Dear Dr Bajaj,
Please find our answers to the editor and reviewer comments:

Editorial and production comments:
Changes to be made by the Author(s):
1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.
done
2. Please reword the title to make it concise.
We don't really see what you expect as our title is shorter than most titles of other JOVE videos in the same field; Ex:Residue-specific Incorporation of Noncanonical Amino Acids into Model Proteins Using an *Escherichia coli* Cell-free Transcription-translation System.
Ex2:Optical Control of a Neuronal Protein Using a Genetically Encoded Unnatural Amino Acid in Neurons
Also we think that shortening the title will make it loose meaning.
It has, however, been slightly modified in response to one of Reviewer's 3 critique
3. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents.
For example: Sutrovax, HiLoad 15/600 Superdex™, GE Healthcare etc.
Done
4. Please expand all abbreviation during the first-time use.
done
5. Please include the goal of the manuscript as well in the introduction section.
Added at the end of the introduction: "In the present manuscript we describe how to synthesize the propargyl-L-lysine, an UAA carrying an alkyne handle, how to incorporate it into a target protein during its translation in a bacteria and finally how to perform conjugation between the modified protein and a hapten carrying an azide function using click chemistry"
6. Please include a single line space between each step, substep and note in the protocol section.
done
7. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., "Do this," "Ensure that," etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as "could be," "should be," and "would be" throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a "Note." However, notes should be concise and used sparingly.
done
8. Please ensure that individual steps of the protocol should only contain 2-3 actions per step.
done
9. Please ensure you answer the "how" question, i.e., how is the step performed?
done
10. Step 2.1: Please briefly describe how the plasmid preparation is done or include citations for both
details were added for the pET24d-mPsaAK32TAG-ENLYFQ-HHHHHH plasmid and a note already indicates "The detailed plasmids information is described in Supplementary MATERIALS." There is no reference available for this plasmid but a reference was already included in the text for pEVOL.
11. To maintain consistency, please use the same terminology throughout. The title suggests combined unnatural amino acid incorporation and click chemistry. However, in the entire manuscript especially protocol/results these terms are not used. Please bring out clarity.
done
12. Please ensure that the protocol fits the 10-page limit including all the headings and spacings.
done

Video:

1. Please remove the university logo on the upper right side from the video.
As the video was made by the video service of University of Nantes on its sole funding it is part of the university charter to display its logo on the video.
2. Please use gloves for performing all the steps involving bacterial culture.
In France the health and safety rules do not include wearing gloves for manipulation of level 1 microorganism. That is why we didn't sear them during the shooting of the bacterial culture steps.
3. Please include the title card both in the beginning and in the end.
done
4. Please increase the homogeneity between the written protocol and the narration in the video. It would be best if the narration is a word for word from the written protocol text.
5. Please ensure that the subsections' titles are the same in both the video and the text.
Done, except for part IV which is a unique section in the video when it corresponds to two sections in the text, because the procedure is really similar for paragraphs 4 and 5 in the text and it would be redundant to show it twice on the video.
6. Please include all figures both in the text and the video.
done
7. Please ensure all the result figures are shown in the video as well.
done

Production comment:
• Please include a single space between Glycoconjugate and Vaccine in the title cards.
done
• Consider leaving the title cards up a little longer- they are hard to read without pausing the video.
The video service could not grant us enough time on our video to redo the whole sound mixing
Check the center alignment on the titles as well- they may be a little offset improperly.
We have checked the alignment of the titles and they are already centered.
• There is interlacing (line "combing") in the protocol footage, editing in a progressive frame mode or deinterlacing footage beforehand will reduce/eliminate these video artifacts.
done
• I'm not sure if the presenter is speaking English around 0:26. It sounds like she switches in mid-sentence. Spoken discussion and VO should be in English unless an exception has been specifically made with JoVE.
We confirm that the presenter is speaking English at 0:26. She is saying "codon suppression method" which is quite difficult to pronounce for French speakers.
• Please remove the Organization's watermark in the upper right of the frame.
same as editor's #1 comment on video

Reviewer #1 :
Major Concerns:
1. Please explain whether the K32Prk mutation affects the stability of proteins.
Added In the result part "The stability of the mPsaA^{K32PrK} thus obtained was assessed by circular dichroism which showed that the structure of the protein was not affected by the mutation of the Lysine 32 into a propargyl-lysine."

2. If the protein is coupled with azido-fluorescein, is there any result that confirms the conjugation? for example, analyse the conjugation efficacy by a fluorescence spectroscopy or scanner.

The conjugation of the protein with the azido-fluorescein is already presented in figure 6A. We have added precisions in the text of the results: “mPsaA WT were then conjugated to the fluoroprobe (**Figure 6A**)”

Minor Concerns:
Please use some figures with high resolution

Reviewer #2:

MAJOR/MINOR CONCERNS

(1) GENERAL. The authors emphasise the generation of "homogeneous" products as one of the major advantages of their protocol. This is based on the general concept and the bioorthogonality of the two reaction partners (propargyl lysine and azide). However, they have not actually experimentally demonstrated the "homogeneity" of their product. This should be made clearer in the manuscript.
The orthogonality of the click chemistry has been demonstrated a long time ago and researchers who use it usually do not perform supplementary experiment to prove its specificity. We have however verified that 1) our mutation was at the correct position by gene sequencing, 2) the protein was modified with the PrK UAA and then the tetrasaccharide was conjugated by mass spectrometry.

(2) MANUSCRIPT

- P7: "Pn14TS-N3" - The abbreviation of the tetrasaccharide needs to be explained, and its source and/or a reference for its synthesis included.
The abbreviation is further explained page 8 by addition of “a tretrasaccharide mimicking the *Streptococcus pneumoniae* serotype 14 capsular polysaccharide”.
The reference 21 was already included in the text to specify the source of the Pn14TS-N3

- P7: "Any carbohydrate antigen containing an azide function can be used." - While this is true in theory, in practice, it is almost certainly not. Cu-catalysed cycloaddition reactions can be notoriously capricious, depending on the exact structure of the substrate. Please rephrase this sentence and/or add a note of caution.
Added:” Theoretically, any carbohydrate antigen containing an azide function can be used”

(3) VIDEO:

- The introduction would be easier to follow if it was illustrated with relevant schematics (e.g. Fig 1 from the manuscript), and not only show the researcher.
added figure 1 at the beginning of the video

- Synthesis: include the names of compounds that are used in the commentary, on the slides (0:56 ff)
- Molecular biology/protein biochemistry: Show the names and quantities of key reagents e.g., in speech bubbles (e.g., at 4:56 min: E coli BL-21 DE3, 100uL)
We thought about adding names and quantities of the reagents used when we prepared the video in the first place but we decided not to because given the number of different reagents used we thought it will fill the image and it might be annoying for the audience to see bubbles appear and disappear all the time. We thought it was the role of the written protocol to give information on the reagent and quantities and that the video is here to show how the solutions obtained after each step should look like and how to perform the gestures. When possible we have tried to write legibly on the tubes and glassware the names of the reagents.

I am also wondering if it might be useful to briefly explain some fundamental operations, e.g., the need

for sterilisation of equipment for the molecular biology/protein expression part, the measurement of protein concentration by UV, or the correct use of a rotary evaporator. Because of the cross-disciplinary nature of the experiment (see comments above), the video will be particularly valuable to scientists who have expertise in e.g., chemistry, but may not be familiar with even basic techniques in molecular biology/protein biochemistry. Alternatively, a slide with relevant links to relevant resources could be included at the end.
There is no need to sterilize equipment for the protein purification.
We think the operations described here are really basic and information can be found easily elsewhere. Also the video will be much longer if we have to explain how to use each instrument and it won't add value to the video as these instruments are not particularly unusual.

Reviewer #3:

Major Concerns:
1. The introduction should be ended with aim of the project/study mentioning the candidate used as representative.
Done as answered to editorial comment #5
2. Results section has a bit of discussion also which may be carefully avoided as the results and discussion sections are written separately in the manuscript.
removed from results section and explained in the discussion section:" However, no conclusion can be drawn since the PrK incorporation site is close to the N-terminus and any truncated form produced is probably degraded by the bacteria »
3. Protocol and Results section do not mention tests like polysaccharide(oligosaccharide) to protein ratio, conjugation yields in terms of protein and oligosaccharide used in beginning versus that received in the purified glycoconjugate. These two are important parameters in characterizing glyconjugates
Added in the text page10: "The conjugation by click chemistry being quantitative the majority of the mPsaA^{K32PrK} was conjugated with the Pn14TS-N_3 as illustrated by the mass spectrometry results (**Figure 6C**)."

4. The discussion section need further elaboration on following points: Next steps e.g. immunogenicity studies; discussion about impact of very high protein to oligosaccharide ratio in the conjugates so obtained in comparison to conventionally obtained conjugates; the conjugation yields in comparison to conventional conjugates and hence cost implications; finally discuss scope to optimize the Protein to Polysaccharide ratio by incorporation of more than one UAAs. The later one seems to be touched upon in abstract but missing in the discussion section.
added at the end of discussion section: The glycoconjugate obtained with the technique described in the present work can then be used to immunize mice. Having such fully-defined and easily modulated glycoconjugate in hands provides unvaluable tools to evaluate the impact of the hapten/protein carrier connectivity on the immune response.[8] Since increasing the hapten/protein ratio is often correlated with enhanced anti-hapten humoral reponse when using short haptens,[30] one might be interested in testing conjugates with multiple haptens. The incorporation of multiple UAAs however needs some adjustments of the protocol as the incorporation of an UAA in the protein tends to decrease the yield of protein production due to the RF1 activity.

Minor Concerns:
1. References need to have same format which is quite mixed in the current format
done
2. The word 'vaccine' may be removed from the title which generally is indicative of the formulated glycoconjugate

The title was reformulated to : “Homogeneous Glycoconjugate Produced by Combined Unnatural Amino Acid Incorporation and Click-Chemistry for vaccine purpose »

3. Not sure of the reason but the title and abstract have some typo differences in very first page and second page of the PDF file
Done, correction of homogenous to homogeneous if this is what the reviewer meant
4. Line 40-41: drug regulatory agencies done; Replace "classical bioconjugation strategies' with 'classical conjugation strategies' done
5. Line 46: ..and efficient in a broad age group including young infants. done
6. Line 47: ..provide the optimal defense.. done
7. Line 53: Apart from a... done
8. Line 56: ..linker, if used... done
9. Line 221-222: share range of the temperature and time which may be required done
10: Line 245: Recheck if the title is really aligned (accessibility and functionality) with the text in section 4, if not, please align
The title is aligned with the content of the section 4 as the point of this procedure is to conjugate by click chemistry a fluoroprobe carrying an azide on the protein mutated with PrK and visualize the conjugate by fluorescence which shows that the PrK was accessible and functional to be conjugated
11. Line 254-255: Mention if 20 and 50 mM are working or final concentrations
Done, it is now specified in the text that it is initial concentrations
12: Line 313-314: It would be useful to provide the amino acid sequence of PsaA highlighing the K32 where modification is done, this could be clubbed in figure 5we don’t see which information necessary to understand the results this new figure would add
13: Fig 5: Cu+ or CuI, Please check
We confirm it is Cu^{I}
14. Fig 6(B): Worth mentioning the differences in apparent variation in mol. wt. of bands in lane 2 vs3,4 and 5 vs 6,7
Added at the end of the results section: “The small increase in the molecular weight of the sample between lane 6 and 7 (**Figure 6B**) indicates a successful conjugation with the tetrasaccharide Pn14TS.”

General comment:
Share the appropriate composition of various buffers unless it is very commonly known in scientific fraternity
Phosphate buffers, click chemistry buffer and TEV buffer are already detailed in the text, we don’t see which other buffer needs to be more detailed.
Use full form of uncommon abbreviations, when used first in abstract and main manuscript
done

Dear Dr Nguyen,

Please find the modifications made regarding the video as an answer to editorial comments:

Changes to be made by the Author(s) regarding the video:

1. If feasible, please convert this editing project settings to Progressive Scan and try to reduce/remove the "combing" effect seen throughout. We are publishing on a website, which does not benefit from interlaced footage.

The previous version of the video was already set to Progressive scan as is the version now resubmitted. We have checked with the service who did the video and they have already changed for a format compatible with a website.

2. The narration sounds good except for the background noise. Please attempt to run noise reduction on the narration audio track.

Background noise has been removed

3. What is the speaker saying at 00:25-00:28? This is not clear. Please re-record this for clarity.
The speaker is saying: “codon suppression method”. As it is not possible for us to record again the speaker we have added a title to the figure (i.e. “codon suppression method”) which appears in the video as she is saying these words. We hope that it will help understand what she is saying.

4. The music in the protocol can be removed.

The music is removed