ELIZABETH NOLAN: Today's recitation will all stem from this reading by Youngman and Green on ribosome purification. But also, we posted an optional-- well, not really optional but an additional reading that's a review that talks about purifying macromolecular machines from the source.

And I guess one thing I'd just like to say about this review, something I like is figure three because it indicates many, many different types of methods that can be used to purify some biomolecule. And I think often it's easy just to think all the time, well, let's just use a tag. And tags have enabled many things, but there's also many possibilities out there and decades worth of work before tags for how to purify proteins. And in some instances, you might not want to use a tag and that's discussed to some degree here.

So I guess I'm curious. What did you all think about this week's paper? Did you like it? Did you not like it? Was it easy to hard read? Did you read the paper?

AUDIENCE: It wasn't too bad. I've purified--

JOANNE STUBBE: You need to speak louder because I can't hear you.

AUDIENCE: It wasn't too bad. I've purified proteins before, so I felt like I could follow along.

ELIZABETH NOLAN: So not too bad means it was easy to understand and follow the text there. Yeah, so compared to the reading for recitations two and three, this one was probably easier to work with, but there's a lot of details in this one too there.

One thing, I guess, I like this paper from the standpoint of it being a methods paper, is the amount of detail they give with how they did this purification and things that didn't work, right? So often, sharing those details with your readers can make such difference for an experimentalist in another lab in another part of the world, right?

And I think, from this, one with biochemistry training could go into the lab and reproduce their purification there. So it's a good example in terms of what details to include and what types of

pitfalls to include.

So how many of you have done a protein purification, either in a lab class or in your research? OK. And so what type of purification did you use?

AUDIENCE: We used a histamine-tag with nickel.

ELIZABETH NOLAN: OK. So you used a His6-tag, probably a polyhistamine tag in a nickel NTA column. For everyone else, has it been that type of methodology or another methodology?

AUDIENCE: I used the same one.

ELIZABETH NOLAN: OK. OK. So would someone like to kind of comment on the basics of affinity tag purification? So how does this work? And why did you do it? So what are the advantages?

AUDIENCE: So you have a light chain of histamines on your protein, and you use nickel, which is a metal that chelates to histamine very well. Is that right?

ELIZABETH NOLAN: Yeah. The nickel's bound to something though. You have the NTA ligand on the resin.

AUDIENCE: I did this over a year ago. Sorry.

AUDIENCE: So if you have whatever the type is, the [INAUDIBLE] bound to some solid substrate. And then you can elute everything that's not bond to that. Elute what it does bind. So you just tag the protein. It's bound to a high concentration of the free ligand.

ELIZABETH NOLAN: Or right, to push it off. So the idea is that you have your biomolecule of interest, whether that be a protein, which is what we're all most familiar with, or the ribosome, and then you attach a tag. And that tag can be any number of things, right?

So in this paper, we saw they used a stem loop structure incorporated into the 23s rRNA. And the idea is that you're going to use that tag to separate your biomolecule of interest from the complexity of the cellular environment there, so all of the other proteins. And so you have some bead or resin that this tag can bind to.

So in the case of the nickel column, the His6-tag will bind to the nickel NTA on the resin. And then you can wash away other components. And then you devise some method to elute the protein you hope to have trapped there.

So what are some advantages of using an affinity tag, just thinking about this generally before we delve into the paper?

AUDIENCE: Easy to install.

ELIZABETH NOLAN: OK. So what do you mean by easy to install?

AUDIENCE: If you wanted to just encode a 6 His-tag at the terminus of your target protein, I don't know if you can say it's trivial. It's easy.

ELIZABETH NOLAN: I'd agree it's easy. There's many plasmids available that you can insert your gene of interest into in order to have this tag genetically encoded. So when you express the protein, the tag's there. So beyond that, from the standpoint of purification--

AUDIENCE: It's more specific.

ELIZABETH NOLAN: Pardon?

AUDIENCE: It's more specific.

ELIZABETH NOLAN: More specific--

AUDIENCE: Pure proteins as opposed to various charges.

AUDIENCE: It simplifies the purification. Instead of doing size exclusion and ion exchange that is much different.

ELIZABETH NOLAN: So the hope is it simplifies the purification because you have some way initially to pull your protein of interest out of your cell lysate there. So that can be a big help.

What are some potential disadvantages of using a tag? So have any of you run into trouble with a tag in the lab?

AUDIENCE: Having a tag can highly deform your protein and change it.

ELIZABETH NOLAN: Yeah. So it might change your protein or deform it. What do you mean by "deform?"

AUDIENCE: It could just cause a conformation change or the tag could make it localize somewhere else based on size.

ELIZABETH NOLAN: Yeah. So that's an example, say if you were doing a study in cells, say, rather than a purification. But maybe you tag a protein and it goes somewhere other than it would go untagged right? And that will affect your observations and your data there.

And it might alter the conformation. So it might affect the folding, right? It might affect the oligomerization. His-tags bind metal ions. So is that a factor to consider there? If you have an enzyme, will the tag affect activity, right?

And these things can be a positive or a negative. Sometimes the tag is helpful in these regards. You can't get soluble protein without the tag, right? And sometimes the reverse. You decide to express your protein or biomolecule with a tag and you find out you get an aggregate, something that the protein shouldn't be.

So these are just things to keep in mind when designing a fusion protein and thinking about how you're going to use an affinity tag to express your protein there and purify your protein. So there's pluses and minuses, right?

And you can always make the choice not to use a tag. So you saw some of that in the review article, right? And I guess one other thing, too, there's this idea of using the affinity tag in the affinity column, which we'll talk about more in the context of the ribosome, but is that always enough? So is the affinity column alone always enough to purify your protein of interest?

So in lab class, that's where it will end, because that's an exercise made for lab class, and it's your first adventure into protein purification for most people, right? Oftentimes, it's not enough. That you do enrich what you've purified with what you want, but often there's contaminants. So you actually do move forward with doing some other type of purification. Like Rebecca mentioned, ion exchange or size exclusion, those are things you can use after the affinity purification there as needed, right?

So contaminations are something to look out for here.

JOANNE STUBBE: What other types of steps do you use for purification besides columns and [INAUDIBLE]. Because people have forgotten all of this. Everybody uses the tag and that's the end of it, and it can be the kiss of death. What other kind of fractionation steps? Do you do any other

fractionation steps? What is it, 535 or something?

AUDIENCE: [INAUDIBLE] salt.

JOANNE STUBBE: The what?

AUDIENCE: The [INAUDIBLE] salt. So some proteins will precipitate. Some will not.

JOANNE STUBBE: Yeah. So that's what you use to precipitate. So that's a mild method. It's fast. It gives you separation on a fair amount of proteins. Anybody know what you use?

AUDIENCE: Ammonium sulfate.

JOANNE STUBBE: Yeah, ammonium sulfate. And then what's the other thing that really is important? What is the other thing you want to remove from your protein when you're using this tag that oftentimes people miss in the literature?

ELIZABETH NOLAN: Yeah, we were getting there.

JOANNE STUBBE: There's another component inside the cell that you need to get rid of that you've been talking about.

AUDIENCE: Where one component has His-tag on the cell, you remove it.

JOANNE STUBBE: So you have the protein. What are the other components in the cell that you need to remove? You can go back and look at your cartoon of the inside of the cell.

ELIZABETH NOLAN: Yeah. So we're getting a little ahead, but that's totally fine. So if you're going to lyse your cell, and then, imagine your protein's soluble, right? So you do a centrifugation to remove the insoluble components. So you have the membrane and all that debris.

And then you take your soluble fraction, and you incubate that on your column, right? And then you elute, you wash, you elute your protein. What might come out with your protein?

AUDIENCE: Nucleic acid.

ELIZABETH NOLAN: Yeah, right. So how do you know if you have nucleic acid contaminating?

AUDIENCE: The A260.

ELIZABETH NOLAN: Right. So A260 will give you a readout of nucleic acids. A280 is what people typically look at for their protein concentration, but you should look at both so you know what's in your protein. You need to look at both.

JOANNE STUBBE: Do that and adaptively repeat stuff out of the literature. Very frequently, you take an absorption spectrum, there's some nucleic acid.

ELIZABETH NOLAN: You have contamination. So a lot of ribosome, DNA.

JOANNE STUBBE: If you don't remember anything else, it's important.

ELIZABETH NOLAN: Yeah. So sure, just something to think about. So Alex noted, it's easy to put on this His-tag. What is actually on this His-tagged protein? So is it just the six histidines are all the same? When you look in the literature, and someone says they put a His-tag on the N terminus of their protein, how do you think about that?

So we have some His6-tag, and then that tag, say, is attached to the protein here. What's going on in between?

AUDIENCE: Maybe a flexible linker of some kind.

ELIZABETH NOLAN: Pardon?

AUDIENCE: It would be like a-- I don't know-- like a flexible linker of some kind.

ELIZABETH NOLAN: Yeah. So maybe some kind of flexible linker. And what might dictate this linker?

AUDIENCE: If you wanted to remove the His-tag after you would want to be able to hydrolyze it or something.

ELIZABETH NOLAN: Yeah, so maybe you want to remove your tag down the road, right? So a protease is often employed. So maybe there's a linker, maybe there's a protease cleavage site. OK. OK.

And we're not going to talk about cloning really in this class, but just thinking back a step, you need some plasmid DNA to ultimately get here that has your gene, right? And so you'll insert

the gene into the plasmid, and many commercial plasmids have something called multiple cloning site. And, for instance, if you want a His-tag or a GST tag, you'll use some different plasmid that has that encoded, right? And then you make a decision about how you put your gene in.

And these multiple cloning sites have multiple sites for restriction enzymes where you can put the gene in, which means, even if the same plasmid is used for 10 different proteins, what's happening here can vary a lot even if you have one protein and put it in different sites. So maybe you have a short linker here because you used a site like NDE1 or SPE1, I'm just making those up, right? Or maybe you have a longer region here between the tag and the protein.

And so it's very important to go look back at the map of the plasmid that was used and ask where was the gene put in and what does that mean? So is this His-tag a 2 kilodalton perturbation. Is it a 5 kilodalton perterbation to your protein?

And some of these plasmids have multiple types of tags, right? So it might have a GST tag and a His-tag, and depending how you put your gene in, you may have two or you may have only one, right? So I'm just pointing out there's a complexity here.

So when someone just writes in their paper, oh, I His-tagged the protein, you need to think beyond just sticking six histamine residues on the N or C terminus there.

Did you have a question?

AUDIENCE: So does that mean that's like another step where they purified the specific plumbing site, one that they wanted? Or does that mean you kind of roll with the heterogeneous--

ELIZABETH NOLAN: No. So you put your gene in particular sites with this type of cloning, right? And so one thing practically, just say you want to express some new protein and you don't know much about this. You might choose to make several different constructs where you put the gene in different sites or maybe you use primers that allow you to add some linker regions because you don't know what will give you better solubility and better yield. And then ultimately, you pick one.

So, for instance, like for me, with working with, say, a His-tag for a protein, there's plenty of plasmids available where you can pick N terminus or C terminus. So one plasmid to put the tag on the N terminus. A different plasmid to put the tag on the C terminus.

I'll clone the gene into both and test overexpression with both and just see if one's better than the other in terms of yield, in terms of solubility there. And then you make a call. Maybe you purify both and see if there is an effect on behavior, like oligomerization or activity if it's an enzyme here.

So if you get into protein purification, it's good to talk to people who have purified many different proteins because the strategy is a little different for each protein. And then you just get more exposure to all of the possibilities and troubleshooting there.

So coming to the paper, what was the big motivation for developing this method to have an affinity tag attached to the ribosome? Right? So Youngman and Green went to quite a bit of effort to devise this new system. What was their motivation? And what really was the big issue they were seeking to overcome?

AUDIENCE: They wanted to get ribosomes with mutations on it. So they want to synthesize bacteria ribosomes with it and purify it.

ELIZABETH NOLAN: Yeah. So they want a mutant ribosome. And they want to make this mutant ribosome in vivo and then purify. So what's the complication with making the mutant ribosome in vivo that they seek to overcome here?

AUDIENCE: You have the wild-type ribosomes in there also.

ELIZABETH NOLAN: So well, right. That was their decision, right? They want to express this mutant ribosome in the background of the wild-type ribosome. So why do they want to do that?

AUDIENCE: Because it could be lethal. The mutant ribosome, it would be a toxic mutant.

ELIZABETH NOLAN: Yeah, toxic mutant. What do you mean by "toxic mutant?"

AUDIENCE: If you create a mutant ribosome, that was the only way for the cell to express them, they'd be toxic and they wouldn't be able to go through translation.

ELIZABETH NOLAN: Yeah. So maybe the mutant ribosome doesn't work very well, and that ends up being lethal to the cell there. So can we imagine why that might be an issue for the types of experiments we've seen in class? So if you're thinking about trying to understand the catalytic mechanism in that function, there's a likelihood the mutations may dramatically affect that activity, right?

If you want to put a point mutation into the peptidyl transferase center, into the decoding center, that could be deleterious to your cell, but it could be very important for your mechanistic study. So they want to avoid this lethal phenotype.

So what is the complication in terms of doing this in the presence of wild-type for some sort of measurement?

AUDIENCE: It gets kind of muddy.

ELIZABETH NOLAN: Gets kind of muddy. Yeah. What does that mean?

AUDIENCE: You don't have a pure mutant in there, and they're not significantly different from the wild-type.

ELIZABETH NOLAN: Yeah. So if you were going to do a standard ribosome purification-- because ribosomes have been purified for many years without an affinity tag-- you're going to have a mixture of your mutant and the wild-type. And so that has a strong likelihood of being a problem for your analysis, right?

So they gave an example in this paper where they actually made a mixture and did some analyses, where you could separate the wild-type from mutant activity but that's not necessarily the case. And so, as pointed out, they're both very large. They're very similar, right? There's no good way to separate a ribosome with a single-point mutation, say, in the peptidyl transferase center from wild-type.

So let's just imagine you have a mixture that's predominantly your mutant ribosome but you have some background contamination of wild-type. Is that an issue? Say you want to measure rates of peptide bond formation.

AUDIENCE: It's going to be an issue.

ELIZABETH NOLAN: Yeah. So why is it probably going to be an issue?

AUDIENCE: Because your mutant might be a lethal function because of your background and resume the function and [INAUDIBLE] mutant [INAUDIBLE].

ELIZABETH Yeah. So imagine you have a mutant ribosome that has very low activity or none, and you

NOLAN: have some small amount of contaminating wild-type that has wild-type activity, right? How do you know-- I mean you might misinterpret your data, and what you're seeing is the wild-type and not the mutant.

And this issue is much more broad than the ribosome. So one of my favorites is the contaminating ATPase. And maybe you have an enzyme that hydrolyzes ATP, but maybe you have a small contamination of an enzyme that does a much better job at hydrolyzing ATP that's in your reaction, right? So what are you seeing there? So that's something to keep in mind in terms of potential contaminations, right?

And so they go through some justification about why they need to do this method based on available methods, and all of those available methods have strengths and weaknesses. So they talked about using systems without the wild-type ribosome. They mentioned in vitro translation, and I'll just say, in passing, those in vitro systems have improved a lot since the time of this paper.

OK. So in terms of their strategy. Let's comment on the various aspects of this strategy. All right. So effectively, they want a way to express the mutant ribosome in vivo in the background of wild-type. They want a way to separate that ribosome. And they want to come up with ribosomes that are active.

OK. So this cartoon basically summarizes their solution to this problem, and we should work through the various components. So the first thing is they attached a tag to either the 23S or the 16S. And we'll focus today's discussion on the 23S because that's what they did more characterization on in the paper.

So how did they decide on the tag and where to place the tag?

AUDIENCE: You don't want the tag somewhere that's going to interfere with function. And you don't want the tag itself to be reactive where it will interfere. So you need to be out of the way and kind of passive so it's not interfering with function.

ELIZABETH NOLAN: So that's one point, right? We don't want this tag to interfere with function. So that's one aspect. What's another aspect?

AUDIENCE: You still have to be accessible

ELIZABETH Yeah, the tag needs to be accessible because something's going to have to bind this tag,

NOLAN: right? So on the basis of those two criteria, we can imagine wanting this tag somewhere on the surface, right? We don't want it where the 30S and 50S interact. We don't want it in a position that's critical. So like maybe if it ended up near where EFTU first binds or EFG, that would be bad. So accessible and in a place where it won't interfere.

Beyond that, the ribosome's huge. So how does one pick where to put this tag? What did the researchers do?

I'll tell you what they didn't do. They didn't reinvent the wheel. So what did they do?

AUDIENCE: Lit review.

ELIZABETH NOLAN: Yeah. They went to the literature. And what did they find in the literature?

AUDIENCE: Someone had installed a tRNA before or something and it didn't interfere with the ribosome function.

ELIZABETH NOLAN: Right. So they took observations that were made from an independent group for an independent project but the observations that were useful to them in their design. So for some reason, this lab stuck a tRNA onto the ribosome and saw the ribosome was still active and functioning well. So the decision was, why don't we use that to place the tag here?

So what about their choice of tag? What did they use? A big tag or a little tag? Any sense of that compared to the size of, say, the 50S? Take a look at figure one.

AUDIENCE: A small.

ELIZABETH NOLAN: Yeah, right. So I'd say very small compared to the size of the ribosome, right? So they decided to take advantage of interaction between this MS2 coat protein and the MS2 RNA recognition sequence, right? So that's one interaction involved here, so ligand receptor interaction.

So here, this is the depiction from the paper showing where they incorporated this MS2 stem loop into the 23S rRNA, here. And so what does that tag need to bind, going back to the cartoon? And what is different about this strategy from, say, what you've done with nickel and TA chromatography and His6-tags?

AUDIENCE: It has three things.

ELIZABETH NOLAN: Three things, yeah. Right? So we have three components, two different interactions, say, between a ligand and its binding partner, right? So here, we have the mutant RNA of the ribosome where there's this MS2 stem loop, OK? That MS2 stem loop binds to the MS2 coat protein, right?

And then there needs to be some way to pull this out. And what they chose to do here was take advantage of a second interaction and one that's commonly used in chemistry and biology, which is looking at an interaction between a protein called glutathione S-transferase and glutathione, here, right?

So effectively, they have a solid support or a resin, so like the nickel NTA column, but in this case, it's modified with GSH. They have to prepare this fusion protein that is a fusion of GST and MS2, and then they have the ribosome with the tag. So why might they have done this with three components rather than two?

AUDIENCE: Since GST/GSH, these are pretty standard affinity tags, it was probably easier to acquire GSH then to try to get MS2.

ELIZABETH NOLAN: Yeah. That's a practical analysis there. So could they have made a resin with MS2? Maybe, right?

So how did they go about doing this affinity purification? What were the steps and why?

So imagine they've done the molecular biology required to express this tagged ribosome. They expressed the tagged ribosome in E. coli. Then what? How are they going to get this tagged ribosome out?

AUDIENCE: They first purify the crude ribosomes [INAUDIBLE] including the [INAUDIBLE] and normal ones. And then they load these crude samples through the column.

ELIZABETH NOLAN: OK. So the crude sample went through the column, right? What happened before that? So can you just put the crude ribosome through the column, if your column is the resin with GSH?

AUDIENCE: You have to preload it with a fusion protein.

ELIZABETH NOLAN: Yes. So what did they preload with the fusion protein?

AUDIENCE: The column.

ELIZABETH NOLAN: Yes. That's what they did, right? We can imagine just looking at this without further details. There's two possibilities with three components. So they could have, as stated and as what they finally did, they could add this fusion protein to the column, right? So the fusion protein binds GSH. And then you take your crude lysate, crude material from the E. coli and run that through the column to trap the ribosomes.

What's the other possibility? They could have taken this fusion protein and put it into the crude mixture, and they talked about that. So what was one of the complications with this fusion protein that led them to incubate it with the column? Was this fusion protein well-behaved?

AUDIENCE: It forms insoluble aggregate.

ELIZABETH NOLAN: Yeah, right? It gave them some headaches. It aggregated. Is that something that can commonly happen?

So they likely tried both ways, and they observed this problem with the fusion protein having aggregation, right? And they avoided that as being a complication to the purification by incubating the column with that fusion protein first.

So after they take their crude sample and have that bind to the resin in the column, how did they get the ribosomes off the column? So what are the possibilities? And what did they end up doing and why?

AUDIENCE: So you could just use an excessive of free MS2 ligands to cause the ribosomes to disassociate from the column. Or you could use an excess of the GST to cause that complex to dissociate from the column.

ELIZABETH NOLAN: Yeah. So thinking about the latter possibility-- so what Rebecca has done is identify the two different ligand receptor interactions, right? We could disrupt this one between MS2 fusion coat protein and the ribosome or between GSH and GST.

So in terms of this one here, does it make more sense to elute with excess protein or excess glutathione, which is effectively a tripeptide?

AUDIENCE: Glutathione.

ELIZABETH NOLAN: Yeah, right? So just that much easier to come by a lot of glutathione than a lot of GSH-- or sorry, GST. So which one did they choose? How did they elute the ribosome off the column?

How long did you each spend on the paper before coming here?

AUDIENCE: GSH.

ELIZABETH NOLAN: Yeah, they used GSH. So they eluted with excess GSH. So what does that mean in terms of the purified ribosome? Is it just the ribosome with the stem loop?

AUDIENCE: They still have the fusion protein.

ELIZABETH NOLAN: Right. We still have the fusion protein on. So if you were making this decision at the bench, you have two different interactions to consider, what would you choose and why? Why and I guess, really, why did they choose to disrupt the interaction between GSH and GST?

AUDIENCE: Because it's difficult to have enough MS2 than to purify with glutathione.

ELIZABETH NOLAN: Here, right? Because if you were going to, say, elute with excess of MS2 stem loop, where would that come from? Or excess MS2 protein, right?

AUDIENCE: Also, it's actually MS2.

ELIZABETH NOLAN: Yes. It's not very practical here, right? At the end of the day, it would be best to have the ribosome without this fusion protein attached, but disrupting this interaction isn't very practical. So it would be quite expensive either way if you were making a lot of some sort of stem loop. And then how would you even know you have the stem loop or the protein here?

OK. So I'm just curious, for those of you who have done like nickel NTA chromatography, do you have a sense of the affinity of the His-tag protein for the resin? So what happens as this tied protein goes down the column?

All right. So you have some column with your resin plus His6 protein.

So was it a strong or weak interaction?

AUDIENCE: Strong.

AUDIENCE: Strong.

ELIZABETH NOLAN: How would you define strong? Or why do you say it's strong?

AUDIENCE: The chelation.

ELIZABETH NOLAN: Well, you're forming a complex, right? You're forming-- the His-tag is binding the nickel NTA.

AUDIENCE: The Kd is probably [INAUDIBLE].

ELIZABETH NOLAN: Is it?

AUDIENCE: I don't know.

AUDIENCE: It is a dynamic process where they're releasing and binding and releasing and binding again.

ELIZABETH NOLAN: Yes. So there's an equilibrium, right? And we talk about binding to the column but how tightly is this tagged protein binding? And is it just binding there and getting stuck? I mean, it needs to stay in your column, right? In this case, it's not very strong. So if you look at reported Kd's for, say, His-tags to nickel NTA, they're on the order of one to 10 micromolar. So orders of magnitude lower affinity than what you just suggested. So why does the column work?

AUDIENCE: Because everything else binds worse than that.

ELIZABETH NOLAN: Well, you hope that. You hope. I mean, sure. I mean, if you know that histamine-- a protein that's histamine-rich it's going to stick, but why does it work? So is it surprising that a micromolar affinity can allow this to be trapped on the column?

AUDIENCE: Is it because you have six histamines tied down [INAUDIBLE]

ELIZABETH NOLAN: Pardon? OK. It's not the amount of histamines. What is in your column?

AUDIENCE: You've got a lot of binding sites.

ELIZABETH NOLAN: Yeah. Right. There's a lot of binding sites in the column. So you're going to have, as Rebecca said, dynamic. This is coming on and off the column, but there's a lot of binding sites. So if it comes off, it can go back on there.

So what about the GST and GSH?

AUDIENCE: I know it's one of the strongest attractions.

ELIZABETH NOLAN: Yeah. This is much stronger than nickel NTA and the His-tag protein. So orders of magnitude higher affinity here for that. OK. But it's something to think about when you're choosing an affinity purification method there and to think about what's actually happening on this column and dynamic process.

So jumping ahead a little bit, they did their experiments first just tagging the wild-type ribosome, OK? And that's very important because they're trying to make a new purification method, and the first thing that needs to be asked is does the wild-type ribosome plus the tag behave the same or differently from the wild-type ribosome without the tag, OK? And you want to know that because if the tag is causing a problem, maybe it's not a good design. And you don't want to go forward making mutants with that kind of modification, OK?

So what do they need to do after doing this purification, right? One is just analyzing the purity of the material that they've come up with. And then the other things they did was look at the subunit integrity, right? And so something to keep in mind is that the ribosome has two subunits. It has many ribosomal proteins. Does putting the tag on only one subunit work well?

And then, of course, they need to think about the activity. And so they presented a number of different assays in this paper ranging from looking at kinetics of peptide bond formation to looking at kinetic studies of release with one of the release factors here.

So let's think about the purity analysis. And what we're going to focus on is there chromatogram, right? So as we know, they took their column of GSH. They first loaded that column with the GST/MS2 fusion protein, and then they added their crude sample and eluted with GSH, right? And so the data they present for the chromatogram for monitoring fractions of that column is shown here, OK?

So what does this tell us? What do we see in this chromatogram? So what are they monitoring? So first you want to read your axes, right? So what are they monitoring.

AUDIENCE: A260.

ELIZABETH NOLAN: Yes, that's the y-axis. And why A260? Going back to 20 minutes ago.

AUDIENCE: DNA.

ELIZABETH Nucleotides, right? Do we want to monitor DNA here? It will work for DNA.

NOLAN:

AUDIENCE: Oh, RNA

ELIZABETH NOLAN: Yeah. So we have the 23S, 16S rRNA. So we're looking at A260, which makes sense. We're trying to purify the ribosome.

Volume, what is this volume?

AUDIENCE: The elution volume.

ELIZABETH NOLAN: Right. The volume eluted from the column. So looking at this trace, what do we see?

AUDIENCE: There's a peak [INAUDIBLE] maybe there's some [INAUDIBLE] introduced later.

ELIZABETH NOLAN: OK. So you've mixed what you see with an interpretation. So let's just stick right now to what do we see in the trace? And then we're going to think about where these things come from. And that's just something important, I think, with the problems we give in this course.

First, you want to ask just what does the data say? And then, how do we interpret this data based on our knowledge of a system here, OK? So do you see what I mean, how you mixed what you see here and a potential interpretation? So just what do we see?

AUDIENCE: Two peaks.

ELIZABETH NOLAN: So there's-- Yeah. Rebecca?

AUDIENCE: Just the large broad peak that results immediately, and then a smaller separate peak.

ELIZABETH NOLAN: So that's a very nice description. We see a broad peak with high A260 absorbance, then it elutes immediately. And then later, there's a peak just after 30 mils that's sharper, right?

So what are these peaks? What came off here?

AUDIENCE: Everything else.

ELIZABETH NOLAN: Yeah. So what's everything else?

AUDIENCE: DNA, [INAUDIBLE].

ELIZABETH NOLAN: Yeah. So things that didn't stick to the column, right? They got washed out. So maybe the native ribosomes, right? Maybe there's DNA in there, tRNAs. Could there be EFTU with tRNA bound, right? And there are things we're not seeing, right?

So what do we think about the second peak?

AUDIENCE: It's much less broad. It's pretty sharp, so that will tell you that it's likely only one thing that has bound to the column a lot. So that would probably be your His-tag.

ELIZABETH NOLAN: Is this a His-tag here? I know we're going back and forth because you're all more familiar with His-tags.

AUDIENCE: Your affinity tag.

ELIZABETH NOLAN: Right. So this is likely what was tagged, right? And with GSH elution, we disrupted the binding interaction with GST, and it came off the column.

Do we know that it's only one thing? No. At this stage, we don't, right? So what are possibilities? What could this be? So we have the tag on the 50S. Because the only-- I mean, it's coming off here just based on the amount of GSH required to push it off the column there.

AUDIENCE: It could be a mixture of intact ribosome, which is the cell that gets tagged.

ELIZABETH NOLAN: Right. Yeah. So we don't know right now in terms of the whole composition of this peak, right? It could be intact 70S because 30S came down. It could be 50S alone. And there's always the possibility of some other contaminant that just for whatever reason came off the column then there.

OK. Would you be excited by this chromatogram if you were the person at the bench doing this work? Yeah. You'd be super excited, right? So it looks quite good.

So of course, there needs to be some more analyses done. And one analysis we're not going to go into in detail but they needed to ask, is this really all tagged ribosome or is there also some contamination of wild-type? And so they designed some analysis using a technique called primary extension to look at that there. And what they saw is that they primarily had the tagged ribosome, which was good news.

So getting at the question in terms of what's actually in this peak, they looked at effectively, say, subunit integrity. And how did they do this? They used centrifugation. And does anyone recall what type of centrifugation they used?

AUDIENCE: Sucrose density gradient.

ELIZABETH NOLAN: Yes. So they used a sucrose gradient, right? And how does that let you do separation?

AUDIENCE: By density.

ELIZABETH NOLAN: Right, by density. And we have the different subunits of different sizes, different density there, right? So this is the data they show. And so, again, we want to look at these data and ask what do we see and what does that tell us, right?

So these are the results from the sucrose gradient centrifugation. And they looked at untied wild-type ribosomes, so isolated 70S and then the tied ribosome.

So what was the key part of the sample preparation here? How did they prepare these samples to be able to look at the subunits individually? Because the question they're getting at is, what is the ratio of the 50S to the 30S in the sample that was purified from the column?

AUDIENCE: They dialyzed using magnesium buffer.

ELIZABETH NOLAN: Yeah. So what was it about the magnesium in the buffer?

AUDIENCE: It causes disassociation between the 30S and the 50S.

ELIZABETH NOLAN: So why? Maybe you said it, and I didn't hear you. What was it about the buffer that allowed this?

AUDIENCE: Is it the positive charge?

ELIZABETH NOLAN: Well, it is the magnesium, but was it low or high magnesium buffer?

AUDIENCE: Oh, low.

ELIZABETH Low, Right? So we learned that magnesium is important for interactions between these

NOLAN: subunits, right? And you've seen some of the experimental details in the paper for recitation two and three, they used different concentrations of magnesium. In this case, they want to separate the two subunits. So they basically used a low magnesium buffer to allow them to dissociate.

So in these data, what are we looking at? The axes aren't shown, but what are they?

AUDIENCE: I have a question. Why not just a no-magnesium buffer?

ELIZABETH NOLAN: Could that be bad for the sample? How low is the magnesium in the buffer?

AUDIENCE: One millimolar.

ELIZABETH NOLAN: One millimolar. So where else might the magnesium go? Is it only important for this interaction?

AUDIENCE: I mean I guess it's useful. It can be used in a lot of different places actually in the cell.

ELIZABETH NOLAN: OK. But we don't have the cell here. We have the purified ribosome.

AUDIENCE: Is it possible that it's holding together the actual conformation of the 50S and the 30S?

ELIZABETH NOLAN: Yeah, right. I mean, that may-- I actually don't know what would happen if this goes into a no-magnesium buffer, right? But I think there's about a dozen contacts where magnesium is used between the two subunits. And you can imagine there's plenty of interactions of magnesium or cations in other places of each subunit.

Joanne, do you happen to know what happens if the ribosome-- I think you get a big unfolded mess.

JOANNE STUBBE: It would be hard to get rid of the magnesium because it has key binding sites along the place where it comes off and goes back on.

ELIZABETH NOLAN: Yeah. So what are the axes? If we were to have them, what are we looking at?

AUDIENCE: I think the gradient.

ELIZABETH NOLAN: Yeah. Right. So we have basically, yeah, the percent sucrose, say, the gradient-- or the density, right? And how are we seeing these peaks?

AUDIENCE: We see two peaks and the upper graph we see the peaks are more similar in size. And then in the second part we see-- well, we see two peaks but one peak has a shift over.

ELIZABETH NOLAN: Right. So just backing up. That's all certainly the case. How are we detecting these peaks? What's the readout? So we have some sucrose gradient here. How do we see the peaks?

AUDIENCE: PUB.

ELIZABETH NOLAN: Well, what, more specifically?

AUDIENCE: Oh, A280.

ELIZABETH NOLAN: Yeah. So here, they're using the absorbance at 280, right? So a little different than the chromatogram, but that's OK, right? There's 280 absorbance here and maybe their instrument for this didn't allow 260.

So as we just heard, if we look at the untagged ribosome, we see that there's two peaks, and they're nicely labeled. It's nicely labeled with the 30S here in terms of percent sucrose in the gradient and 50S here, right?

And so we see kind of in our standard control, the peak for the 30S is smaller than the 50S, and this is where they're placed. And so what we want to do is compare this data to the data here for the tagged ribosome.

OK. So what are the two major observations, two major differences? Rebecca.

AUDIENCE: The second one's enriched for the proteasome, there's relatively higher concentration of 50S.

ELIZABETH NOLAN: OK and--

AUDIENCE: And it's [INAUDIBLE].

ELIZABETH NOLAN: Yes. So or another way to say that, maybe, it's depleted in the 30S, right? But I think you're coming across the same observation, right? So first we see there is a peak corresponding to the 30S and then there's this peak here which is shifted relative to what we saw for the 50S

and the untagged ribosome. And the peak heights or peak areas, however you want to analyze it, that ratio is very different than what we see up here. So why is this shifted and why is there more 50S?

AUDIENCE: Can I ask a question? So can you definitively say that it's enriched or depleted in 30S? I don't know a lot about biochemistry in general, but can you say that possibly the multiple histamine-- oh, it's not histamine.

AUDIENCE: Is it just tagged because of the shift. I mean, it seems like such a small derivation.

ELIZABETH NOLAN: OK. So what else is there in the sample? So we have that stem loop tag, but based on how they eluted this, what else is there?

AUDIENCE: The fusion.

ELIZABETH NOLAN: The fusion protein, right? So then the question is-- and this is just getting to the point of needing to look at all the details in terms of what's done in someone's experiment to try to figure out what's happening. Is the MS2 coat protein alone enough to cause a shift? So what else might that be?

AUDIENCE: Could it be the GST?

ELIZABETH NOLAN: Well, you know that the GST/MS2 is there because it was GSH that was used to elute that whole thing. So the ribosome and the GST/MS2 fusion. So I guess the question is, just thinking what is the size of the fusion protein? And then how does that compare to the ribosome? And what does that mean in terms of a shift to a different percent sucrose?

So just what are possibilities? We learned that the fusion protein had some problems, that it liked to aggregate. Is it possible that contributes? Is it possible that not all of the 70S was dissociated, right? So there is not a label for where the 70S would come here. These are just things to think about when looking at the data here and to look at their explanation.

So is this good news or bad news? And is there a solution? Yeah?

AUDIENCE: Sorry. I had a question. Since they're monitoring the A280, so the protein absorption, since the 50S would have the fusion protein associated with it still, wouldn't that affect the A280? Can we definitively say that it's actually depleted in the 30S or is maybe just the absorbance of the 50S higher for the tagged protein because of the fusion?

ELIZABETH NOLAN: Yeah. So that's a good point, right? The fusion protein is going to have some absorbance defined by its extinction coefficient. And then the question is, what is that in magnitude? And how does that compare to, say, the total extinction coefficient of the 50S, right? So there are a number of ribosomal proteins, 20 to 30, and we don't know how many of those got through this purification, but they're there.

So that's how I would go about analyzing that. I actually don't know what the relative absorbance of the fusion protein to the intact 50S ribosome is but that's a caveat to consider and could contribute.

So what do we think? What are you going to do for an assay?

If we were to set these ribosomes up, the wild type and the mutant one, and, let's say, do a peptide bond formation reaction, like what they presented in the paper as is, what would you expect? Are you going to see the same activity or less activity? Working under the model that we don't have enough 30S here to fully form 70S ribosomes.

AUDIENCE: You'd expect the 30S to act like the limiting agent in peptide bond formation where they come together. And you can only make as much ribosome as you have 30S. So you would get less peptide bond formation.

ELIZABETH NOLAN: Right. So you'd have fewer 70S assembled ribosomes that can do translation. Right. So what was their solution to this problem? Or if you don't remember, what would you do?

What happens if we don't have enough of a given component? We'll finish on this note.

AUDIENCE: You just add more of the 30S.

ELIZABETH NOLAN: Yeah, right? So you can imagine adding in more 30s there. We know how to purify wild-type ribosomes, can dissociate the subunits and imagine purifying 30S and adding that into the reaction to have the correct stochiometry there. So that's exactly what they did in their assays.

And so I encourage you to take a look at the assays they did for peptide bond formation and for peptide release. And in the posted notes, there's a schematic overview of everything that was done to get the components of their syringes. So these were quench flow experiments, like what you talked about in recitation in prior weeks and we talked about with EFP.

And there's also an example where they used puromycin, like what we saw with EFP, in the

experimental setup, so that antibiotic that causes change termination there. And so what they found is that these tagged ribosomes really work quite well in terms of the kinetic comparison there. And they moved on with this methodology to use it to purify new ribosomes for other studies. So it turned out to be a useful method for their lab, and I would argue, what would be a useful method for other labs, based on the information they presented in their paper there. OK? So you're off the hook, and I'll see you in recitation next week.