

Characterization of low affinity Fcγ receptor biotinylation under controlled reaction conditions by mass spectrometry and ligand binding analysis

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The supplementary material contains additional figures and a table. Figures contain additional SPR sensorgrams, MS data (graphs, chromatograms, MS and MS/MS spectra), graphs supporting the statistical analysis of the DoE. A table with the chymotryptic peptides for quantitation is included in the supporting information.

File name: Supporting information minimal biotinylation.pdf

ABSTRACT

Chemical protein biotinylation and streptavidin or anti-biotin based capture is regularly used for proteins as a more controlled alternative to direct coupling of the protein on a biosensor surface. Upon biotinylation an interaction site of interest may be blocked by the biotin groups, diminishing apparent activity of the protein. Minimal biotinylation can circumvent the loss of apparent activity, but still a binding site of interest can be blocked when labeling an amino acid involved in the binding. Here we describe reaction condition optimization studies for minimal labeling. We have chosen low affinity Fcγ receptors as model compounds as these proteins contain many lysines in their active binding site and as such provide an interesting system for a minimal labeling approach. We were able to identify the most critical parameters (protein:biotin ratio and incubation pH) for a minimal labeling approach in which the proteins of choice remain most active towards analyte binding. Localization of biotinylation by mass spectrometric peptide mapping on minimally labeled material was correlated to protein activity in binding assays. We show that only aiming at minimal labeling is not sufficient to maintain an active protein. Careful fine-tuning of critical parameters is important to reduce biotinylation in a protein binding site.

KEYWORDS

Protein-protein interactions, ligand binding, oriented immobilization, minimal labeling, Fcγ receptor

Statement for broader audience

Protein-protein interactions are studied in vitro to understand the protein function in a biological environment. Most techniques to study these interactions require one of the interactions partners to be immobilized onto a solid surface. The immobilization strategy should be chosen as such that interaction sites are not blocked or masked. Minimal labeling only is not sufficient to keep the binding site intact and modification of the binding site should be avoided by optimizing reaction conditions.

INTRODUCTION

Protein binding analysis in biosensor experiments relies in many cases on appropriate immobilization or capture of one of the interaction partners on a solid surface. Many different approaches for protein immobilization are generally applied throughout ligand binding studies. However, a key requirement in such a study is that the immobilized or captured ligand remains active, in other words, the interaction site involved in binding should not be blocked or masked.¹

A commonly used immobilization strategy is based on amine coupling of the ligand directly to the sensor surface.² Coupling through amine groups is considered to be a random process, hence orientation and cross-linking of the ligand on the sensor surface is less well controllable. For ligands that have many lysines in or around the interaction site of interest, this may result in a marginally active or inactive surface. Other direct coupling chemistries, such as thiol coupling, may face similar problems, as any amino acid that is directly coupled to a sensor surface may have an impact on the protein binding characteristics.

Alternative approaches are mainly based on protein capture, in which a protein can be captured to the surface in a highly selective manner. The orientation of the ligand to the surface will be more site-directed and when carefully constructed one can design the capture in such a way that the interaction site is not blocked. Capture approaches that are often used include the capture of his-tagged proteins by an anti-histidine antibody^{3,4} or the capture of biotinylated proteins by a streptavidin surface.² A disadvantage of most capture approaches is that in general the ligand will be regenerated after interaction

measurements, and ligand capture has to be repeated with each analysis. The regeneration and recapture of ligand is not necessary when the biotin-streptavidin capture is chosen, because the affinity of biotin to streptavidin is high^{5,6} and virtually no dissociation takes place. Next to this high affinity, the capture is specific, therefore only biotinylated proteins will be captured in most well-defined buffer systems.

Biotinylation of proteins is most elegantly performed by incorporating an AviTagTM into the protein,⁷ a peptide sequence that can then be specifically biotinylated by bacterial biotin ligase (BirA) in vitro or in vivo. However, when a protein is not expressed with such a tag, chemical biotinylation provides a robust alternative. Chemical biotinylation can generally be performed on primary amines, where similar considerations are applicable as in direct coupling on a sensor surface. A number of groups have shown that controlling the pH during the coupling reaction may drive preference for reactions on α -amino groups or ϵ -amino groups.^{8,9} Selo et al.⁹ were able to selectively biotinylate α -amino groups over ϵ -amino groups, which then results in biotinylation at a peptide's N-terminus instead of randomly on various lysines in the peptide sequence. However, these experiments were performed on small peptides with a limited number of amine groups on lysines present and may therefore differ from reaction conditions in entire proteins with a large number of lysines. Papalia et al.¹⁰ have reported a minimal labeling approach, in which they chose the reaction conditions in such a way that only a limited number of lysines in a protein is biotinylated, thereby reducing the chance of biotinylation in the binding site of interest in a protein. Furthermore, they concluded that low levels of biotinylation lead to less cross-linking of the ligand on the surface, resulting in high-density surfaces with optimal activity.

Here we describe the biotinylation of Fc γ receptors, a class of cell surface receptors that is important in the binding of IgG to effector cells in a human body.¹¹⁻¹³ Five different subclasses of Fc γ receptors are known, with different isoforms and natural variants,^{14,15} all of which carry many lysines in or close to the IgG binding site.¹⁶⁻²² As an alternative to amine groups (lysines), coupling may be performed on carbohydrates, however the presence of carbohydrates depends on the expression platform^{23,24} and moreover Fc γ receptor glycosylation plays a crucial role in IgG binding.²⁵

Hence these receptors provide an interesting group of proteins in which the impact of minimal biotin labeling may be studied. A minimal degree of biotinylation is defined here as a maximum of one biotin attached to a single protein molecule, which corresponds to a degree of labeling of approximately 1. A subset of Fc γ receptors was selected to explore the influence of the biotinylation reaction conditions on IgG binding, which included the factors protein:biotin ratio, reaction pH and reaction time based on previous publications.^{9,10} Experimental setup and analyses of the data were executed under a statistical design of experiments (DoE). Furthermore, using Fc γ RIIb as a model system, we investigated whether certain reaction conditions could be correlated to remaining ligand binding activity, defined as binding to IgG and to biotinylation at certain lysine residues (ϵ -amines) or at the N-terminus (α -amine) within the protein. Therefore, surface plasmon resonance (SPR) binding assays and mass spectrometry (MS) analyses were performed respectively. Results of the different SPR and MS analysis were used to identify the most critical parameters in chemical biotinylation impacting Fc γ receptor activity after minimal labeling.

RESULTS AND DISCUSSION

IgG binding studies to biotinylated Fcγ receptors

The Fcγ receptors were biotinylated under controlled conditions (Table 1) to be used as ligands in IgG binding studies. Our previous experiences showed that direct immobilization of the Fcγ receptors minimizes their activity for IgG binding and only a limited stability on the surface is obtained (data not shown). The IgG binding sites of Fcγ receptors contain a number of lysines,¹⁶⁻²² hence, upon amine coupling on a sensor surface the interaction with IgG will be affected. In our experiments degrees of labeling of the different samples were determined at values between 0.36 and 1.78 indicating that on average a minimal amount of biotins had been attached to the proteins.

Table 1

Immobilization of all samples to a streptavidin SensEye® sensor surface was performed, followed by analysis of IgG binding to the Fcγ receptors. The ligand density on the sensor surface expressed in resonance units (RU) was plotted against the degree of labeling [Figure 1(A)]. The amount of ligand that was immobilized on the sensor surface is independent of the degree of biotinylation indicated by the apparent random distribution of points in this plot. This was verified by a regression analysis of the ligand density versus the degree of labeling where no significant effect on the slope of the regression was found ($P=0.58$, $P=0.12$, $P=0.63$ and $P=0.19$ for FcγRIIIa, FcγRIIIb, FcγRIIa and FcγRIIb respectively). As a control, non-labeled proteins were also applied to the surface under the same conditions resulting in no immobilization of ligand with FcγRIIIa. In case of the other three ligands, the RU determination from the

camera image calculated 36 to 60 RU of ligand, which is too low for proper functional ligand coupling. This was confirmed by injecting IgG samples over these spots which resulted in no measurable interaction [Figure 1(B)]. This indicates that no relevant amount of functional ligand adheres non-specifically to the surface. The immobilization levels in each of the biotinylated samples are higher compared to the control samples, except for 1 sample which was present in a blocked flowchannel.

Figure 1

Papalia et al.¹⁰ have shown that similar surface densities are reached at different degrees of labeling, but that the chance of cross-linking at the surface becomes higher at higher labeling degrees. We were interested to see whether possible cross-linking at the surface could have an influence on the remaining activity of the receptors on the surface. Binding of IgG to immobilized Fcγ receptors at similar ligand densities was measured in an SPR measurement (Fig. S1). The binding of IgG is expressed as the maximum response after 20 min association time and this value is plotted against the degree of labeling [Figure 1(B)]. An actual steady state is not reached in any of the sensorgrams which is related to the biphasic binding of IgG to Fcγ receptors.^{25,26} Hence, for comparison of the remaining activity under different biotinylation conditions the maximum response at 20 min post injection was chosen as measure for binding.

Figure 2

Higher responses are measured on those receptors with a lower degree of labeling and lower responses are measured at higher degrees of labeling [Figure 1(B)]. No IgG binding is measured on the spots where we immobilized non-labeled control samples. It

must be noted that non-labeled protein contributes to the calculated degree of labeling, yet is not immobilized and henceforth does not contribute to either immobilized ligand response or IgG binding response. The significant factors contributing to this trend were found in a statistical analysis of the IgG binding as an output in the DoE analysis (example of FcγRIIb shown in Figure 2). The statistical analysis indicated that significant factors on the IgG binding response are the protein:biotin ratio during biotinylation ($P < 0.0005$), the interaction between the protein:biotin ratio with the incubation pH ($P = 0.001$) and the interaction between protein:biotin ratio with the incubation time ($P = 0.015$). The main driver for significant differences in remaining ligand activity is the protein:biotin ratio. Within each of the different protein:biotin ratio conditions that were tested, also the combination with either of the two other input variables has a significant influence. However, the incubation pH and incubation time on its own do not have a significant influence on the IgG binding to biotinylated Fcγ receptors. The adjusted model where non-significant interactions of factors were excluded has a predictive R^2 of 98%. Appropriateness of the statistical model was evaluated by analysis of the normalized residuals (Fig. S2).

These data suggest that either cross-linking of the ligand at the surface may occur, as proposed by Papalia et al.¹⁰ or that the biotins are present at lysines in or near the interaction site involved in IgG binding.

Biotin distribution by mass spectrometry

In the paper by Papalia et al.¹⁰ high degrees of labeling are related to more ligand cross-linking at the sensor surface. For cross-linking at the surface, the biotin loading on

a single protein molecule should be at least two or more. To determine whether more cross-linking may occur under certain biotinylation conditions, we determined the biotin distribution on the protein molecules by intact mass spectrometry analysis. An average degree of labeling of 1 can be obtained when exactly each protein carries a single biotin, however in theory it may as well be possible that a percentage of the proteins carry two or more biotins and the other percentage is not labeled at all. On average, both samples may give a degree of labeling of 1. Subsequently, unlabeled protein molecules will not be immobilized on a streptavidin sensor and only the proteins with multiple biotins will be immobilized in the latter case, which may be prone to cross-linking in contrast to a protein that only carries a single biotin. FcγRIIb was taken as a model system for further investigation.

Figure 3

In the intact mass analysis of FcγRIIb after deglycosylation we could distinguish peaks that correspond to the mass of the intact protein [Figure 3(A)] and to masses of the intact protein with one, two or three biotins attached (the peak with three biotins attached not being visible in the mass spectra that are shown). The calculations of biotin distribution on FcγRIIb were based on the ion intensities of the corresponding peaks in the mass spectra which were then used for a relative quantitation [Figure 3(B)]. Similar experiments were performed on the other three Fcγ receptors and data are shown in supporting information (Fig. S3). The ion intensities of the unmodified proteins may be overestimated compared to the biotinylated proteins due to differences in ionization efficiency between the different species. This leads to lower degrees of labeling based on the MS data compared to the fluorescent biotin quantitation kit [both values are

indicated in Figure 3(B)]. Although the percentage of unmodified protein may be overestimated, the distribution of the peaks among the different samples can be compared to each other from species to species as all samples were analyzed with the same settings and in a single analysis. The degrees of labeling from fluorescent determination by the FluoReporter kit are used for quantitation purposes and MS data are only used for comparison of biotin distribution on the proteins.

The distribution of biotins on the protein molecules is different between the eleven biotinylation conditions [Figure 3(B)]. Highest biotin loading is reached in the samples with the highest protein:biotin ratio. Only under these conditions a substantial percentage of protein with two biotins attached is formed and only very limited protein with 3 or more biotins are found overall (<0.5%). The distribution of protein species with only a single biotin and without biotin modification varies between the applied reaction conditions and only a minor difference in IgG binding is measured in these samples [Figure 3(C)] whereas lowest IgG binding is measured for the samples with double-biotinylated species. Together these data suggest that a decrease in IgG binding is most likely not dependent on ligand cross-linking at the surface, or at best plays a minor role under the investigated conditions.

Biotin localization by peptide mass mapping

Our second hypothesis for the reduced ligand activity considered biotinylation of lysines that are present in or near the interaction site involved in IgG binding. The Fcγ receptors have many lysines in their IgG binding site^{19,20} (Figure 4), and consequently we were interested to identify the actual positions that are biotinylated to determine whether this

IgG binding site is influenced upon biotinylation. As the four low affinity Fcγ receptors are relatively similar proteins we chose FcγRIIb as a model to identify the biotinylated residues by peptide mass mapping analysis.

Figure 4

The biotinylated samples and a non-modified sample of FcγRIIb were subjected to deglycosylation, reduction, alkylation and trypsin or chymotrypsin digestion to generate short peptides of the proteins. We chose a chymotrypsin digestion over a trypsin digestion for quantitation, because the biotin modification is expected on lysines. The more commonly used trypsin enzyme cleaves at lysines, but does not recognize biotinylated lysines²⁷ which would complicate the calculation of modified residues. Chymotrypsin on the other hand cleaves a protein into relatively small peptides because it cleaves at several different amino acids (Tyr, Trp, Phe, Met, Leu and His²⁸). The use of chymotrypsin prevents the presence of lysines at the C-terminus of the peptide which simplifies the data analysis for quantitation compared to a tryptic digestion.

Chymotryptic peptides on the other hand are less facile to fragment in MS/MS analysis, which is necessary to identify the exact location of the biotins. A trypsin digestion for the localization at specific residues was therefore included.

First of all we used MS/MS fragmentation data of the tryptic digests to determine which lysines were biotinylated and whether the N-terminal alanine was biotinylated as well (Fig. S4, S5). The tryptic peptides T112-117 (DKPLVK) and T112-125 (DKPLVKVTFQNGK), which contain the lysines that are present in one of the active binding sites of FcγRIIb, are modified at Lys¹¹³ and Lys¹¹⁷ (Fig. S4). In the MS/MS

fragmentation spectra we could localize the biotin at Lys¹¹³ in peptide T112-117 and at Lys¹¹⁷ in peptide T112-125. The latter one explains the missed trypsin cleavage of this peptide, as trypsin does not recognize the lysine upon biotinylation and cannot cleave at this lysine.²⁷ The MS/MS fragmentation spectra of biotinylated peptides T1-4 and T1-8 were used to determine whether the lysine or the N-terminal alanine was biotinylated (Fig. S5). MS/MS fragmentation of biotinylated T1-4 proves that the N-terminal alanine of this peptide is biotinylated and trypsin cleaves at the non-modified lysine (Lys⁴) (Fig. S5a). Fragmentation of biotinylated peptide T1-8, which contains a missed trypsin cleavage site, matches with the expected fragment ions of the peptide with the biotin conjugated to the lysine at position 4. In this peptide the N-terminal alanine is not biotinylated as expected fragment ions for this modification are absent (Fig. S5b).

After we confirmed that the N-terminal alanine and each of the lysines in the amino acid sequence were biotinylated, we quantified the percentage of biotinylation at each position from the chymotrypsin digestion. A short cleavage time was used for chymotrypsin to maintain moderately sized peptides which are generally well ionized in mass spectrometry analysis (Table S1). A few peptides contained more than one lysine in the amino acid sequence of the chymotryptic peptides. Quantitation of these peptides was performed on the masses corresponding to peptides with one, two or three biotins attached. The exact location of biotin on a particular lysine could not be determined in these chymotryptic peptides, but trypsin data proved that all of the lysines and the N-terminal alanine in FcγRIIb were biotinylated, only not all to the same extent. Quantitation was based on the peak area in extracted ion chromatograms of the unmodified and modified peptides in the chymotrypsin digestion. Ionization efficiencies

of non-modified peptides and of biotinylated peptides are most likely different, due to the modification. The calculated percentages of modification should therefore only be used to compare the reaction conditions among each other, rather than as absolute numbers.

The extracted ion chromatograms of peptides C1-7 (APPKAVL) and C111-120 (KDKPLVKVTF) in the non-modified sample and one of the biotinylated samples are used to illustrate the quantitation (Fig. S6, S7, respectively).

Figure 5

Peak area percentages of biotinylated peptides were calculated for each of the eleven DoE runs [Figure 5(A)]. Two peptides, C1-7 and C111-120, have the highest levels of biotinylation irrespective of the labeling conditions that were applied, as the highest percentage of modification is detected at both peptides in each of the eleven samples that have been analyzed. In DoE runs 1 and 9 (lowest IgG binding), these peptides are modified at levels over 30%. These peptides contain residues Ala¹ and Lys⁴ and residues Lys¹¹¹, Lys¹¹³ and Lys¹¹⁷ respectively. All other lysines that are present in the amino acid sequence are modified with biotin to some extent, but modification levels are below 10% in each of the reaction conditions. To quantify biotinylation levels at Ala¹ we used the trypsin digestion. Only for this position the tryptic peptide mapping could be used, as we measured both the non-modified tryptic peptide T1-4 and the biotinylated peptide T1-4, which was biotinylated at Ala¹ as previously shown in the MS/MS fragmentation spectrum. We expected a higher level of biotinylation at Ala¹ in the DoE runs incubated at pH 6.5 since other groups^{8,9} have published that incubation at lower pH (e.g. pH 6.5) would preferentially incorporate the biotins at α -amines (i.e. the N-terminus). If the reaction would shift to the α -amine at pH 6.5, we would expect an

increase in biotinylation levels at peptide T1-4 (Ala¹) or peptide C1-7 (Ala¹/Lys⁴) and a decrease in biotinylation levels at any of the other peptides when incubated at pH 6.5. However, we do not see a shift of biotinylation at ϵ -amines (i.e. lysines) towards biotinylation at the α -amine when incubating at lower pH. The biotinylation levels at peptides T1-4, C1-7 and C111-120 are similar when compared between each of the tested conditions [Figure 5(A)]. Runs 1 to 4 have been incubated at pH 6.5 and no increase in biotinylation level at peptide C1-7 or peptide T1-4 compared to e.g. peptide C111-120 is observed in one of these runs. Both groups^{8,9} have performed the experiments on short peptides rather than on intact proteins, which may explain the apparent discrepancies with our results as the environment of the biotinylation site is different.

Lysines at positions 111, 113 and 117 in the protein are present in one of the interaction sites known for IgG binding^{19,21} (Figure 4). The samples with highest biotinylation levels on Lys¹¹¹, Lys¹¹³ and Lys¹¹⁷ have a higher degree of labeling and lowest responses in the IgG binding assay on SPR. We hypothesized that the lysines that are more prone towards biotinylation may be easier accessible, for example because they are more surface exposed. The three dimensional model of Fc γ RIIb (Figure 4) displays all lysine residues which are all present on the outside of the protein model, making all of them easily accessible for modifications. This does not explain the preference of biotinylation at certain residues. Possibly the local electrostatic environment around Lys¹¹¹, Lys¹¹³ and Lys¹¹⁷ is preferred for the biotinylation reaction.

The percentage of biotinylation at peptide C111-120 (KDKPLVKVTF), containing Lys¹¹¹, Lys¹¹³ and Lys¹¹⁷, was used as an output in a statistical analysis of the DoE. Both

protein:biotin ratio ($P < 0.0005$) and the incubation pH ($P < 0.0005$) have a significant impact on the level of biotinylation at this specific position, as well as the interaction between the two parameters ($P = 0.004$) [Figure 6(A)]. Appropriateness of the statistical model was evaluated by analysis of the normalized residuals (Fig. S8). However, similar significance in these factors was observed for the other peptides e.g. C1-7 ($P < 0.0005$ for ratio and for incubation time, $P = 0.002$ for the interaction between both). The same factors that have significant influence on the degree of labeling at individual peptide level drive the total degree of labeling [Figure 6(A), 6(B)].

Figure 6

This suggests that these factors relate to the degree of labeling, in other words the pattern of labeled peptides is the same for each of the tested conditions [Figure 5(A)]. This is also illustrated by the co-linearity of the IgG binding and % modified peptide [Figure 5(B)] of peptides C1-7, C111-120 and T1-4. All three peptides show similar correlation between IgG binding and the percentage of modification which is in line with the correlation found in the degree of labeling and IgG binding presented in Figure 1. Linear regression analysis of percentage of modified peptides versus IgG binding [Figure 5(B)] shows that there is a significant deviation from zero, i.e. correlation between the two variables ($P = 0.048$, $P = 0.051$ and $P = 0.0093$ for C111-120, C1-7 and T1-4 respectively). However, when we exclude the two highest levels of modification, then the linear regression is not significantly different from zero ($P = 0.35$, $P = 0.31$ and $P = 0.53$ respectively). These highest two points originate from the only two reaction conditions where a substantial amount of double-biotinylated species are formed [Figure 3(B)]. These results suggests that reaction conditions that lead to a substantial

percentage of FcγRIIb protein with 2 biotin molecules attached most likely have a reduced binding activity.

Steady state equilibrium affinity determination

Minimally labeled material for each of the four low-affinity Fcγ receptors was taken for further assay development. The receptors were biotinylated at a protein:biotin ratio of 1:0.75, incubation time of 30 minutes and an incubation pH of 6.5. Degrees of labeling were determined between 0.38 and 0.59. No mass spectrometry analyses were performed on these newly prepared ligands, but based on results in Figure 3(B) we assumed that there are no substantial amount of double-biotinylated species present in these preparations. IgG1 samples between 9 nM and 20 μM were injected for steady state equilibrium (SSE) affinity determination (Figure 7). Affinities determined from these minimally labeled samples were compared with values from literature^{15,29} (Table 2) determined at 25°C. The paper of Bruhns et al.¹⁵ reports K_A value which were converted to K_D values for our comparison ($K_D=1/K_A$). Both references used the steady state equilibrium determination for affinity. Bruhns et al.¹⁵ directly immobilized the Fcγ receptors with an amine coupling; Patel et al.²⁹ immobilized the IgG on the sensor surface by amine coupling and used the Fcγ receptors as analyte.

Figure 7

Affinities that we determined with the minimally labeled material are highly similar compared to the two references; all three values are of the same order of magnitude (Table 2). These results indicate that a minimal labeling of ligands provides a robust

alternative for routine use of ligands that are problematic to immobilize by direct coupling.

Table 2

CONCLUSIONS

Protein biotinylation is applied in many cases where a protein needs to be immobilized on a sensor surface for protein interaction measurements. In certain cases, direct immobilization is not preferred because the protein becomes partially inactive or the protein conformation changes upon direct coupling. One of the possibilities to overcome these issues is by capturing a protein by the high affinity biotin-streptavidin interaction.

However, chemical biotinylation of one of the interaction partners may be required, which may interfere with the binding site and reduce activity. Here we have shown that aiming at a minimal protein labeling is successful to maintain protein activity. In the examples of Fcγ receptors that we showed here, we were able to obtain low levels of biotinylation while similar ligand densities were obtained on the sensor surface.

Statistical evaluation of a design of experiments revealed that a very low protein:biotin ratio is a key parameter to obtain the most active protein on the surface while still sufficient amounts of protein are labeled in order to immobilize these.

However, even when low levels of biotinylation (between 0.3 and 1.8) were obtained under various conditions, differences in protein binding were measured while similar ligand densities were obtained. This is likely due to partial inactivity of the captured ligand. Further characterization of biotinylated Fcγ receptors by mass spectrometry showed that proteins that have two or more biotins attached on a relatively large fraction

of the total protein resulted in less active proteins on the sensor surface. This may be due to ligand cross-linking at the surface, however, the highest biotin loading that we found was two biotins on a protein and only very little cross-linking is expected. On the other hand, localization of the biotins in the protein sequence of FcγRIIb by peptide mapping mass spectrometry showed that under all of the tested reaction conditions one of the lysine containing peptides is preferentially biotinylated. The lysines at positions 111, 113 and 117 in the protein sequence (peptide C111-120), which are present at one of the IgG binding sites of FcγRIIb, were most prone towards biotinylation. We have shown here that biotinylation of these residues has a direct impact on the remaining activity of the protein on the sensor surface.

The level of biotinylation at these positions can be minimized, most notably by choosing conditions in which limited proteins with two coupled biotins are formed. Although the reaction cannot be directed to the α -amine at the N-terminus, biotinylation at the IgG binding site can be minimized by selection of reaction conditions that will mainly result in a single biotin on each protein, simply by reducing the chance of attaching a biotin in the IgG binding site. The aim should be to have the lowest amount of double-biotinylated species to have the most active ligand, while at the same time the highest amount of proteins is labeled with a single molecule to keep an efficient labeling procedure. In our example with FcγRIIb, the level of biotinylation is mainly driven by protein:biotin ratio and incubation pH. Most active ligand is mainly achieved by protein:biotin ratios of 1:0.5 and to a lesser extent at protein:biotin ratios of 1:1.25. However, this only impacts the total level of biotinylation and not the position of the coupled biotin. This supports the finding that minimizing the amount of proteins with two biotins attached is key in

retaining activity. In addition, although pH was found to influence the degree of labeling, it was not found to be a significant factor in IgG binding. These conditions may vary from protein to protein, and therefore it is recommended to perform a small design of experiments study on other proteins.

In the end we tested whether the minimally labeled material will give us reliable affinity data in an IgG binding experiment. Steady state affinity measurement on minimally labeled Fcγ receptors matched with values that have been reported in literature. This finding strengthens our conclusion that a very minimal labeling of protein is recommended to retain the most active ligand on a sensor surface and excludes random immobilization by a direct amine coupling method.

MATERIALS AND METHODS

Expression and purification of Fcγ receptors

Fcγ receptors were expressed and purified at Synthon Biopharmaceuticals BV (Nijmegen, the Netherlands). The amino acid sequences of the extracellular domains of the human Fcγ receptors were derived from Uniprot: FcγRIIIa (FCGR3A) from entry P08637 amino acids 17-208, FcγRIIIb (FCGR3B) from entry O75015 amino acids 20-208, FcγRIIa (FCGR2A) from entry P12318 amino acids 36-218 and FcγRIIb (FCGR2B) from entry P31994 amino acids 46-217. A leader sequence and a hexa-histidine-tag were included at the N-terminus and C-terminus respectively. DNA synthesis was performed by GeneArt [part of Thermo Fisher Scientific (Waltham, MA USA)]. The synthesized DNA fragments were cloned into a pcDNA3.1 (Life Technologies, part of Thermo Fisher Scientific) based vector. Plasmid preparations were made using the

EndoFree Plasmid Maxi Kit [QIAGEN (Hilden, Germany)]. The Fcγ receptors were expressed using the Expi293 Expression System (Life Technologies). Transient transfections were performed according to the manufacturer's protocol.

Histidine-tagged Fcγ receptors were purified from cell cultures by Immobilized Metal Affinity Chromatography (IMAC) on a 5 mL Chelating Sepharose Fast Flow column on an ÄKTA explorer 100 system (GE life sciences (Eindhoven, the Netherlands)). The column was charged with 100 mM copper(II)sulfate after which the supernatants were loaded. The column was washed with PBS pH 7.4 / 20 mM imidazole after which the target protein was eluted in a gradient elution with PBS pH 7.4 / 20mM imidazole to PBS pH 7.4 / 500 mM imidazole.

Biotinylation

Biotinylation reactions were performed with EZ-link Sulfo-NHS-LC biotin [Thermo Scientific (Waltham, MA USA)] which was dissolved in MQ water. Multiple factor levels of incubation time (30, 135 and 240 minutes), incubation pH (pH 6.5, pH 7.0 and pH 7.5 in case of FcγRIIa and FcγRIIb; pH 6.5, pH 7.5 and pH 8.5 in case of FcγRIIIa and FcγRIIIb) and protein:biotin ratios (1:0.5; 1:1.25 and 1:2) were applied as per DoE based setup (Table 1). Fcγ receptors were diluted to 1 mg/mL in PBS buffer at the indicated pH. The reaction volumes were kept constant at 200 μL and 10 μL biotin stock solution was added to the desired protein:biotin ratio. Biotinylation reactions were performed on ice and after incubation free biotin was removed using PD G-25 minitraps (GE life sciences) according to manufacturer's instructions. The degree of labeling was

determined by FluoReporter Biotin quantitation kit (Invitrogen (Waltham, MA USA)) according to manufacturer's protocol.

Surface Plasmon Resonance analysis

Biotinylated Fcγ receptors were immobilized on G-Strep SensEye® sensors (Ssens BV (Enschede, The Netherlands)) in the continuous flow microspotter (CFM) (Wasatch Microfluidics, Salt Lake City, UT, USA) using a print time of 5 minutes and a 50 mM sodium acetate buffer pH 4.5/0.05 w/v% Tween 80. Interaction measurements between Fcγ receptors and monoclonal antibody (Synthon Biopharmaceuticals BV, Nijmegen, The Netherlands) were performed on an IBIS MX96 SPRi instrument (IBIS Technologies BV, Enschede, The Netherlands) in HBS buffer pH7.2 / 0.05 w/v% Tween 80. A baseline of 1 minute was followed by an association time of 5 to 20 minutes and dissociation at 1 μL/second in 1 step for 4 minutes. Regeneration was performed with 25 mM phosphoric acid pH 3.0 in a single step of 30 seconds.

Mass spectrometry analysis

An UPLC-ESI-qTOF (Waters, Milford USA) was used to analyze intact proteins and for peptide mapping. Intact mass analysis was performed on a polymeric reversed phase column (PLRP-S, 50x4.6mm, 5μm (Agilent, Santa Clara, CA, USA) operated at 60°C and a flow rate of 400 μL/min was used. Mobile phase A and B consisted of 0.05% TFA in Milli-Q and 0.05% TFA in 50% acetonitrile respectively. A linear gradient from 60% A to 1% A in 35 minutes after a baseline of 2 minutes at 60% A was followed by 1% A for 3 minutes and an equilibration step at 60% A for 2 minutes. Online UV detection was measured at 280 nm. Online MS detection with an MS scan method was used from 400

to 4500 m/z with a scan time of 0.98 seconds and an interscan time of 0.02 seconds.

The following conditions were used for the MS: capillary voltage of 3 kV, sample cone of 25 V, source temperature of 120°C, desolvation temperature of 200°C and a desolvation gas flow of 600L/hr. Intact mass analysis was performed on Fcγ receptors, that were deglycosylated by overnight incubation with PNGase F at 37°C.

A reversed phase C18 column (Shim-pack XR-ODS II, 150 x 2.1 mm, 2.2 μm (Shimadzu, Kyoto, Japan)) at 30°C and a flow rate of 250 μL/min was used for the peptide mapping analysis. Trypsin digestion of Fcγ receptors was performed at 37°C overnight and chymotrypsin digestion of Fcγ receptors was performed in the presence of CaCl₂ at 35°C for 4 hrs after deglycosylation, reduction and alkylation of the protein.

The separation was performed with a baseline at 96% A for 2 minutes followed by a linear gradient from 96% A to 30% A in 30 minutes. Then the %A decreased to 1% in 2 minutes and was kept at 1% for 5 minutes, followed by returning to 96% A in 3 minutes and equilibration for 3 minutes. Online UV detection was measured at 214 nm and 280 nm. Online MS detection with an MSE method was used from 100 to 1700 m/z mass scan and a scan time of 0.5 seconds and interscan time of 0.1 seconds. The second mass scan was from 100 to 2000 m/z with a scan time of 0.5 seconds and interscan time of 0.1 seconds using a collision energy ramp from 19 to 30 eV for peptide fragmentation. The same MS settings as described before were used for the peptide mapping analysis. Data acquisition and data analysis was performed in MassLynx software (version 4.1) (Waters, Milford, MA, USA).

Statistical analysis

A 2-level full factorial Design of Experiments (DoE) was created in Minitab v17 (Minitab Ltd, Coventry, UK) which consisted of three factors at 2 levels and three replicates of center points (Table 1). In total the DoE consisted of eleven independent runs (a run is defined as a biotinylation reaction on one Fcγ receptor) for each Fcγ receptor (a total of 44 runs). Statistical analysis of data for output variables was performed in Minitab. Output variables included the degree of labeling, ligand density on the sensor surface, IgG binding to immobilized Fcγ receptor and in addition for FcγRIIb, biotin distribution and relative amount of biotinylated residues from peptide mapping quantification.

SUPPLEMENTARY MATERIAL

Supplementary material contains additional figures and tables supporting the data presented in this manuscript.

ACKNOWLEDGEMENTS

We thank George Ye for the purification of Fcγ receptors and performing the DoE on biotinylation on all samples. We would also like to thank Dr. Mark Eggink for the critical review of the manuscript, especially the mass spectrometry part, and Dr. Siem Heisterkamp for the helpful discussions on the statistical data analysis and interpretation. We thank EFRO Province of Gelderland and Overijssel, the Netherlands for giving us the opportunity to financially support the research project.

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TABLES

Table 1 Experimental conditions for biotinylation

Run no	Protein:biotin ratio	Incubation pH*	Incubation time (min)
1	1:2	6.5	240
2	1:2	6.5	30
3	1:0.5	6.5	30
4	1:0.5	6.5	240
5	1:2	7.5 / 8.5	30
6	1:1.25	7.0 / 7.5	135
7	1:1.25	7.0 / 7.5	135
8	1:0.5	7.5 / 8.5	30
9	1:2	7.5 / 8.5	240
10	1:1.25	7.0 / 7.5	135
11	1:0.5	7.5 / 8.5	240

* pH 6.5-7.0-7.5 in case of FcγRIIa and FcγRIIb; pH 6.5-7.5-8.5 in case of FcγRIIIa and FcγRIIIb

The “Run no” column shows the order of experiments after randomization by Minitab software

Table 2 Steady state equilibrium affinity determination

Receptor	K_D (μM) (this paper)	K_D (μM) ²⁹	K_D (μM) ¹⁵
Fc γ RIIa	0.79 ± 0.11	0.80	0.19
Fc γ RIIb	4.07 ± 0.21	3.10	8.33
Fc γ RIIIa	0.38 ± 0.03	0.85	0.85
Fc γ RIIIb	2.70 ± 0.17	1.90	4.55

FIGURE LEGENDS

Figure 1. Effect of biotinylation on ligand immobilization and remaining activity for four Fcγ receptors. a) Ligand density in resonance units (RU) is plotted against the level of biotinylation expressed as degree of labeling. b) Maximum IgG binding response in RU plotted against the level of biotinylation expressed as degree of labeling.

Figure 2. Pareto chart of the factors used in the design of experiments (DoE) for IgG binding activity to FcγRIIb as output variable. Significant factors for the IgG binding response are identified.

Figure 3. Biotin distribution under various biotinylation conditions. a) Deconvoluted mass spectra of the control sample (unlabeled FcγRIIb; top) and one of the biotinylated samples (pH 7.5, 240 minutes, 1:2 protein:biotin ratio; bottom). b) Relative intensities of the peaks in the mass spectra corresponding with the number of biotins on the protein. The X-axis represents the degree of labeling (DOL) as determined in the fluorescence assay (FR) and as determined by mass spectrometry (MS) and the run number of the DoE (Run). C) Correlation between the percentage of modified protein and the IgG binding response in SPR binding assay for species with 1 or 2 biotin molecules attached.

Figure 4. Three-dimensional protein model of FcγRIIb with lysine residues indicated as colored amino acids. Below is the amino acid sequence of FcγRIIb with the IgG binding site from Hulett et al²¹ in red and underlined and the lysine residues in blue.

Figure 5. a) Biotinylation levels on the different modified residues. Extracted ion chromatograms from peptide mass mapping chromatograms were integrated. The peak area of the modified peptide relative to the total peak area of a particular peptide was used to calculate the percentage of biotinylation at each position. Run numbers correspond to the different DoE samples and reaction conditions areas indicated in Table 1 and the degrees of labeling are indicated between brackets. b) Correlation between IgG binding in the SPR assay and % of modified peptides C1-7 and ,C111-120 and T1-4 (representing total biotin modifications, i.e. 1 and 2 biotins per peptide relative to total peptide).

Figure 6. Pareto chart of the factors used in the DoE based on the percentage of modified peptide C111-120 (a) and total degree of labeling (b).

Figure 7. Steady state equilibrium (SSE) affinity determination of IgG1 on four low affinity Fcγ receptors immobilized on a streptavidin sensor after minimal biotinylation. FcγRIIa in black, FcγRIIb in blue, FcγRIIIa in green and FcγRIIIb in red. Sensorgrams of a triplicate concentration series between 9 nM and 20 μM are shown on the left side,

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concentration vs response curves for SSE affinity determination are shown on the right side. Analyses were performed at 25°C for comparison with values from literature.

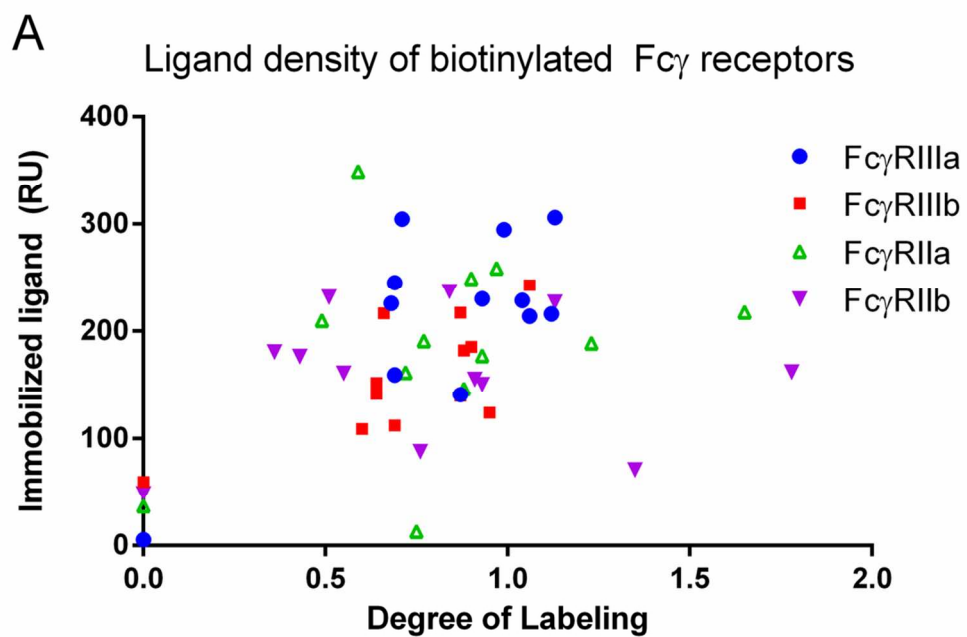


Figure 1A

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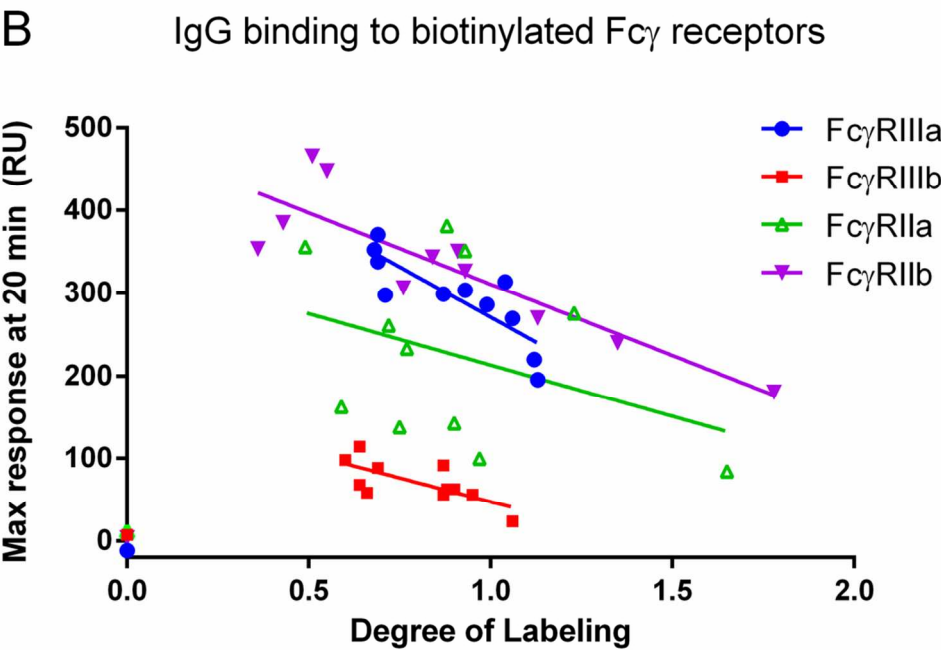


Figure 1B

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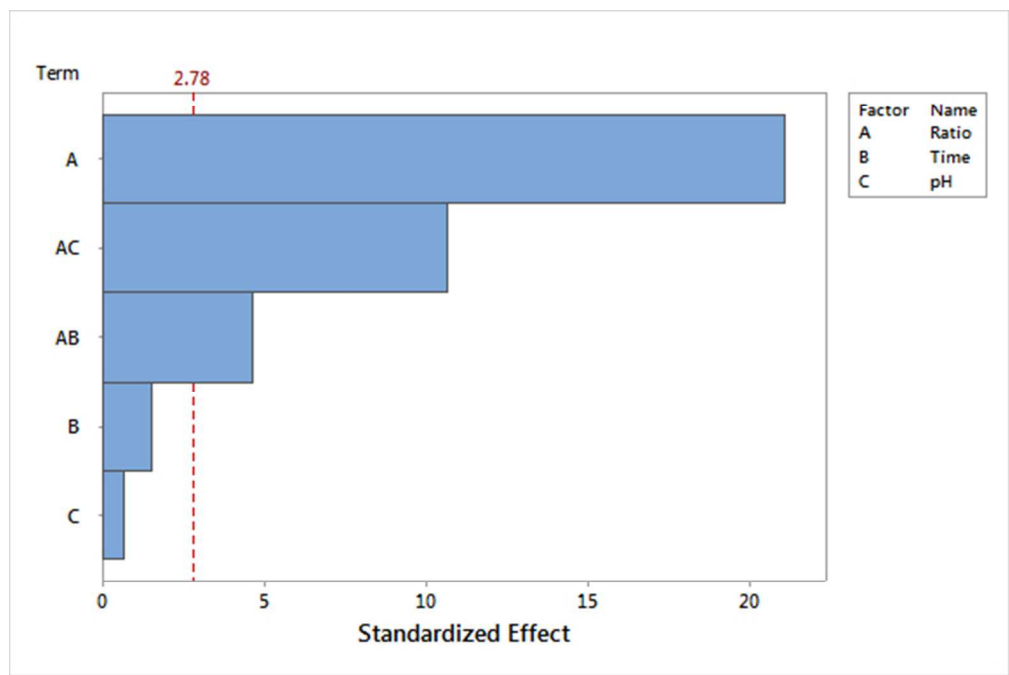


Figure 2

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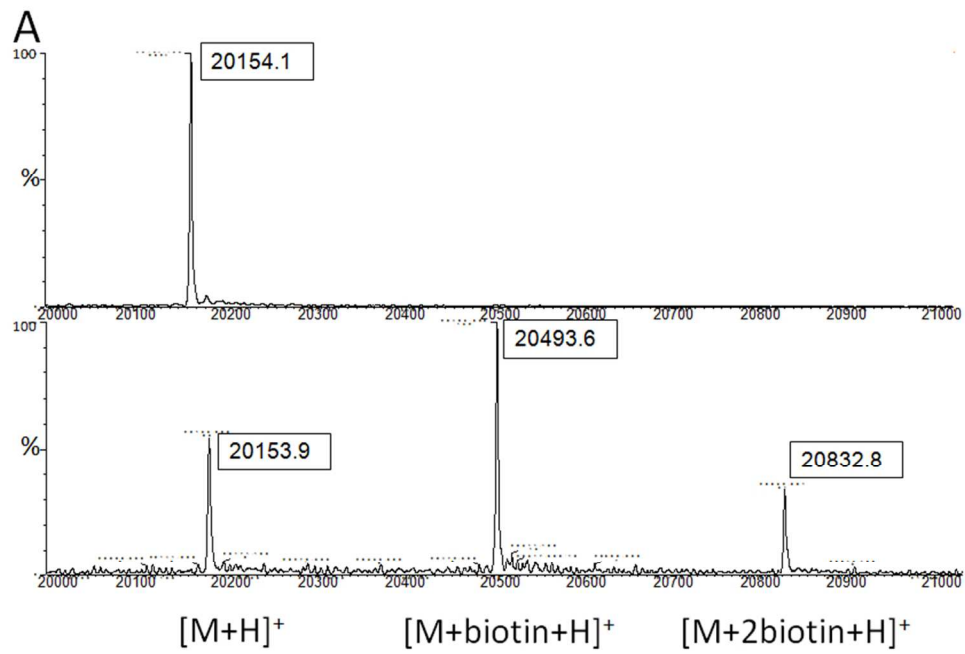


Figure 3A

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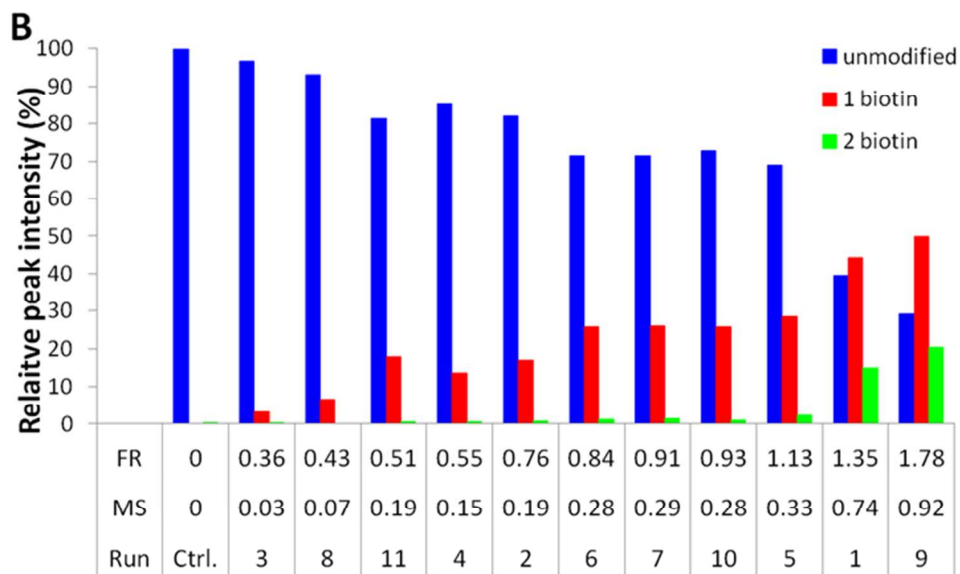


Figure 3B

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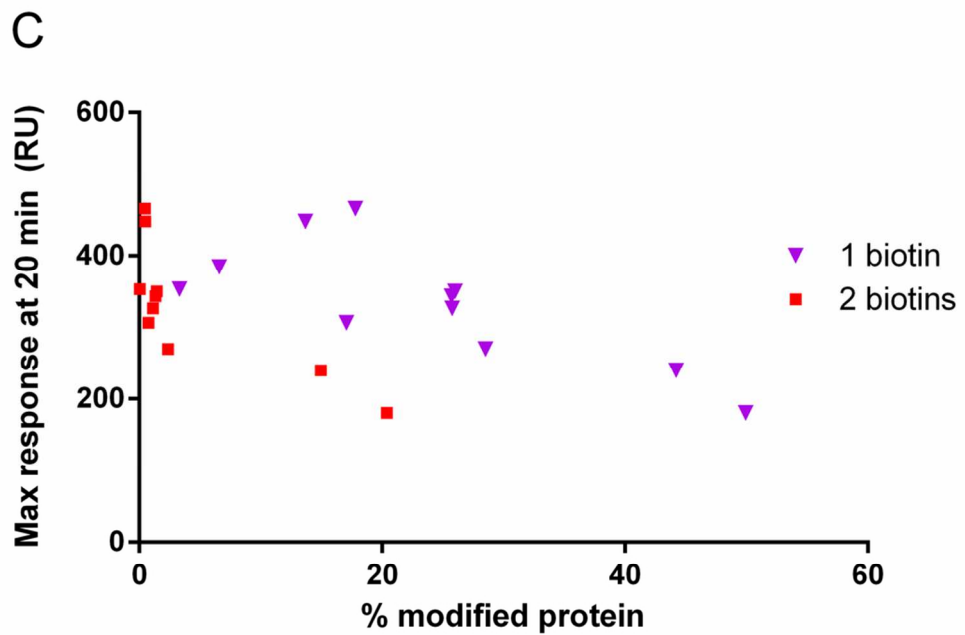


Figure 3C

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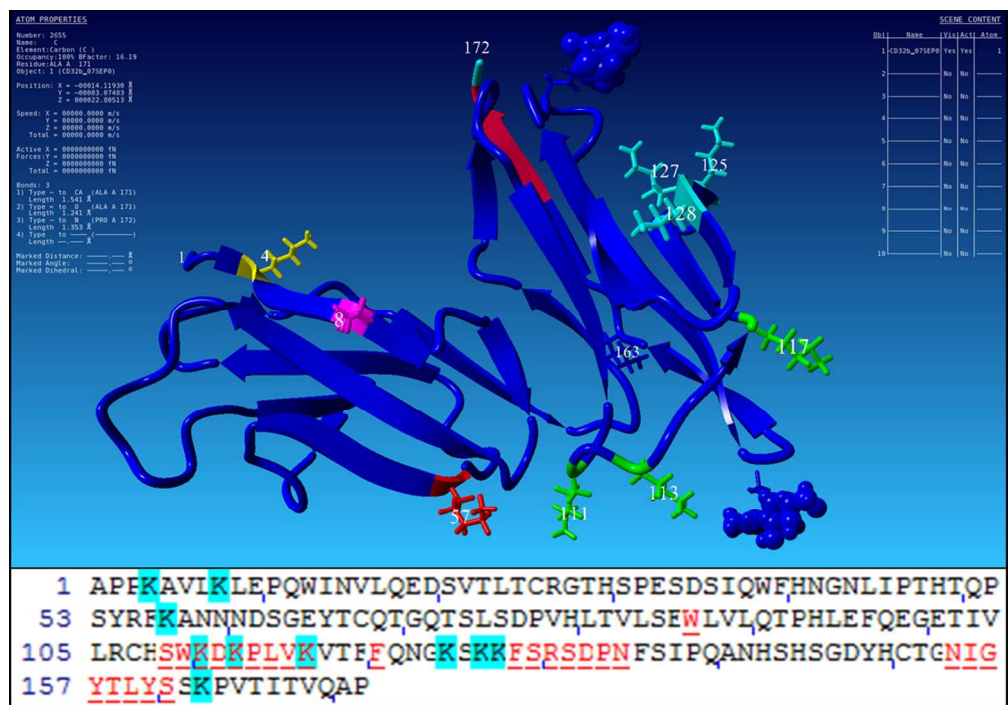


Figure 4

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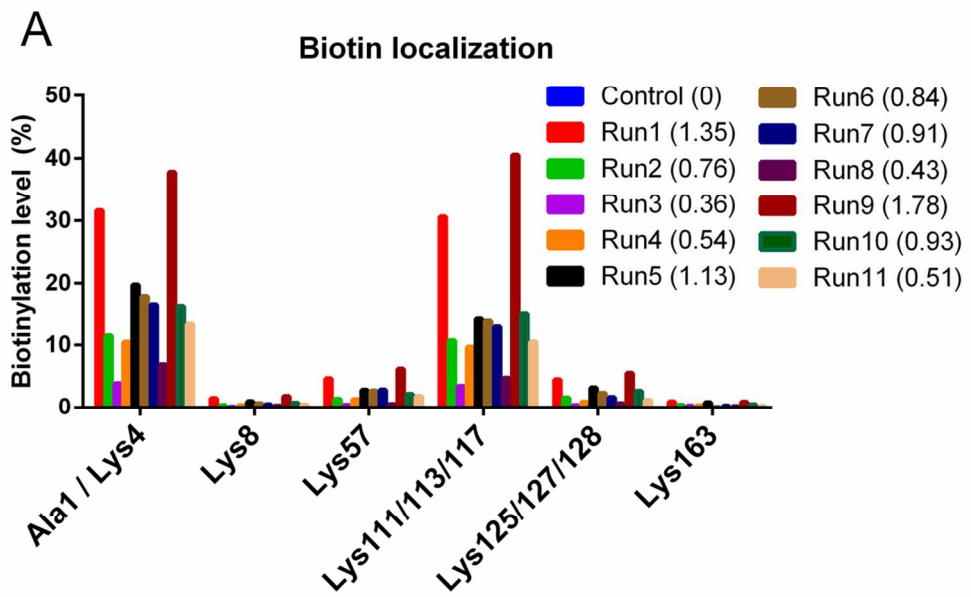


Figure 5A

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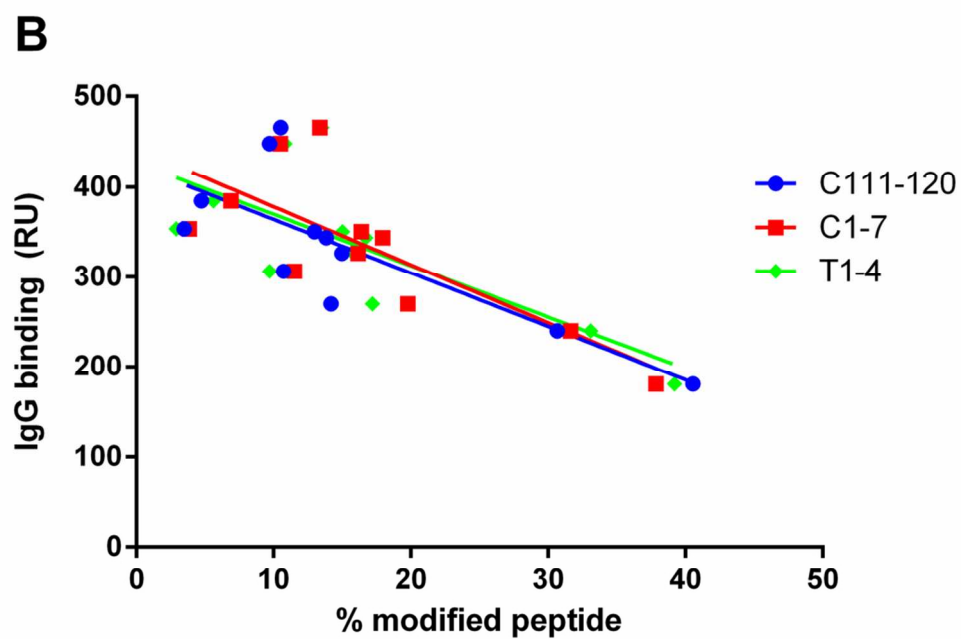


Figure 5B

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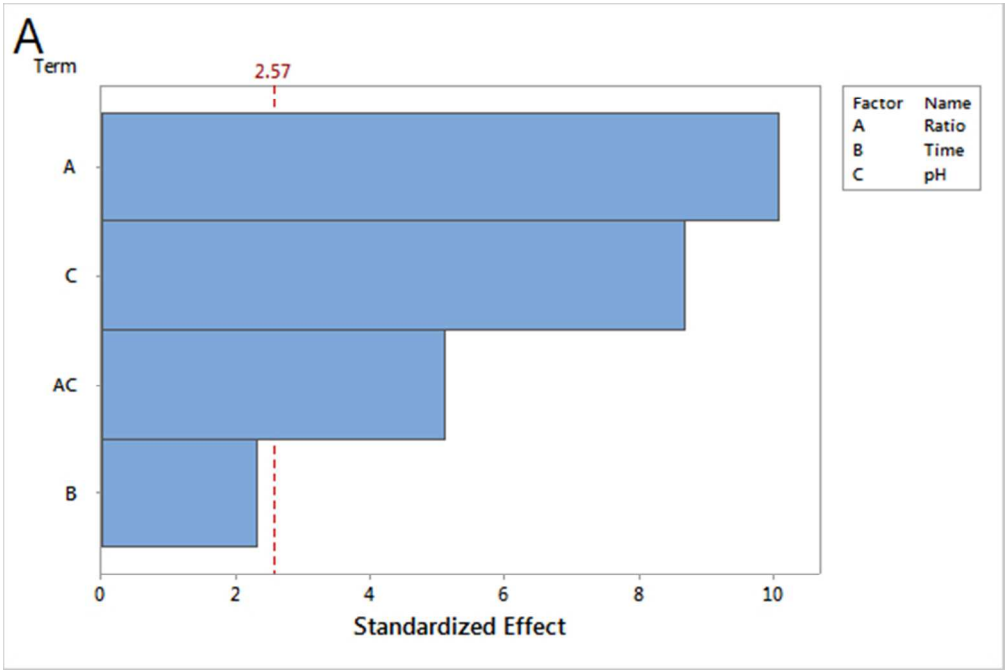


Figure 6A

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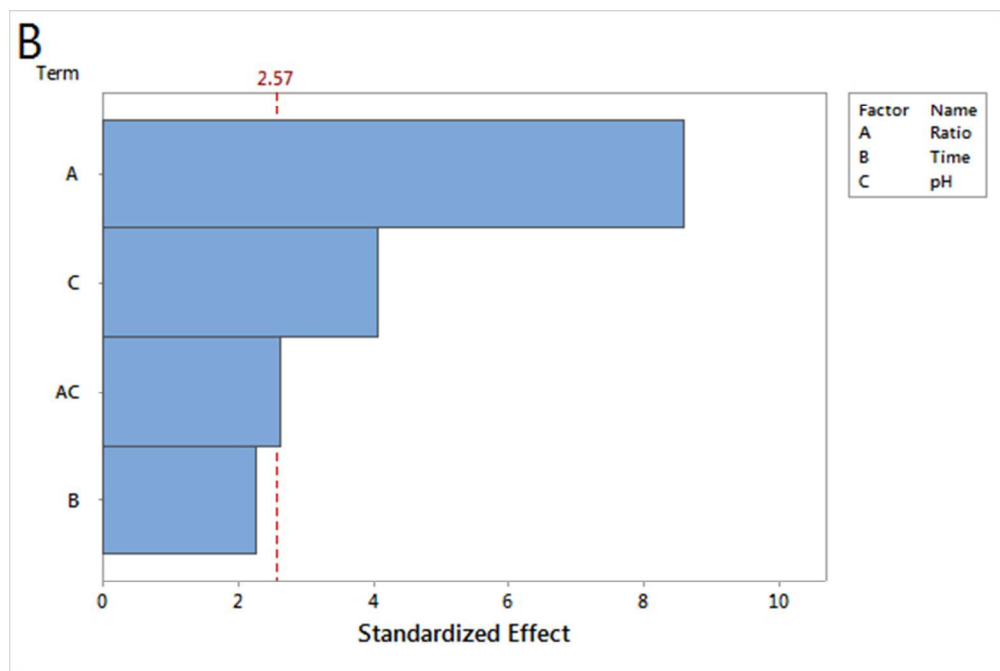


Figure 6B

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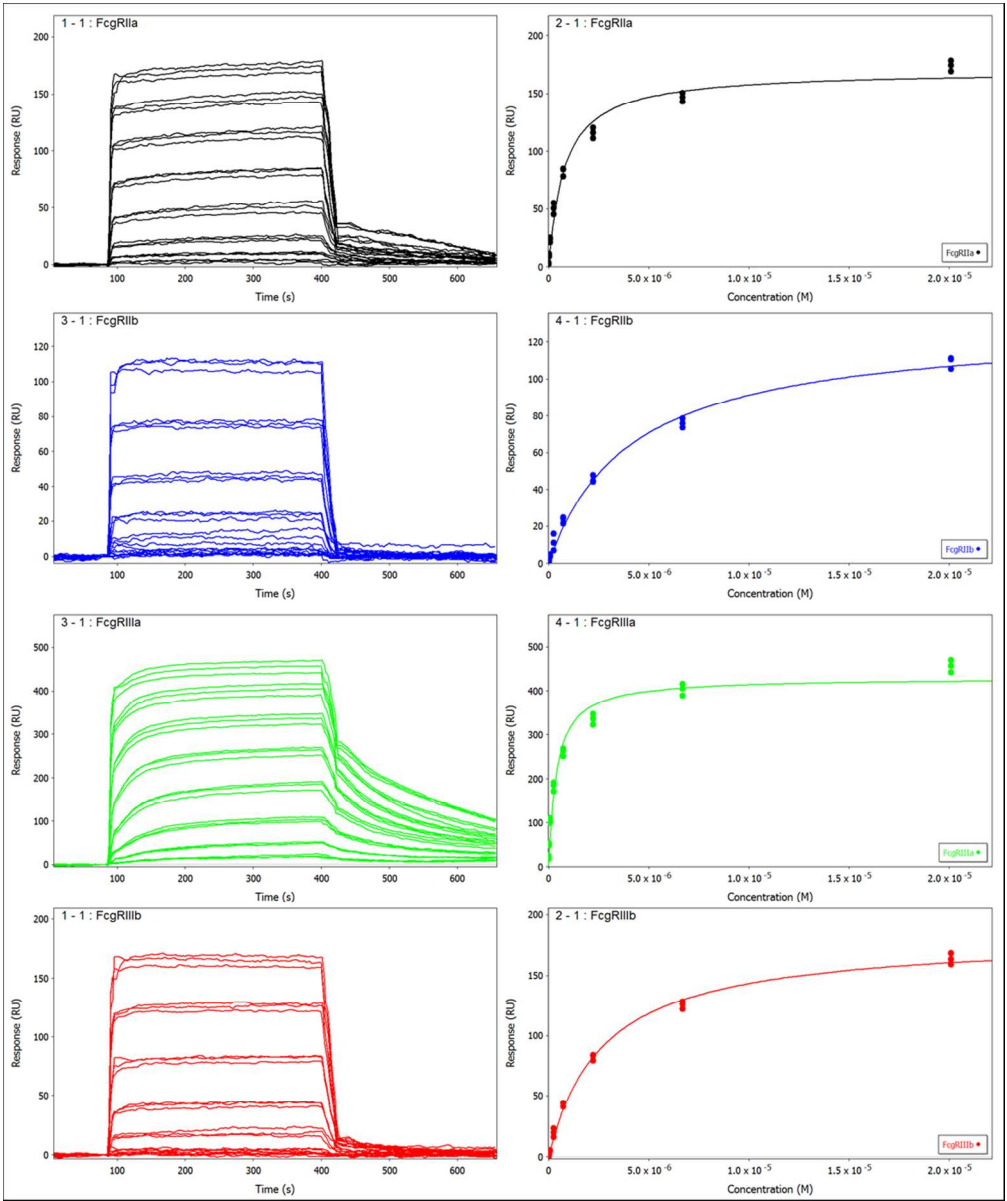


Figure 7

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Characterization of low affinity Fc γ receptor biotinylation under controlled reaction conditions by mass spectrometry and ligand binding analysis

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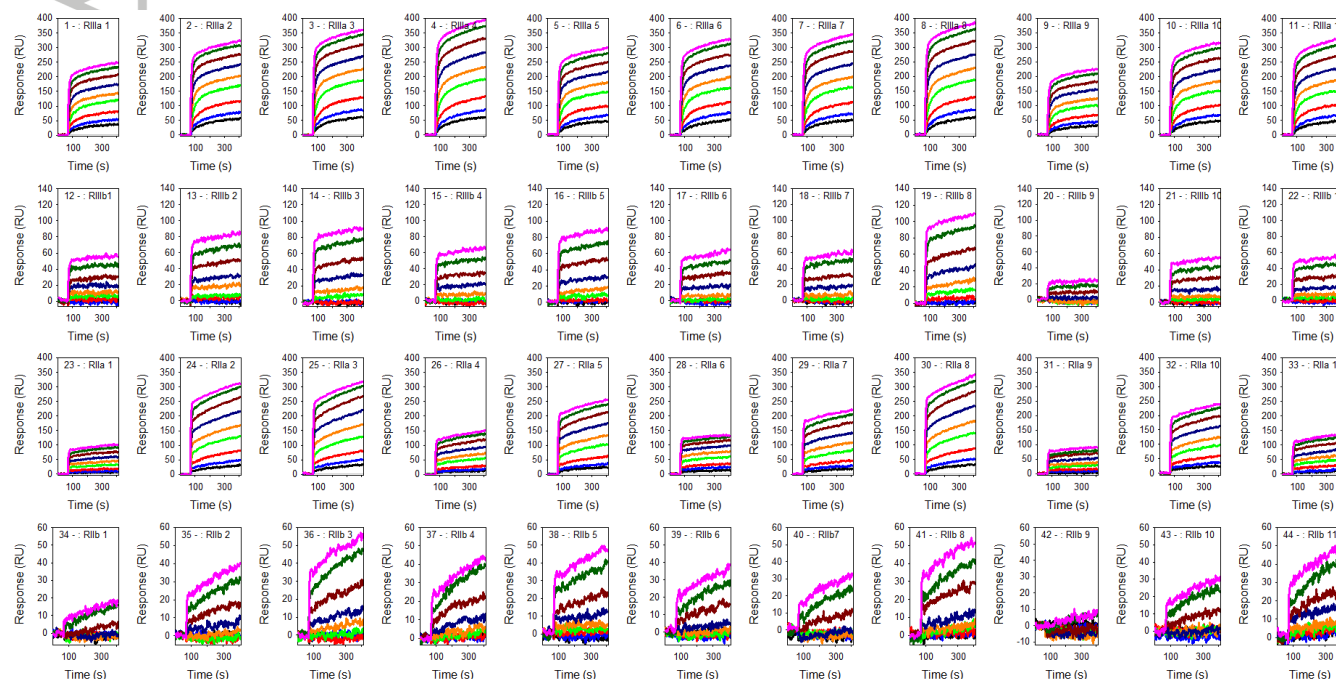


Figure S-1. Sensorgrams of IgG binding to immobilized biotinylated Fc γ receptors. Dilution series of IgG between 25 nM and 6.65 μ M were injected on each of the Fc γ receptors. Samples that were biotinylated in eleven different biotinylation conditions of Fc γ RIIIa (row 1), Fc γ RIIIb (row 2), Fc γ RIIa (row 3) and Fc γ RIIb (row 4) were immobilized on a streptavidin sensor at similar ligand densities. X-axis displays the time in seconds; response in resonance units (RU) is displayed at Y-axes where per row the axis is at the same scale. Intensities after 15 minutes were compared between biotinylation conditions, although no steady state was reached at that point.

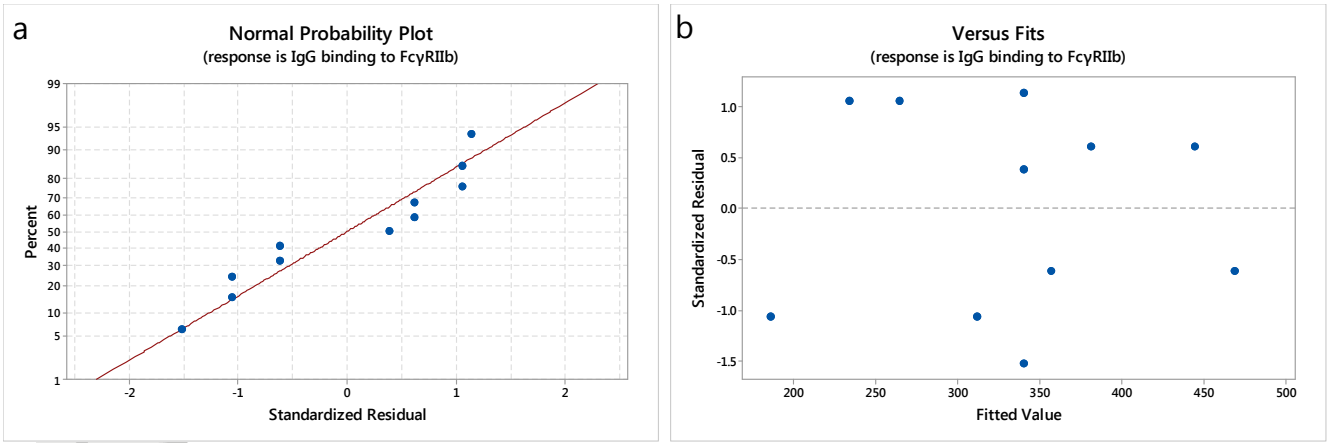
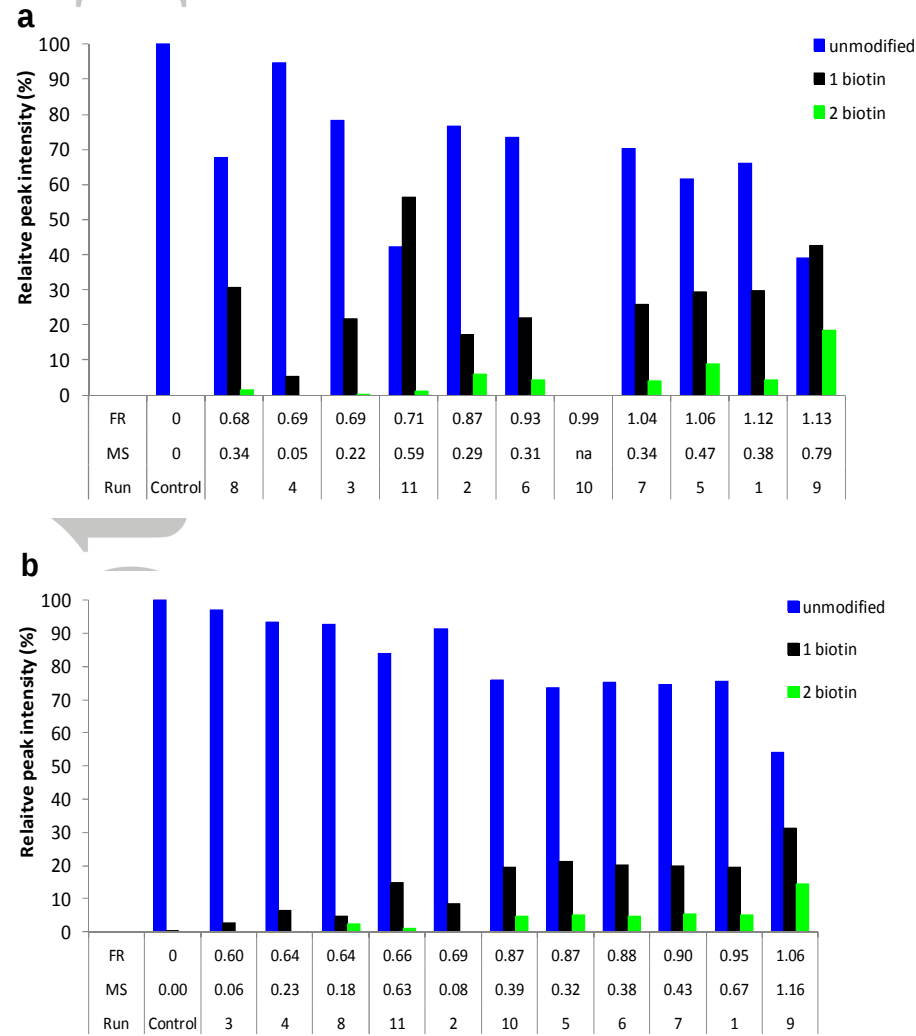


Figure S-2. Normal probability plot (a) and plot of standardized residuals versus fits (b) are shown to support the appropriateness of the statistical analysis of the DoE for IgG binding to FcγRIIb.



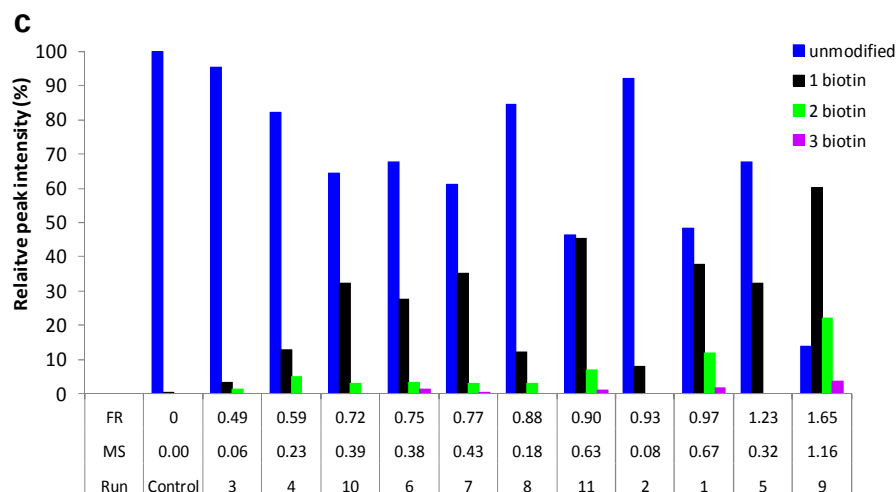
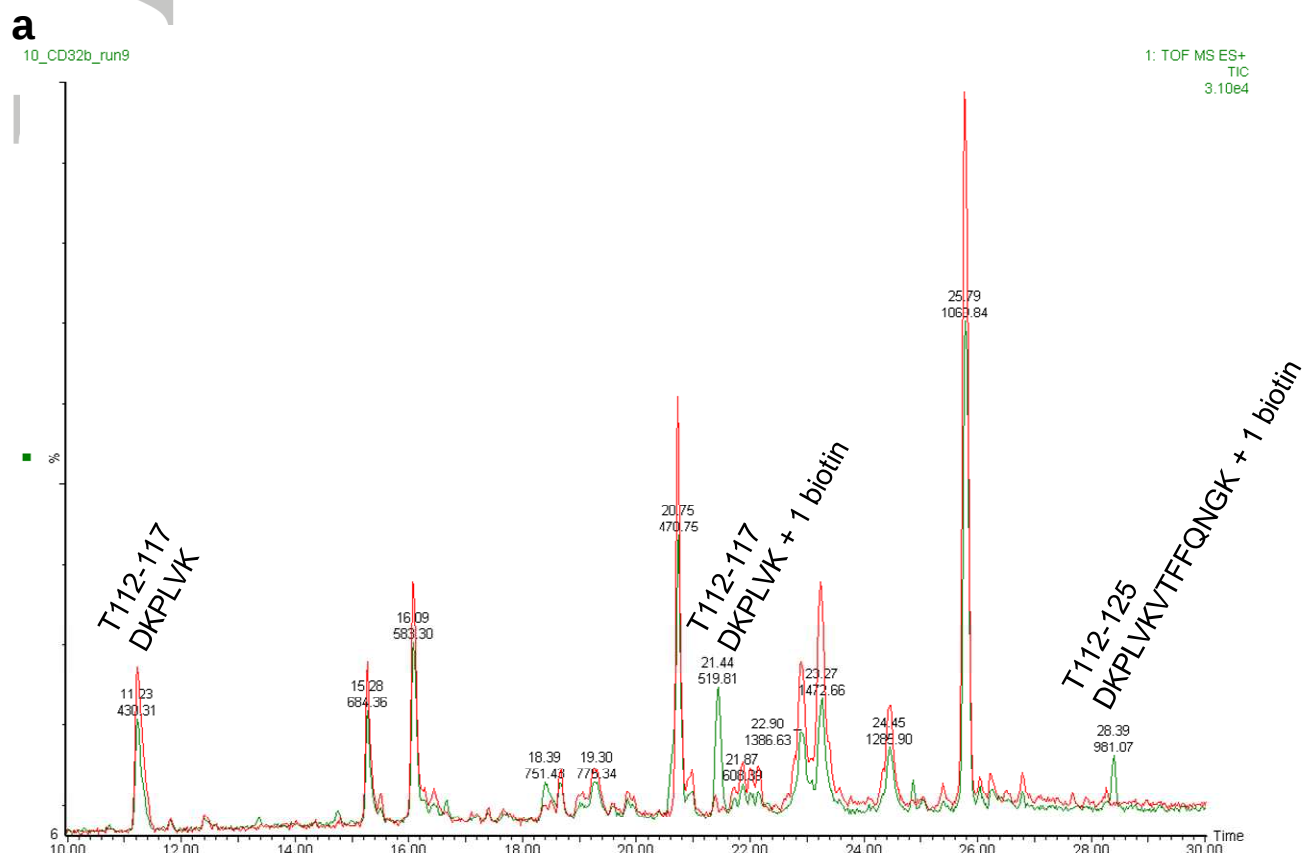
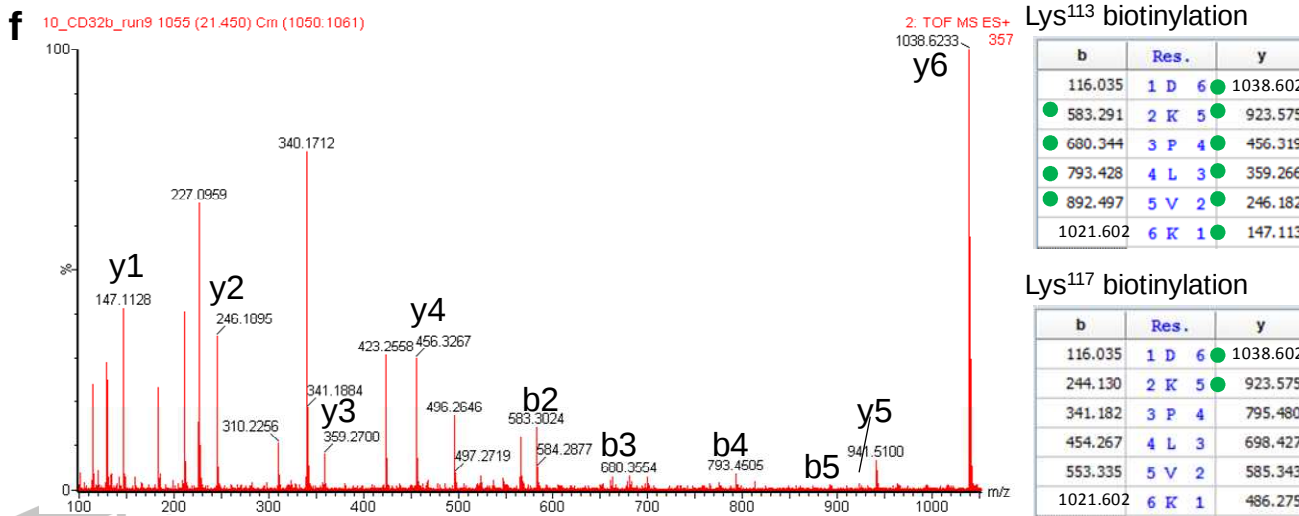
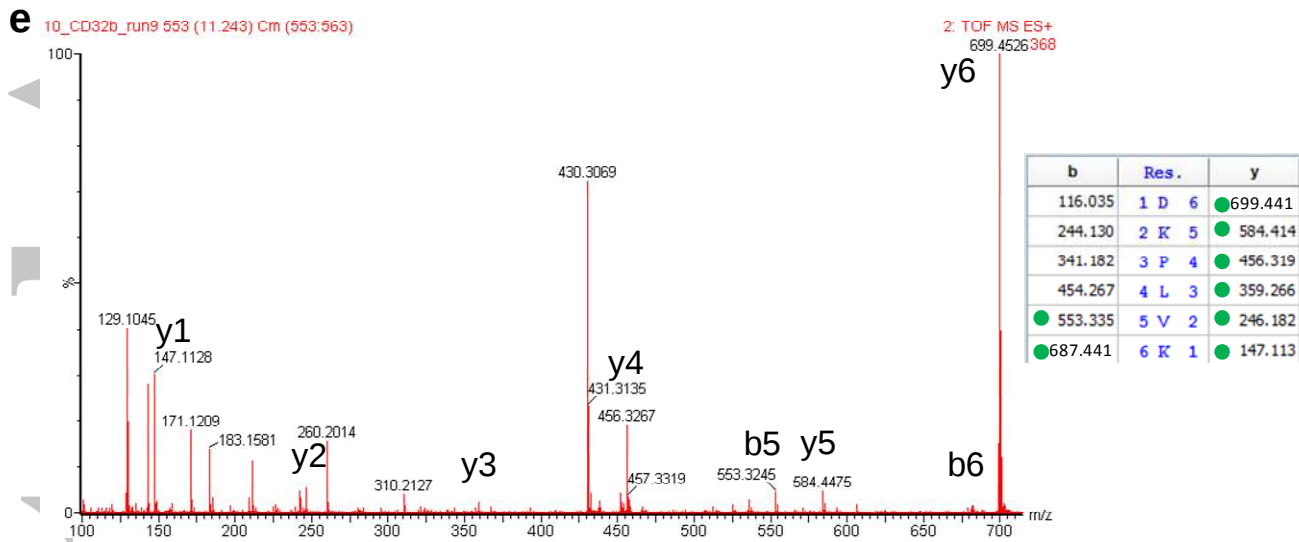
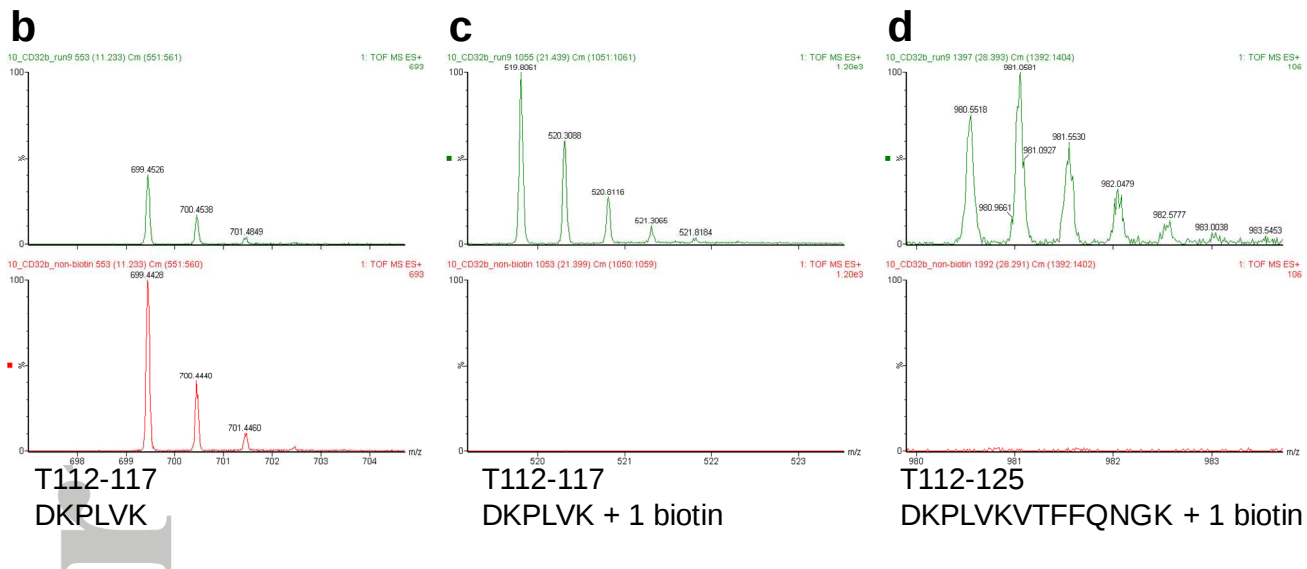


Figure S-3 Biotin distribution on FcγRIIIa (a), FcγRIIIb (b) and FcγRIIa (c) expressed as relative intensities of the peaks in the mass spectra corresponding with the number of biotins on the protein. The X-axis represents the degree of labeling (DOL) as determined in the fluorescence assay (FR) and as determined by mass spectrometry (MS) and the run number of the DoE.





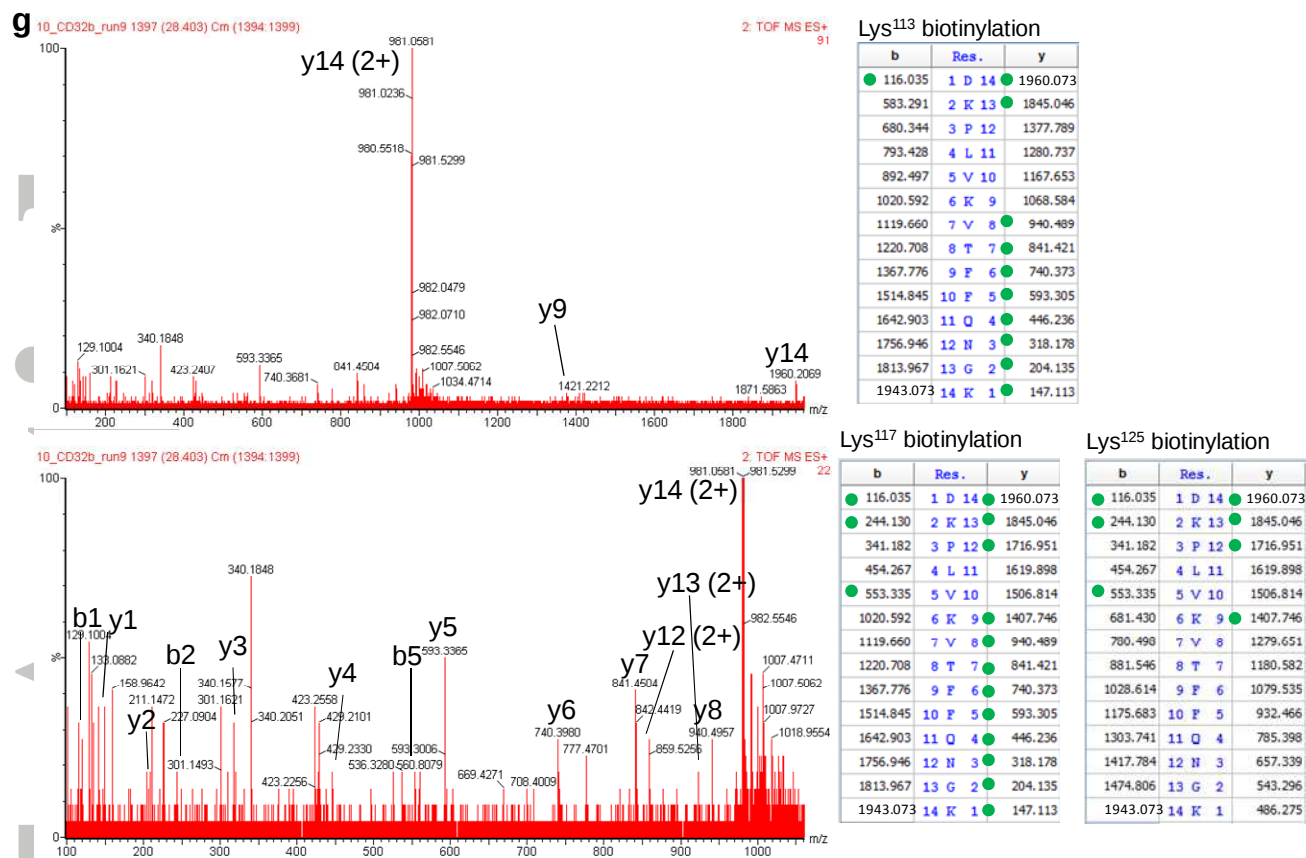


Figure S-4 a) Part of the tryptic peptide mapping chromatogram (10-30 minutes) of the control sample (red) and a biotinylated sample (green) showing the same peptide T112-117 without biotin (11.2 minutes), T112-117 with a biotin (21.4 minutes) and in a missed cleavage site T112-125 with a biotin (28.4 minutes). Mass spectra corresponding to the peaks at 11.2 minutes (b), 21.4 minutes (c) and 28.4 minutes (d) of the control sample (red) and the biotinylated sample (green), showing a decrease in peak intensity of the unmodified peptide T112-117 in the biotinylated sample, and no peaks of the biotinylated peptides T112-117 and T112-125 in the control sample. MS/MS fragmentation spectra of the unmodified peptide T112-117 (e) and of biotinylated peptides T112-117 (f) and T112-125 (g) including the expected fragment tables with b- and y- ions for the different potential biotinylation sites are shown at the right side. Green bullets indicate the fragment ions that are detected. The unmodified peptide T112-117 is confirmed by the full y-series that is detected (e). Biotinylation in peptide T112-117 (f) is confirmed at Lys¹¹³ by the full y-series and 4 out of 6 b-series ions. The alternative biotinylation site (Lys¹¹⁷) can be eliminated as only 2 y-ions are detected, which are equal to the Lys¹¹³ biotinylation. Biotinylation in peptide T112-125 (g) is confirmed at position Lys¹¹⁷ by the nearly complete y-series. Both Lys¹¹³ and Lys¹²⁵ biotinylation sites can be eliminated for this peptide due to the poor coverage of ions in the fragmentation spectrum.

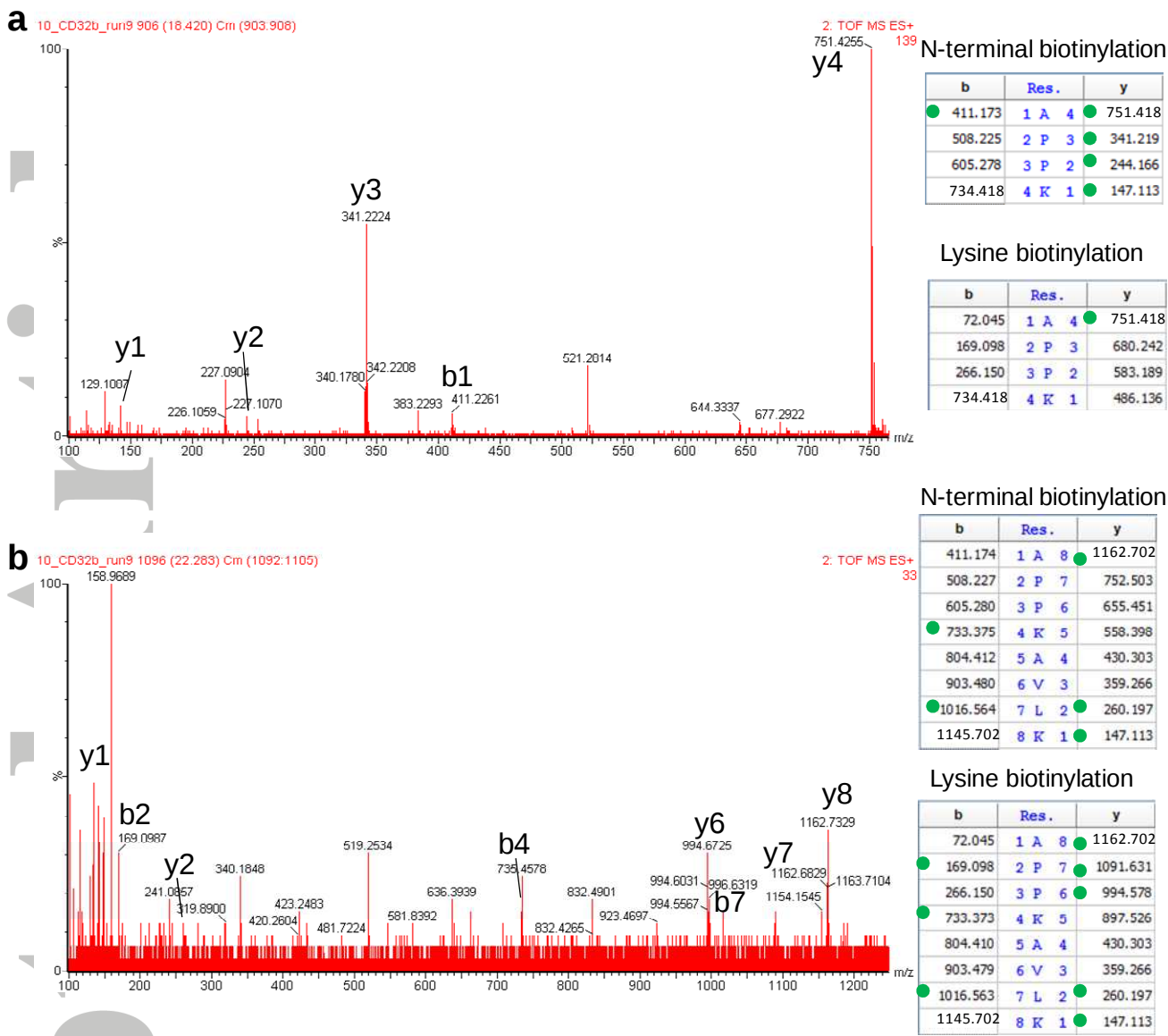


Figure S-5 MS/MS fragmentation spectra of peptide T1-4 modified with biotin (a) and peptide T1-8 modified with biotin (b). Fragmentation tables based on N-terminal biotinylation or lysine biotinylation are shown at the right side. The green bullets indicate which ions are found in the MS/MS spectrum, showing that the N-terminal alanine is biotinylated at peptide T1-4 as all y-ions can be assigned in the MS/MS spectrum and that Lys⁴ is biotinylated in peptide T1-8 because of ions y6, y7 and b2 that can be assigned in this fragmentation spectrum only.

Table S-1 Chymotryptic peptides of FcγRIIb which are modified with biotin, the corresponding masses and retention time in the EIC chromatograms

Peptide	Amino acid sequence	Residue(s)	modification	[M+H] ⁺	m/z	charge	tR (min)
C1-7	APPKAVL	Ala1	-	695.4	695.4	1	16.4
		Lys4	1 biotin	1034.6	517.8	2	23.5
C8-24	KLEPQWINVLQEDSVTL	Lys8	-	2012.0	1006.5	2	29.8
			1 biotin	2351.2	1176.1	2	32.7
C57-66	KANNNDSGEY	Lys57	-	1112.4	556.7	2	10.1

			1 biotin	1451.6	726.3	2	17.4
C111-120	KDKPLVKVTF	Lys111 Lys113 Lys117	-	1174.8	587.9	2	20.0
			1 biotin	1513.8	757.4	2	23.7
			1 biotin	1513.8	757.4	2	24.3
			1 biotin	1513.8	757.4	2	24.6
			2 biotin	1853.0	927.0	2	27.3
			2 biotin	1853.0	927.0	2	27.9
			2 biotin	1853.0	927.0	2	28.3
C121-129	FQNGKSKKF	Lys125	-	1083.6	542.3	2	12.3
		Lys127	1 biotin	1422.8	711.9	2	18.7
		Lys128	1 biotin	1422.8	711.9	2	19.6
C161-173	SSKPVTITVQAPH	Lys163	-	1364.8	682.9	2	16.6
			1 biotin	1704.0	852.5	2	21.5

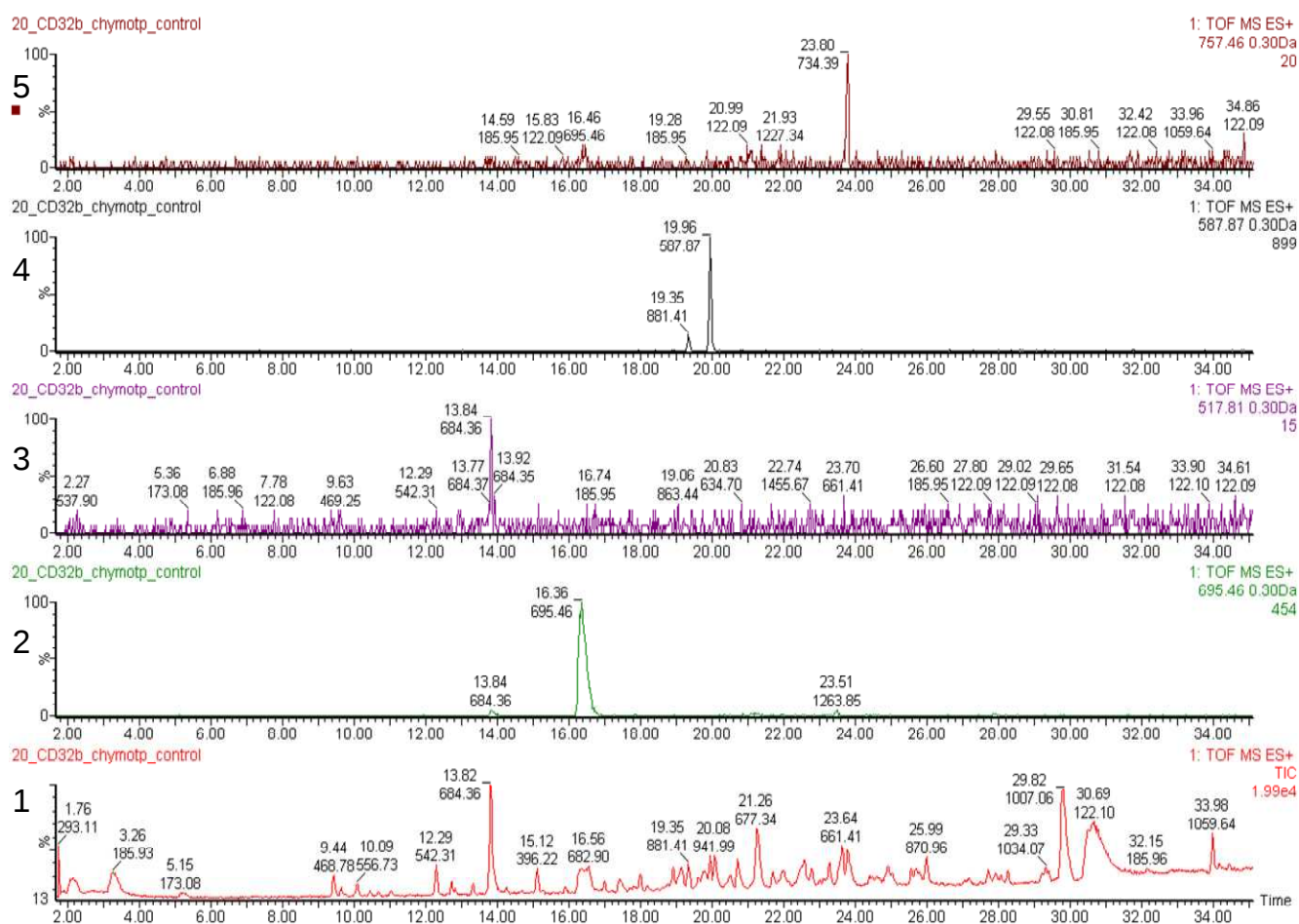


Figure S-6 Chymotryptic digestion of FcγRIIb control sample. Total ion current chromatogram (1) and extracted ion currents of peptide C1-7 without biotin (2) at 16.4 minutes and C1-7+biotin (3) where no peak with the correct mass is detected, peptide C111-120 without biotin (4) at 19.9 minutes and C111-120+biotin (5) where no peak with the correct mass is detected.

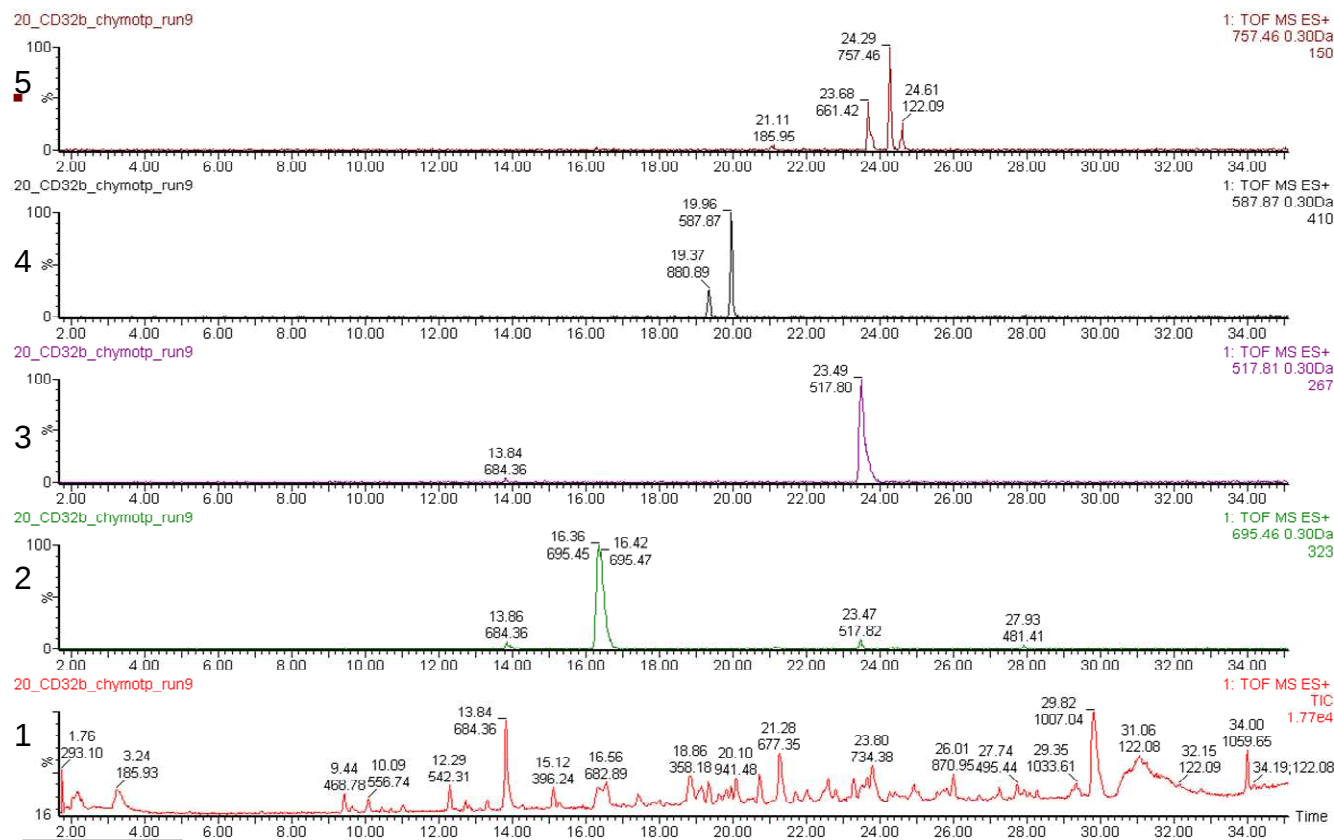


Figure S-7 Chymotryptic digestion of FcyRIIb biotinylated sample. Total ion current chromatogram (1) and extracted ion currents of peptide C1-7 without biotin (2) at 16.4 minutes and C1-7+biotin (3) at 23.5 minutes, peptide C111-120 without biotin (4) at 19.9 minutes and C111-120+biotin (5) at 23.9, 24.3 and 24.6 minutes. Peptide C111-120 contains three lysines that can possibly be biotinylated and these can be resolved on the reversed phase C18 column, appearing as three separate peaks with the same mass.

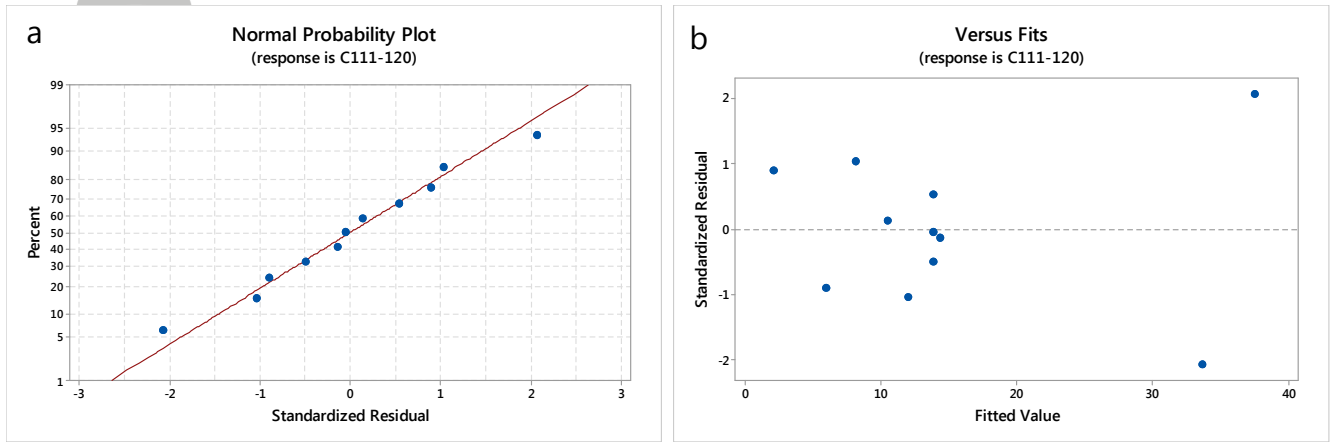


Figure S-8 Normal probability plot (a) and plot of standardized residuals versus fits (b) are shown to support the statistical analysis of the DoE. The standardized residuals in the normal probability plot (a) are linearly distributed and the standardized residuals plotted against the fitted values are randomly distributed (b). Both graphs indicate a proper statistical evaluation of the peptide biotinylation