So a unique binding site. So in thinking about how to do this, you want to think about the ribosome structure.

And we know that the 16S rRNA is involved in binding to the mRNA at the beginning of the initiation step. So what was done was to mutate the 16S and come up with an orthogonal ribosome.

So this has been done. That's not a solution to the problem of RF1 terminating translation on its own. So then the next question is, if we can have just this orthogonal ribosome and orthogonal mRNA, can we improve that system to minimize RF1 mediated chain termination?

So effectively what we want to do is prevent RF1 from binding to the A-site of the orthogonal ribosome. But it's still going to do its job for the endogenous ribosome here. So what needs to

happen? And we'll go through the steps.

This is just the schematic in cartoon form. So imagine we're starting with native ribosomes and orthogonal ribosomes. And we have tRNAs and RF1. And nothing has been done to this orthogonal ribosome so RF1 can still bind there.

And so we want to have some evolution. So mutagenesis and selection of the orthogonal ribosome such that only the tRNA goes to the A-site. And RF1 only goes to the wild type ribosome.

So there's other possibilities. One possibility I'll just throw out there is using rather than a triplet, a quadruplet codon there, which we won't talk about. There's more than one solution to the problem. But where we're going to focus on is work done to minimize RF1 and how to think about doing that from the standpoint of what we know of ribosome structure and the interactions. OK?

So the name of this new O ribosome is Ribo-X and so what did they do? They started with the orthogonal ribosome. OK? And so, the first is that there needs to be some mutation to the ribosome, so libraries of mutants.

There needs to be some selection process. So effectively, there is a requirement for of activity from the ribosome. and. When there's this, there needs to be some sequencing. Or identity determination, so where is the mutation? Here. And then with some mutant in hand, that looks like it's a good option, there needs to be assays to study it.

And we're pretty much going to focus on step four. I'll briefly say something about steps one, two, and three here. So the first thing is, if we want to mutate this O ribosome, how do we think about designing a mutant library?

And so, what we need to think about in this case because the goal is to minimize RF1 mediated chain termination and enhance tRNA getting into this A-site, we want to look at how the ribosome interacts with RF1, how it interacts with the tRNA, and also think about the mRNA there.

And so, there's crystal structures available. There might be biochemical information available. But really to ask, where does that make sense to make mutations? And so if we think about the stop codon being recognized by the tRNA and RF1 in the A-site, somehow we want to

mutate the ribosomal RNA in that region to give us the desired outcome.

So what they did is mutate 16S rRNA to favor suppression of the amber stop codon by the tRNA. And crystal structures guided the library design. And so they looked at crystal structures where tRNAs are bound to the A-site or where RF1 is bound to the A-site. And from these, they selected seven different positions of the RNA and randomly mutated them.

So that gives you some new mutants to study. Then there needs to be a selection process. So the mutant needs to be active. Some of these mutations might cause the ribosome to be inactive and that won't be very helpful.

And so they developed an assay based on antibiotic resistance to select. And effectively, an enzyme that provides resistance to chloranfenicol, which is an antibiotic that blocks translation and was put under the control of the O ribosome. So you can imagine using antibiotic resistance as a selection there.

And then the sequencing, once we've selected first some mutants, we have to ask where is the mutation? And so what they found after going through this work is that for Ribo-X it's only a double mutant in the 16 S rRNA. So two positions, U3531G and U534A.

So these mutations in proved suppression of the amber stop codon, and I also point out these mutations are very unusual. So, at least at the time of this work, no sequenced natural ribosome had these two mutations here. And they're found in very few examples of sequenced RNase here.

So, I mean, just to think about the ribosome's so huge and just two point mutations can make this change here. So what's seen in terms of some characterization. What do we need to ask in terms of characterization. Bless you.

So something we want to ask about is fidelity here. So if we think about fidelity, one, we can ask, if we're using this to express some protein, what is the protein yield and how does that compare to the native ribosome?

We want it to incorporate amino acids correctly with high fidelity and incorporation of the unnatural amino acid. So doesn't this incorporate amino acids? So that's the question we need to ask. OK? And then of course, we need to ask about amber stop codon suppression efficiency.

And so, in thinking about this what is the point of comparison? So we can imagine in all of these comparing this new orthogonal ribosome Ribo-X to the starting orthogonal ribosome. Right? Here. So what are the experiments? So first let's think about protein yield.

And I'll just say, I have a pet peeve when people don't report their protein yields in experimental. So if you're doing biochemistry, always think about doing that there. So what they did is an experiment where they made a plasmid. So we have an orthogonal DNA that will give orthogonal mRNA.

So this gets transcribed... to give the orthogonal mRNA and then it gets translated by either the O ribosome... or Ribo-X. And the result of this is a fusion protein where we have a protein called GST, glutathione S-transferase, and then MBP, which is maltose-binding protein.

And as we move forward, it will become clearer why they use this fusion. OK so just the first question is, how does the yield of protein compare? Are they doing a similar job or were these two mutations detrimental?

So here's the result from this experiment one looking at protein yield. OK, so again we're looking at an SDS page gel that's being stained for the protein. And we see that this GST and BP fusion has a molecular weight of 71 kilodaltons, right?

And what we see up here are the components that were in each of the experiments for each of the lanes. So here in this lane, we have no O ribosome, no Ribo-X but the plasmid was included. Here, we have the orthogonal ribosome in the plasmed, here Ribo-X in the plasmid. So what do we see? Pardon?

AUDIENCE: Are they all the same yield?

ELIZABETH NOLAN: Are they all the same yield? There's three lanes.

AUDIENCE: [INAUDIBLE]

ELIZABETH NOLAN: Yeah, so no orthogonal ribosome, no translation. And that's a good thing to see, right? That tells you that this orthogonal mRNA is not being translated by the endogenous ribosome. That's an important observation.

And then I think what you meant to say, is that in these two lanes where we have either the

starting orthogonal ribosome or Ribo-X, what we see is what appears to be a very similar amount of protein. So here, you know you assume and you look at the experimental, the same volumes were loaded, all of these things. We're getting the same amount of protein yield. So that's a great result here.

So that's good news. What's the next experiment? And we'll close, I think on this experiment. So the next experiment is amino acid misincorporation. So again, what they did is they used this GST MBP fusion protein. And there's a linker region here. And in this linker region, they engineered a protease cleavage site here. So for thrombin here. And why did they do this to look at amino acid misincorporation, whether that's happening. Effectively, they took advantage of the fact that GST contains cystine, whereas maltose binding protein has no cystine.

So their idea was let's use radio labeled cystine as a probe and monitor for radioactive cystine incorporation. So effectively, what can be done is that this can be expressed and purified in the presence of the radio labeled cystine. Thrombin can be used to cleave. And then you can look and ask is there radioactivity associated with GST? And we hope the answer is yes. And is there radioactive activity associated with maltose binding protein.

And so where we'll begin on Friday is looking at the data from this assay. But until then, what I'd like you to think about is in terms of amino acid misincorporation, kind of strengths and limitations of this assay. Right so the choice of using one amino acid to take a look there. OK? So I'll see you Friday.