Novel approach for immunoassay capture antibody immobilization – Click chemistry with expanded genetic code

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Abstract

Antibodies are commonly used in biosensors for diagnostics and research due to their ability to specifically bind certain molecules. However, current manufacturing techniques leave biosensors to use randomly oriented capture antibodies. This results in decreased antibody function as some antibodies denature, capture sites are blocked or antibodies wear off. Site specific immobilization has been studied before, but not with expanded genetic code.

In this study E.coli C.321.deltaA.exp -strain was used to produce fragmented antibodies (Fab) with non-canonical p-azido-l-phenylalanine amino acid incorporated in protein. These fabs were immobilized covalently in DBCO magnetic beads via bio-orthogonal azide-alkyne cycloaddition reaction. Coated beads were compared to passively and randomly coated beads by saturating them with labeled antigen.

Results show that Fabs can be modified by adding p-azido-l-phenylalanine into C-terminal end of protein chain without any compromises to antibody functionality or stability.

Introduction

Antibodies are well known for their ability to specifically bind to different molecules. This ability is used widely in in-vitro-diagnostics (IVD) as immunoassays. Immunoassays are commonly used tools to detect different molecules in solutions and most common uses are to help diagnose and manage diseases and conditions as well as detect toxins in the environment. One of the most recognizable examples of such tool is the immunologic pregnancy test available for consumer use throughout the world. Its function is based on antibodies which detect human chorionic gonadotropin, a hormone produced in high amount by the placenta during the first weeks of pregnancy. (1)

In an immunoassay, capture antibodies are immobilized on to the surface of a biodetector. As a sample (e.g. blood or urine) is introduced to the biosensor the capture antibodies attach to the analyte binding it to the surface. Everything that did not bind can be washed away and a labeled antibody is introduced to the biosensor. This antibody with a label attached to it binds to the same analyte allowing a visual detection of the analyte. Usually the label is a fluorescent molecule, enzyme or radioisotope and it can be detected with various methods. (1) However, most of the currently used techniques to immobilize the capture antibodies are not site specific and they rely on weak hydrophobic or polar interactions. This results to decreased antibody function as some of the antibodies denature, capture sites are blocked, or antibodies wears off in the later stages of assay. (2)

Ideally all the antibodies would bind covalently the detector surface with similar orientation that preserves the antibodies' function. This increases the biosensor capacity to bind molecules and has a potential to increase the sensitivity of the assay. Some studies show that uniform antibody orientation increases the test signal as high as 200-fold compared to a test utilizing randomly oriented capture antibodies. (3)

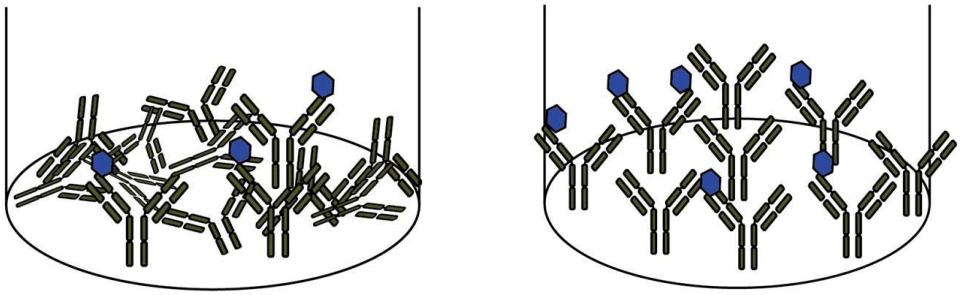

Figure 1. *On the left is a conventional test surface where some of the antibodies are unable to bind analyte due to denaturation or blocked binding site. On the right is an ideal test surface where all the antibodies are functional and uniformly oriented.*

Expanded genetic code is a technique where one of organism's codons have been reprogrammed to code for a non-canonical amino acid (ncAA) (4). This lets us to introduce features in proteins that would not otherwise be possible or very difficult. One of the possible features is to attach a molecule needed for bio-orthogonal binding reaction in the tail-end of an antibody. Bio-orthogonal reactions are specific reactions that do not occur in any living systems and happen only when both molecules are present in reaction. (5, 6)

The aim of this study is to modify Anti-Digoxigenin and Anti-Dengue NS1 fragmented antibodies (fab) by introducing p-azido-L-phenylalanine in their protein sequence with expanded genetic code and to immobilize Fabs with strain promoted azide-alkyne cycloaddition (SPAAC) into Dibenzocyclooctyne (DBCO) functionalized surface and to compare the surface binding function to conventional non-site-specific bound surface.

Materials and methods

Genes, Plasmids, E.coli strains

Harmonized Anti-Digoxigenin fab (Digox-Fab0) gene sequence (7) and Anti-Dengue fab (Dengue-Fab0) sequence (Supplementary document) were received from University of Turku. All gene variants were ordered from Integrated DNA Technologies Inc, USA.

All protein expressions were done with pLK04 plasmid vector (8). Cloning was done with E.coli C321.deltaA.exp (Addgene #49018) (9), normal protein production was done with E.coli Xl-1 Blue, and production with expanded genetic code was done with E.coli C.321.deltaA.exp transformed with pEVOL-pAzF plasmid (Addgene plasmid # 31186) (10) containing $tRNA_{CUA}$ and aminoacyl-tRNA synthetase for p-azido-l-phenylalanine.

Antibody sequence design

Received sequences were used to search for 3D models of humanized fab from NCBI Blast Protein database. 5I17_A was used as a light chain model and 2VXS_H was used as a heavy chain model. 3D models were searched for constant domain amino acids that had solvent accessibility value greater than 0.3 and large side chain. For each gene variant ordered, one codon was changed to TAG-codon and a tail of six histidine codons were added to the end of heavy chain. (Supplementary document)

In total there were six variants of anti-digoxigenin fab genes ordered: Digox-Fab0, Digox-FabL108, Digox-FabL191, Digox-FabL216, Digox-FabH243 and Digox-FabH249. Also, four variants of anti-dengue fab genes ordered, were: Dengue Fab0, Dengue-FabL153, Dengue-FabH196 and Dengue-FabH221.

Naming of the variant follows guidance as follows: Fab binding target – "Fab" Heavy (H) or Light (L) chain modification and number of the modified codon.

Fab expression vector production and cloning

Each ordered gene variant and pLK04 vector were dual digested with SfiI and HindIII FastDigest enzymes (ThermoFisher) according to manufacturer's instructions. Reaction mixes without other digestion enzyme were used as controls. Products were separated with 1% agarose gel (Tris-Borate-EDTA buffer (Medicargo AB), 1 % (w/v) agarose (ThermoFisher), Midori green direct (NIPPON Genetics) stain (0.5 µL per well) and FastGene 1kp DNA Ladder (NIPPON Genetics) as a size marker) with 70 V voltage until visible dyes were in the bottom fourth of the gel.

Correct bands according to expected sizes were cut from gel and purified with Macherey-Nagel NucleoSpin Gel and PCR Clean-up kit with manufacturers guide.

Purified products were ligated with T4 DNA ligase (ThermoFisher) according to manufacturer's guide with 1:3 molar ratio of vector backbone (pLK04) and insert (ordered gene). Two ligation controls with either insert or vector backbone missing were also used. 1 µL of ligated DNA was transformed into 25 µL electrocompetent (11) C321.deltaA.exp cells. Bio-Rad GenePulser was used with 1 mm chilled cuvette and 1.8 kV voltage setting. After electroporation cells were moved into 37 °C LB medium (ThermoFisher) and were incubated in 37 °C 250 rpm for 45 min before plating into LA ampicillin plate (1.5 % (w/v) agarose diluted in LB medium with 100 µg/mL ampicillin).

With each successful transformation plates three colonies were inoculated in 5 mL of LB medium and was put into 37 °C 250 rpm o/n incubation. After the incubation, part of the growth was used to make glycerol preparations and from the rest plasmids were extracted from the cells with Macherey-Nagel NucleopSpin EasyPure kit as instructed by manufacturer.

To confirm ligation success one plasmid extraction product originated from each transform LA plate were digested with FastDigest SfiI enzyme (ThermoFisher) according to manufacturer's guidance. Digestion products were separated in 1% agarose gel as before.

To confirm correct constructs, plasmids were sent with primers (Supplementary document) to Eurofins Scientific for sequencing. All samples that had frame sifting insertions or deletions were discarded.

Antibody production

Each of the remaining plasmid with correct fab gene inserted was transformed together with pEVOL-pAzF plasmid into C321.deltaA strain, except Digox-Fab0 that was transformed into XL-1 Blue strain, with same protocol as before. To control transformation, all transformations were done also either with fab gene plasmid or pEVOL-pAzF. After transformation cells were put into 5 ml of LB medium and incubated as before. After incubation, C321.deltaA.exp cells were put into LA plate with ampicillin (100 µg/mL) and chloramphenicol (25 µg/mL) and XL-1 Blue were put into LA plate with ampicillin (100 µg/mL) for o/n.

One colony from each plate was picked for o/n culture as previously. After incubation, 1 µl of cells were put into 20 ml SB medium (30 g/L Tryptone, 20 g/L Yeast extract, 10 g/L MOPS (3-[N-morpholino]-propanesulfonic acid), 100 µg/mL ampicillin and 25 µg/mL chloramphenicol) for preproduction culture and the rest was used for glycerol stock and were put into -80 °C.

Preproduction cultures were incubated for o/n in 37 °C 250 rpm shaking. After incubation precultures were diluted in 200 ml of SB medium for OD600 of 0.1. This production culture was incubated in 37 °C 300 rpm shaking until it reached OD600 of 0.8. Then tRNA synthetase production of pEVOL-pAzF plasmid was induced adding arabinose to a final concentration of 0.5 % and p-azido-l-phenylalanine diluted in 0.1 M NaOH to a final concentration of 1mM. 10 minutes after tRNA synthetase induction fab production was induced by adding Isopropyl β-D-1-thiogalactopyranoside (IPTG) to a final concentration of 0.5 mM. Cultures were incubated in 26 °C 250 rpm shaking for o/n.

After incubation, cells were centrifuged in 6000 g 4 °C for 15 min. Supernatant was removed and cells were washed by resuspending them into modified NPI-10 solution (50 mM NaH2PO4 sodium phosphate, 300 mM sodium chloride, 10 mM imidazole, pH 8) and centrifuged as previously. Cell pellets were freeze -20 °C for o/n.

Antibody purification and analysis

Before and after each purification step a sample was taken for fab yield assay.

Cell pellets were thaw and weighted. Pellets were resuspended in lysis buffer (50 mM NaH2PO4 sodium phosphate, 300 mM sodium chloride, 10 mM imidazole, 10 mM MgCl2, 25 U/mL benzonase, 0.4 mg/mL lysozyme, pH8) on ice. 5 ml of solution was used for every 1g of pellet. Cells were lysed by freezing in -20 °C and thawing in 40 °C water bath for three times. Then cells were centrifuged in 20 000 g for 45 min in 4 °C.

Fabs were purified with Macherey Nagel Protino Ni-NTA Agarose according to manufacturer's gravity-flow under native conditions protocol. Eluted fractions' protein concentration was measured with ThermoScientific NanoDrop ND-1000, zeroed with elute buffer. Fractions containing most proteins were pooled and buffer was exchanged to PBS (Medicargo AB) with CentriPure P10 (EMP Biotech Gmbh) desalting collums as instructed by manufacturer. All Ni-NTA purification was done in darkroom.

Ni-NTA purified Fab0 produced with XL-1 Blue was additionally purified with size exclusion chromatography (SEC). Sample was concentrated with 10 000 MWCO ultrafiltration spin column, 4000g, 3 min, +4 C. SEC was done with Superdex® 200 Increase 10/300 GL column according to manufarturers instruction with flow rate 0.2 ml/min and fraction size 0.5 ml. Fraction protein concentration was measured with NanoDrop ND-1000 (ThermoFisher) and fractions containing most proteins were pooled.

2-methyl-4-isothiazolin-3-one was added in every fab sample to a final concentration of 0.05 % as a preservative.

To confirm the success of purification, 2 µg of protein from each sample were run with SDS-PAGE (12), until the visible color stain from the sample buffer (62.5 mM Tris-HCl pH 6.8, 2.5 % SDS (Sodium dodecyl sulfate), 0.002 % Bromophenol Blue, 0.7135 M (5%) β-mercaptoethanol, 10 % glycerol) reached the bottom 1.5 cm of the gel. Also, one empty well was filled with random amount of Fab0 and sample buffer to fill the well.

To confirm the incorporation of p-azido-l-phenylalanine into amino acid chain, 1 µg of each fab variant were labeled with 13 pmol DBCO-Cy5.5 (Jena Bioscience GmbH) by incubating proteins mixed with label in 4 °C for o/n. Samples were run with SDS-PAGE as described previously and gel was visualized using Li-COR Odyssey at 700 nm bandwidth.

Fab yield and activity assay

Fab yield assay was done with 96-well plate coated with Goat Anti-Human IgG (manufactured in University of Turku) and Eu-2A11 mAb (HyTest Ltd). To make standard curve, samples containing 0 ng, 10 ng, 50 ng, 100 ng, 150 ng and 300 ng of standard fab (University of Turku) were diluted in Assay Buffer (Kaivogen Oy). Each sample from purifying process were diluted 1/10, 1/100 and 1/1000 in Assay Buffer.

Each sample had three parallel wells. Plated samples were incubated in slow shaking for 30 min in RT. After incubation, unbound sample was washed with Delfia Plate Wash (Wallac Oy) by using Wash Buffer (Kaivogen Oy)

Samples were labeled with 50 µg Eu-Labeled 2A11 antibodies (HyTest ltd.) and incubated in slow shaking for 30 min in RT. After incubation, wells were washed as previously, and Eu-labels were enhanced with 10 min RT incubation in 200 µl Europium Fluorescence Intensifier (EFI) -buffer (Kaivogen Oy).

Samples time-resolved fluorescence was measured with VICTOR multilabel reader (PerkinElmer). All results were handled with Microsoft Excel. Average signal and coefficient of variation was calculated from each parallel samples. Fab activity and yield were calculated from the standard curve.

Immobilizing antibodies to DBCO surface

To compare the site specific and random immobilization unmodified Fab0s were DBCO functionalized with NHS chemistry. This was done with NHS-PEG4-Azide (ThermoScientific) according to manufacturer's instructions and 40-fold molar excess of NHS-PEG4-Azide. Non-reacted NHS-PEG4-Azide was removed with NAP-10 desalting column (Sigma-Aldrich) according to manufacturer's instruction.

Due to highest reactivity with DBCO earlier in test labeling Fab H249 and DBCO functionalized Fab0 were chosen for comparing specific and random immobilization. Also, one control coating was done passively by using non-functionalized Fab0s. DBCO Magnetic Beads (Jena Bioscience GmbH) were mixed with 2 x molar excess of Fabs in PBS -buffer total volume of 500 µl. Mixture was incubated in RT for 1 h. Then beads were washed with 1 ml and 500 µl PBS, 2 x with 250 µl Kaivogen wash buffer and 2 x with PBS as before. Each wash step was done as follows: Magnetic holder was placed under the wash well to hold beads in place, supernatant was removed, and wash buffer was added in well, beads were then mixed into solution with shaker.

DBCO-Fab-Beads were suspended in PBS to a final concentration of 0.5 µg/ml according to original bead mass.

Bead and antibody characterization

To test antigen binding capability of coated beads an immunoassay was performed by using Cy5-labeled dsDNA-Digoxigenin (University of Turku) as an antigen and flow cytometry to read Cy5 -label signal. Reaction consisted of 3.6 million coated beads, 100nM Cy5-dsDNA-Digoxigenin in a final volume of 500 µl. Also, control reaction with uncoated DBCO-Magnetic beads and label was done.

Reactions were incubated for 1 h in orbital shaker. Beads were washed as previously with buffer containing 0.5 % Tween in PBS and beads were diluted in 500 µl PBS.

Labels were red with DB Acuri B6 flow ctometer with flow rate 14 µl/min, core size 10 µm and Cy5 settings. In total there were 100 000 counts per assay analyzed. Further data analysis was done with BD FlowJo -program.

Also, Fab0s' and FabH249s' thermal stability were compared with Prometheus NanoDSF (NanoTemper). Measures were done with 5 ng of Fab in PBS buffer according to devices instruction.

Results

Fab expression vector production and cloning

Plasmids that were transformed into 321.deltaA.exp cells tended to recombine into multimers due to this strain retaining RecA activity. This was seen from agarose gel electophoresis (Figure 2) as bands from Digox-FabL216 and Digox-FabH243 were significantly larger (>10 kbp) than they should have been (1.2 kbp & 4 kbp). Also, their sequencing with primers used, failed. This led to discarding Digox-FabL216 and Digox-FabH243 variants.

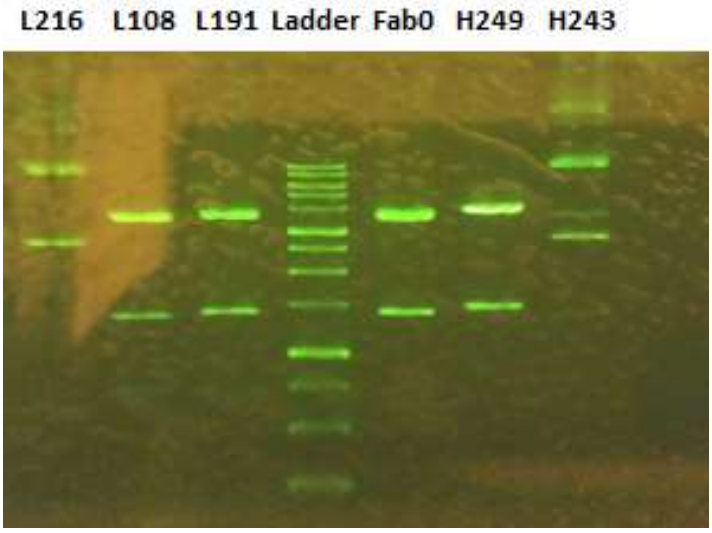

Figure 2. *Purified plasmids after transformation containing a different variant of anti-digox fab digested with SfiI and HindIII. L216 and H243 digestion products are larger than expected.*

Also, all cloning and production of the dengue fab failed or the sequencing results showed that inserted genes had insertions or deletions that resulted translation frame shift. Thus, ending the dengue part of the study. This happened probably due to using a gene that is not as well codon optimized as anti-digoxigenin Fab for the production organism. Instead of using Anti-DengueNS1 better known Fab gene should have used.

Digox-Fab0, Digox-FabL108, Digox-FabL191 and Digox-FabH249 genes were successfully inserted in pLK04 plasmid.

Antibody production, purification, and analysis

It was essential, that all Ni-NTA purification with protein that contain azido-group is done in a darkroom, as nickel is acting as a catalyst in a reaction between Azido-group and Aryl Electrophile. This reaction occurs under blue light. (10, 13).

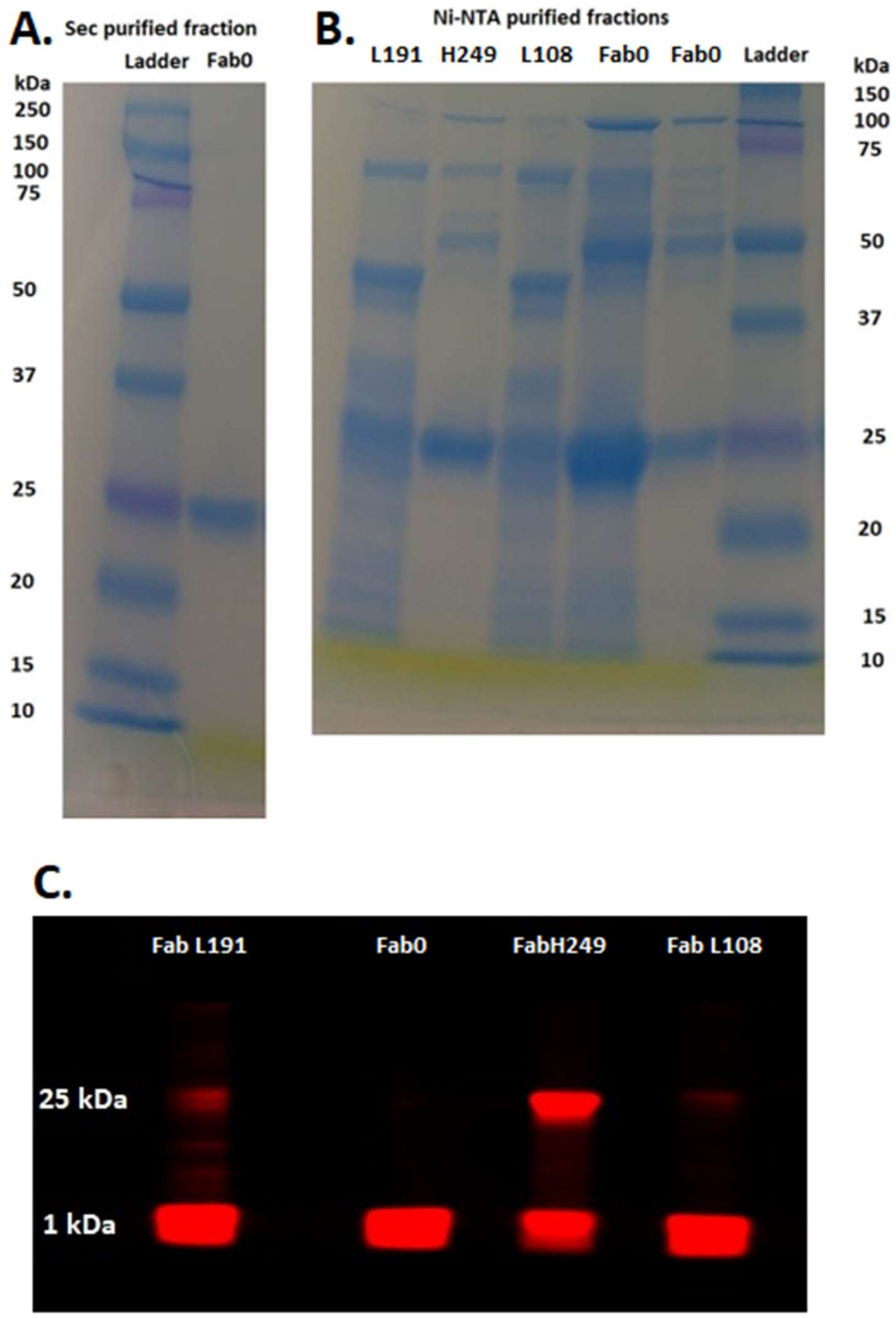

Figure 3. *Antibody purification and test labeling results. 50 kDa band represents complete Fab and 25 kDa bands represent light and heavy chains A) SEC purified Fab0 in SDS-PAGE. B) Ni-NTA purified FabL191, FabH249, FabL108 and Fab0. C) DBCO-Cy5.5 labeled fabs. Unbound label is seen in 1 kDa band and some unspecific binding is seen in all the samples, but most of the label has bound to modified fab and almost none is bound into unmodified Fab0*

Results from antibody purification are presented in Figure 2. SDS-PAGE separates proteins according to their molecular weight and separates dimeric proteins to monomeric (12). All modified and unmodified antibodies were produced and purified successfully. Bands in 50 kDa represent Fabs' heavy and light chains

and 25 kDa band represent heavy and light chains independently as β-mercaptoethanol separates them in gel.

Fab0 went through additional SEC purifying step (Figure 3 A), as its NH_2-sidegroups are DBCO functionalized with Azide-PEG_4-NHS and thus they were needed to be purer than azido modified fabs.

DBCO-Cy5.5 labeling was also successful (Figure 3 C). Some unspecific label binding is seen in all samples, but modified Fabs are all labeled – FabH249 being the strongest – while unmodified Fab0 has barely noticeable band in 25 kDa lane. This suggests that E.coli C.321.deltaA.exp had incorporated non-canonical p-azido-N-phenylalanine into the protein sequence of the fabs. However, DBCO-Cy5.5 label had also some unspecific binding. This has two plausible explanations: Cross reactivity with cysteine and hydrophobicity. DBCO-molecule has some low-level reactivity towards available cysteines which might explain additional labeling. Also, DBCO-molecule is quite hydrophobic, so it can bind to other proteins with hydrophobic interactions.

Fab yield and activity assay

Standard curve is presented in Figure 4. 150 ng and 300 ng standard fab datapoints were discarded as the curve saturated quickly after 100 ng datapoint. Due to these datapoints being removed the curve relies only on three datapoint making it not an ideal for reliable concentration determination. However, it was still considered for a usable tool to compare total antibody production and purification yields between Fab variants.

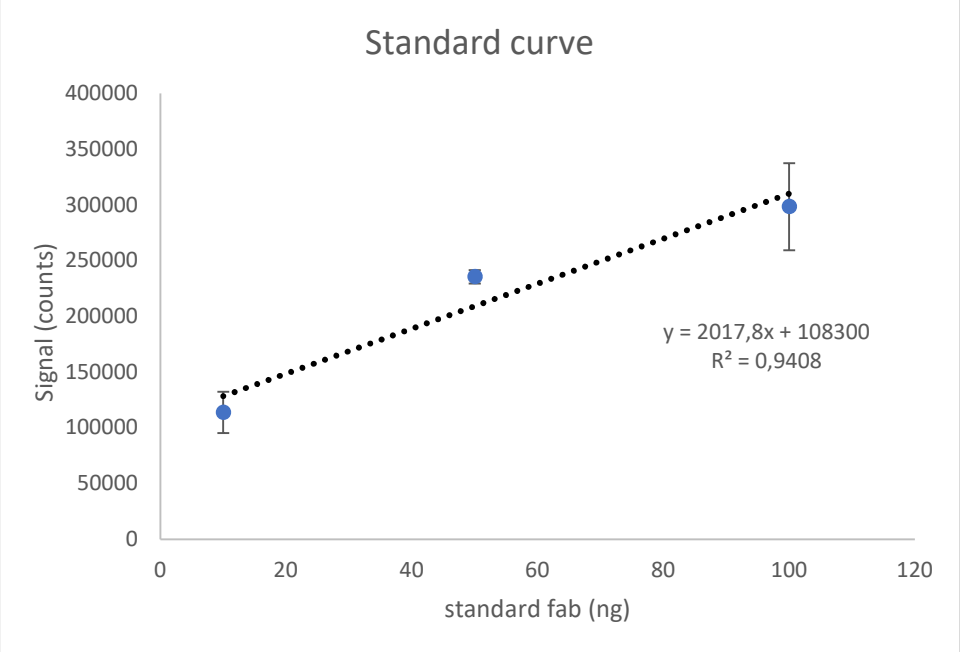

Figure 4. *Standard curve, R^2-value and its equation used calculate Fab activities and yield.*

Fab amounts per well and original sample concentrations are presented in table 1. Fab0 was produced with E.coli XL-1 Blue strain that is well known and optimized for cloning and expression, so it is not a surprise that it had the highest production yield. However, Azido-modified Fabs produced with C.321deltaA.exp had much better yield than was expected. Especially FabH249 proved to be produced almost as well as Fab0 in a well proven production strain.

Table 1. *Fab variant sample concentration as calculated from standard curve.*

Fragmented antibody	Amount per well (ng)	Sample concentration (ng/µl)
Fab L108	63	28,6
Fab L191	88	39,9
Fab H249	139	63,1
Fab 0	166	75,6

Results indicate that closer the C-terminal end the modification is , the higher the yield is. This might be explained with low affinity of p-azido-l-phenylalanine carrying $tRNA_{uag}$, ribosome stalling and function of tmRNA and ArfA. As p-azido-l-phenylalanine tRNA has lower affinity than its natural counterparts, it might cause ribosome stalling and activation of tmRNA resulting ribosome recycling and destruction of incomplete protein. However, when the modification is in near of the stop codon it is possible that ArfA has a higher chance to rescue the protein. (14)

All other samples did not contain any protein of interest, but this cannot be used to lead any conclusion about total purification yield as the volumes of purification step flow through and eluate was not recorded.

Bead and antibody characterization

The fabs in the beads binds to digoxigenin, so more Cy5 signal per bead means there are more fabs in bead. Also, as site-specific immobilization allows fab to attach on only one bead, they should be more uniform than their randomly immobilized counterparts.

Results from flow cytometry immunoassay are presented in Figure 5. When comparing size and shape of beads with DBCO-Fab0-beads and FabH249-beads, the latter are more uniform (Fig 5 A and B) but they still form aggregates. When comparing Cy5 signal levels in all measured counts, the assay with just magnetic beads gives lowest signal, assay with passively attached Fab0s gives the second lowest.

FabH249 coated beads gave higher signal than previously mentioned, but there also seems to be more variation in signal per count (Fig 5. A B). When aggregated beads are gated away and comparing the monomeric beads, they give highest average signal and has second lowest signal variation. (Fig 5 C)

Signal variation in DBCO functionalized Fab0 assay is quite more than expected. Signal peaks several times, first peak is at the signal level of the uncoated beads, second one is at the level of the passively coated and the highest peak is at the same level than site-specific coated beads. They have highest peak signal levels, but also the highest variation in signal levels. (Fig 5 A, B) When aggregated beads have gated away, the beads still give three peaks, highest being at the same level than FabH249 coated beads. (Fig 5 C) This three peaked histogram is likely due to beads left without antibodies and some beads being coated passively.

These results suggest that the beads with site-specifically immobilized capture antibodies do not aggregate as much as non-specifically immobilized counterparts. This is probably due to antibodies were not able to crosslink multiple beads at once due to their univalent reactivity. They also gave significantly higher signal than bare or passively coated beads, indicating that antibodies are well functional. Also, non-specifically coated antibodies had more likely to leave beads empty or coat passively with hydrophobic interactions. This is probably due to NHS-chemistry failing to BDCO-functionalize the Fab0s.

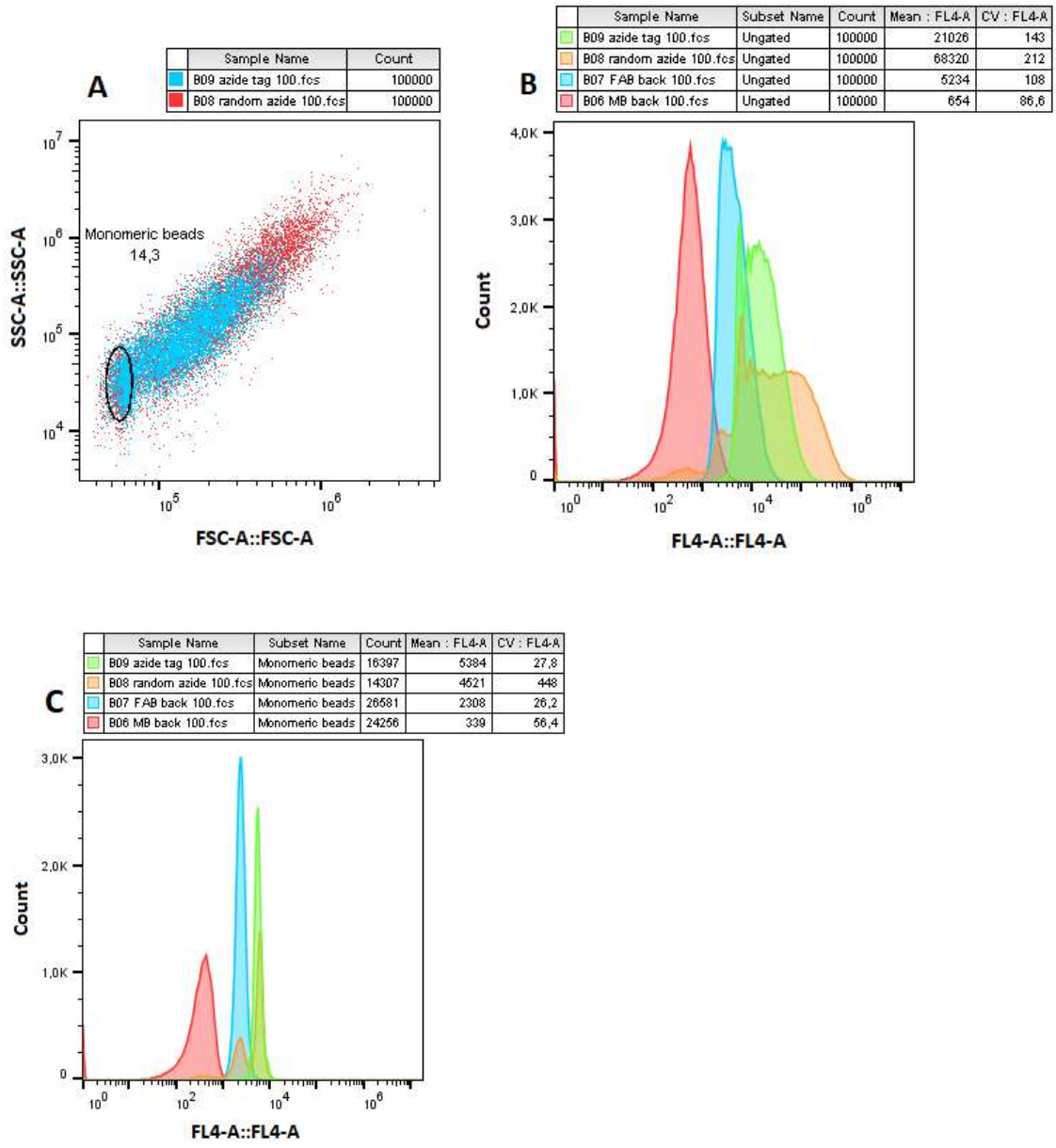

Figure 5. *Flow cytometry immunoassay results. A) Forward scatter (FSC) represents the size and Side scatter (SSC) represents the shape of the count. Each count is marked with Blue (Fab H249 coated bead) or Red (DBCO functionalized Fab 0 coated bead) dot. Black circle represents the gated counts presented in part C. B) Histogram of fluorescence intensity of Cy5 at 700 nm (X-axis) and amount of counts (Y-axis). Red area represents uncoated Beads, Blue area represents passively Fab0 coated Beads, Orange represents DBCO functionalized Fab0 coated beads and Green area represents FabH249 coated beads. C) Histogram of monomeric gated bead fluorescence intensity of Cy5 at 700 nm (X-axis) and amount of counts (Y-axis). Colors are as in part B.*

Results from thermal stability measurements are presented on Figure 6. The FabH249 and Fab0 both melt around 65 °C, so minimal if any changes in stability is observed. However, after melting FabH249 goes through reaction that does not occur in Fab0. This reaction is probably due to UV-spectrum light used to measure fluorescence initiates the photocrosslinking reaction (15) as the tertiary structure of protein unfolds. However, this reaction's significance to intended use of antibody is minimal.

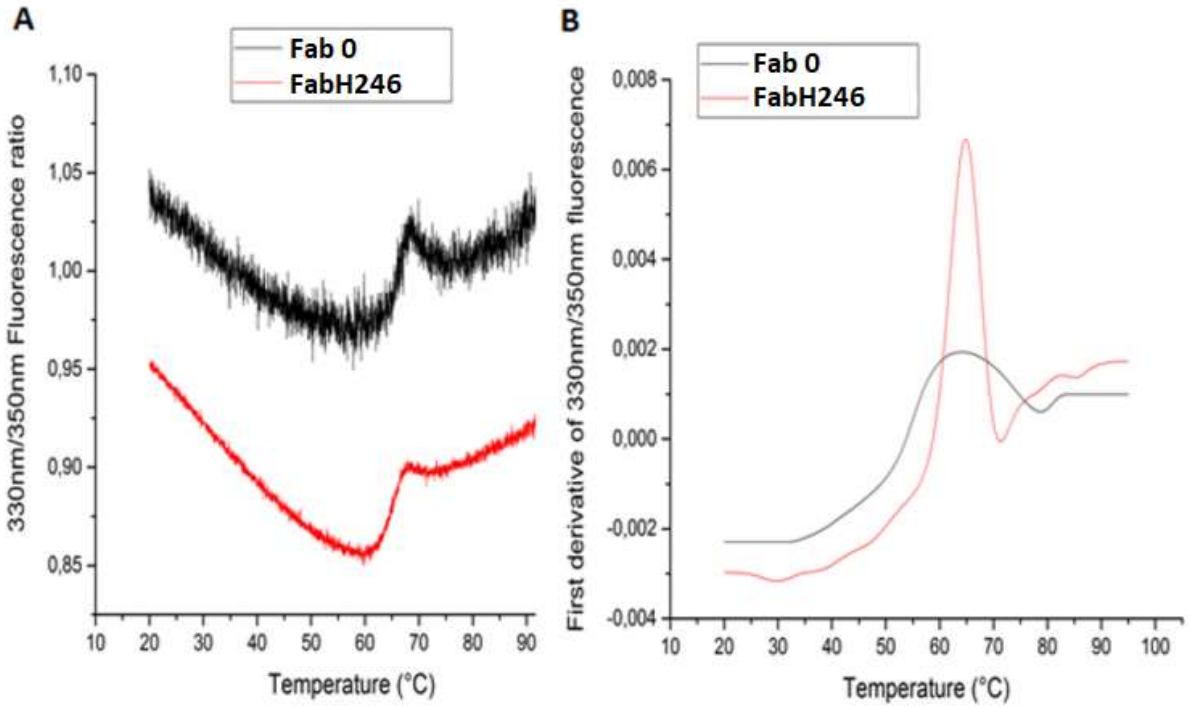

Figure 6. *Thermal stability measurement results. A) Melting temperature of both Fabs sets around 65 °C. B) FabH249 temperature/fluorescence curve is making noticeable wave right after 60 °C is passed, same phenomenon does not occur in Fab0.*

Discussion

All-in-all this study can be considered as a success. Results suggest that fragmented antibodies can be modified with non-canonical p-azido-l-phenylalanine Amino Acid and produce them with E.coli C.321deltaA.exp strain. It also seems that the best place to add new reactive azido group is the C-terminal tail end of the Heavy chain. However, this study does not proof any performance benefits for site-specific immobilized capture antibodies over normal assay with passively or biotin immobilized capture antibodies, but further research is needed. Especially the performance in capture antibody coated well based ELISA or DELFIA platform would be interesting target of research which we could not address due to the unexpected lack of labeled antigens and unavailability of DBCO-functionalized microtiter wells.

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References

1. Wheeler MJ. Immunoassay Techniques. In: Hormone Assays in Biological Fluids. Humana Press; 2006. p. 1-21.

2. Welch NG, Scoble JA, Muir BW, Pigram PJ. Orientation and characterization of immobilized antibodies for improved immunoassays (Review). Biointerphases. 2017;12(2):02D301.

3. Trilling AK, Harmsen MM, Ruigrok VJB, Zuilhof H, Beekwilder J. The effect of uniform capture molecule orientation on biosensor sensitivity: Dependence on analyte properties. Biosensors & bioelectronics. 2013;40(1):219-26.

4. Chin JW. Expanding and reprogramming the genetic code. Nature (London). 2017;550(7674):53-60.

5. Raliski BK, Howard CA, Young DD. Site-Specific Protein Immobilization Using Unnatural Amino Acids. Bioconjugate chemistry. 2014 Nov 19,;25(11):1916-20.

6. Lang K, Chin JW. Bioorthogonal Reactions for Labeling Proteins. ACS chemical biology. 2014 Jan 17,;9(1):16-20.

7. Kulmala A, Huovinen T, Lamminmäki U. Effect of DNA sequence of Fab fragment on yield characteristics and cell growth of E. coli. Scientific reports. 2017 Jun 19,;7(1):3796-10.

8. Huovinen T, Julin M, Sanmark H, Lamminmäki U. Enhanced error-prone RCA mutagenesis by concatemer resolution. Plasmid. 2011;66(1):47-51.

9. Lajoie MJ, Rovner AJ, Goodman DB, Aerni H-, Haimovich AD, Kuznetsov G, et al. Genomically Recoded Organisms Expand Biological Functions. Science (American Association for the Advancement of Science). 2013;342(6156):357-60.

10. Chin JW, Santoro SW, Martin AB, King DS, Wang L, Schultz PG. Addition of p-azido-L-phenylalanine to the genetic code of Escherichia coli. Journal of the American Chemical Society. 2002 Aug 7,;124(31):9026.

11. Dower WJ, Miller JF, Ragsdale CW. High efficiency transformation of E. coli by high voltage electroporation. Nucleic acids research. 1988 Jul 11,;16(13):6127-45.

12. He F. Laemmli-SDS-PAGE. Bio-protocol. 2011;1(11).

13. Konev MO, McTeague TA, Johannes JW. Nickel-Catalyzed Photoredox-Mediated Cross-Coupling of Aryl Electrophiles and Aryl Azides. ACS catalysis. 2018 Oct 5,;8(10):9120-4.

14. Garza-Sánchez F, Schaub RE, Janssen BD, Hayes CS. tmRNA regulates synthesis of the ArfA ribosome rescue factor. Molecular microbiology. 2011 Jun;80(5):1204-19.

15. Fleet GWJ, Porter RR, Knowles JR. Affinity Labelling of Antibodies with Aryl Nitrene as Reactive Group. Nature (London). 1969 Nov 1,;224(5218):511-2.