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# Peer Review File

## Generation of binder-format-payload conjugate-matrices by antibody chain-exchange

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This manuscript has been previously reviewed at another journal. This document only contains reviewer comments, rebuttal and decision letters for versions considered at Nature Communications.

Parts of this Peer Review File have been redacted as indicated to remove mentions of previous journals.

**This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.**

**Attachments originally included by the reviewers as part of their assessment can be found at the end of this file.**

Version 0:

Reviewer comments:

Reviewer #2

(Remarks to the Author)

[**Editorial note: Reviewer 2 provided their comments as an attachment.**]

Reviewer #4

(Remarks to the Author)
I was reviewer 4 of the manuscript that was originally submitted to **[redacted]**. In the manuscript now under evaluation in ncomm, the authors responded in full to all the comments I made originally (where my recommendation was "minor revision". Therefore, my present recommendation is to accept.

I will not comment on the evaluations of the 3 other reviewers. This is up to them.

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Referee #1 (Remarks to the Author): This team has published (Nat Commun 2020) an antibody-chain exchange technology approach for the production of binder-format matrices of bispecific antibodies (ForCE). Here the concept of antibody-chain exchange was extended to antibody-drug conjugates (ADCs) to generate a platform the authors term (pair-ForCE). The authors transfer different payloads from Fc donor-payload conjugates to binder-acceptors by chain exchange. Using this approach, they generated a matrix of ADCs targeting HER2. This matrix was designed to include ADCs targeting HER2 that differ in specificity, format, avidity, site of conjugation, type of payload and number of payloads per antibody. These were tested using simple immunoprecipitation, flow cytometry for internalisation and cell viability. 1. In terms of technological advance this current submission does not add relative to the previous study with the only difference being the use of Fc domains that are previously conjugated with linker+drugs. There remains many issues with creating the next-generation of more tolerable and efficacious ADCs. However, pair-ForCE fails to truly deliver new insight for the development of ADCs. For example, finding that avidity (bivalent) ADCs internalise better and thus are more cytotoxic in cells is not new neither unexpected.

It is well known and accepted in the antibody-engineering field that the format of bispecific and/or multi-functional antibodies has a strong influence on their functionality. The same aspect, however, has not yet been thoroughly addressed for ADC formats. We do agree with the reviewer that "…there remains many issues with creating the next-generation of more tolerable and efficacious ADCs…". However, we disagree with the conclusion that pair- ForCE fails to truly deliver new insight. The stated argument "… that avidity (bivalent) ADCs internalize better and thus are more cytotoxic in cells…" is indeed neither new nor unexpected. That individual finding does indeed validate our technology. The reviewer may, however have overlooked the additional unexpected data enabled by our technology, which define binder/format/payload combinations for the same target with similar activities, without avidity effects. Additional new aspects described in our work include combinations of exchange technologies with transglutaminase-coupling and click-chemistry enhanced conjugation. We've now better explained the novelty and versatility of our technology, and highlighted the surprising findings in the results and discussion sections (see pages 16 and 19). We also provided additional experiments that demonstrate the activity of pair-ForCE-generated HER2-binding MMAE-ADCs on cell lines with different HER2- expression levels (see page 16 and Figure S12). These additional experiments were conceptualized and performed by Michaela Fischer. For this reason we added her to the author list.

The Referee #1 states correctly, that the presented technology does not address or provide a solution to *in vivo* toxicity, the major problem/difficulty in ADC development. However, as the authors state, pair-ForCE does allow format optimization, which is also a critical aspect in ADC development, especially in terms of cellular uptake. In the presented case, the finding that bi-valent HER-2 is more potent may be not surprising. However, not every receptor tolerates bivalent binding modes for internalization. Therefore, I find the argumentations of the authors reasonable.

2. Furthermore there is a lack of characterisation of the constructs: there is no mass spectrometry or binding data (BLI or SPR), and there is no correlation with activity in vivo. There are no statistics for the data or information of replicates. The graphs on the paper do not show the data points and / or have information regarding biological replicates, and even more important, there are no statistics

We agree with the reviewer that binding kinetics of the ADCs can be included as a control, even though (and in contrast to many other ADCs) our technology assures that binding regions should not be affected by conjugation. Therefore we measured the binding kinetics of the HER2-targeted, pair-ForCE-generated ADCs by SPR. We have clearly stated those findings in the results section (see page 12) and included the corresponding data as supplementary Figure S9. The SPR experiments show that pair-ForCE does not influence the binding kinetics or affinity to HER2. This demonstrates a major advantage of pair-ForCE, namely that the site-specific conjugation that we employ does not interfere with the antibody binding regions. The SPR experiments were designed, performed, and analyzed by Jack Bates. For this reason, we've added him to the author list. We've also provided additional information about statistics and replicates in the figure legends.

I disagree in this point with both, the referee #1 and the authors. The idea of pair-ForcCE is not that molecules are generated for analytical characterization like binding affinity, stability, etc.. The technology allows a fast screening of a wide array of different ADC formats and linkers in a functional assay as shown in the cytotoxicity measurement data. Binding affinity, homogenicity, stability, and other parameters can be measured once the optimal format is found. It is important to note that the optimization of the different building blocks of an ADC are often done individually. Here the authors focus on the format, and only on the format.

3. Homogeneity is studied by mass spectrometry and there is no mass spectrometry disclosed only HIC which is insufficient.

We would like to refer the reviewer to Table 1 in the original manuscript, where we already provide a summary of the mass spectrometry DAR determination. We also provide a detailed description of the mass spectrometry protocol in the materials and methods section. Additionally, we agree with the reviewer about the inclusion of MS spectra. Since it is not feasible to include all of the raw MS data in the manuscript, we have now included exemplary annotated MS spectra for MMAE-conjugated Fc donors and HER2-targeting ADC products in Figure S6. Along with the MS spectra, we also added information about which peaks were used for DAR calculation in Fig. S6.

The authors are correct, they provide MS data with a sufficient method description. As I mentioned under 2., the MS date are here only to show that the pair-ForCE allow the generation of the desired molecules but are later not part of a screening experiment. Homogeneity is only of limited relevance at this point.

4. The figures are difficult to read because the quality of the images is very poor.

We apologize for this issue. The quality of our figures may have been compromised during the PDF-conversion process of the submission file. We have now improved the image quality of the figures in the manuscript PDF and will also upload each figure additionally as individual high quality files

I had no issue with the figures. However, I am not the one to judge on that. The editors will know what is acceptable.

Referee #2 (Remarks to the Author):

1. Reviewer report: Generation of binder-format-payload conjugate-matrices by antibody chain-exchange

A. Summary of the key results: The article describes a further development, called pair-ForCE, of a previously presented technology ForCE, which allows the format optimization of bispecific antibodies. In a first set of experiments the authors recapitulate results from the ForCE publication by demonstrating the successful exchange of dummy chains resulting in functional molecules with Fab fragment fused at the N-terminus, C-terminus or both. Next, the two anti-Her2 antibodies Trastuzumab and Pertuzumab were used as examples to generate antibodies labelled with pHAb, which carried a Fab fragment at the N-terminus, C-terminus or both. Thereby the pHAb dye was introduced by the empty, non-Fab carrying chain, which was chemically randomly conjugated with the dye and purified. As a result a set of 3 different pHAb labelled antibody for each of the two anti-Her2 antibodies was received. Mass spectrometry analysis of the 2x 3 constructs showed comparable pHAb to antibody ratios for each the construct. This demonstrated that the technology allows homogeneous labelling for various different constructs. On a side note it was shown that the technology allows the labelling of an anti-EGFR antibody with HRP or biotin, which demonstrates the versatility of possible labels of the technology. Along this line it was shown that monovalent Trastuzumab-derived molecule with C-terminal GFP fusion can be produces using pair-ForCE. This molecule specifically recognized HER2 positive cells, while the starting materials did not stain the cells. The introduction of a Q-tag at two different positions in the Fc, allowed the transglutaminase mediated site-specific conjugation of a chemically bio-orthogonal azid group. Using click chemistry MMAE was attached in each of the postions individually and simultainiously. Four sets of antibody variants were generated with Fab fragment fused at the N-terminus, C-terminus or both, resulting in 4Ab x 3Formats x 3Conjugations = 36 different constructs. As expected, the conjugation to antibody ratio was very similar between all constructs, depending on single or double conjugation. Interesting, the variability was not significantly smaller then for the randomly conjugation of the pH sensitive dye using EDC/NHS chemistry. This questions the effectiveness of the two step labeling approach. In uptake experiments with SK-BR-3 cell, using the anti-HER2 antibody constructs labelled with the AF488, it was shown that the molecules with the C- and N-terminal Fab fusion were most effectively internalized, especially at low concentrations. The MMAE labelled constructs were compared in a cell proliferation assay. Non-surprisingly, the double conjugated, N- and C-terminal Fab fused constructs showed

the highest potency. Interestingly, it was found the potency dependent strongest on the number of MMAE conjugation, then the format, while the choice of the antibody had almost no impact.

B. Originality and significance: if not novel, please include reference Antibody drug conjugates are an emerging class of therapeutics not only in the field of cancer but also in infectious disease There is a revival of ADC going on in the field of cancer. Better understanding of target proteins, availability of bispecific antibodies technologies, improved conjugation and linker chemistry allowed the development and clinical testing of several ADC in the last couple of years. I share the authors opinion that screening of different antibodies, conjugation sites, linker chemistries and payloads are essential for the development and optimizations of ADCs. The proposed pair-ForCE technology, which is an improvement of the previously published ForCE technology, is a step into the right direction. From my perspective, the strength of the technology comes with the homogeneity of the labeling among the constructs, which allows a direct comparison. Another plus is that the same antibodies can be conjugated in various ways, so that they can be used in various different assays (e.g. ELISA, flow cytometry, SPR, proliferation assay). This allows a fast characterization of different binders in combination with various payloads. In contrast to the authors, I do not see the EDC/NHS chemistry as problematic. Most antibodies do not have free lysins in or in close proximity to their CDRs or other functional domains like FcRn or FcgR recognicion sites. Note, that thousends of antibodies have been conjugated using random EDC/NHS chemistry without losing their binding property.

C. Data & methodology: validity of approach, quality of data, quality of presentation In general the data has a good quality and the methods are described accurate and in reasonable detail. For the constructs used for the proliferation assay it would be great if SDS-PAGE and analytical SEC data could be provided. The amount of functional molecule after chain-exchange has a direct impact on the assay readout. A brief description of the gating strategy for the flow experiments would be helpful.

We agree with the reviewer and have now provided CE-SDS and analytical SEC data for the Fc donors, the HER2- binding acceptor modules, and the resulting pair-ForCE products used in the proliferation assay (see supplementary Figure S7 and page 12 in the main text). The data confirms the high quality of the pair-ForCE ADC products. Note that we could not determine the monomer purity of MMAE-conjugated constructs using our standard analytical SEC HPLC column. This is because the hydrophobic MMAE moieties interact with the column material and cause peak broadening and a shift in retention times. In order to properly determine monomer purity of these molecules with aSEC, one would need to test specialized HPLC columns which interact minimally with hydrophobic payloads – a costly and time-intensive effort. However, quantification of the percentage of aggregates is still possible with our standard column, and we demonstrate that there are no aggregates detected in the majority of samples. A few samples have only a very minimal percentages of aggregates (0.3% - 0.5%). Overall, these controls show that the molecules in the proliferation assay are of high quality. We have explained the QC outlined above in detail in the figure legend of Figure S7. We also

now provide information about the gating strategy for the flow cytometry experiments in the materials and methods section.

The authors address my criticism with reasonable arguments and additional experiments. I regard this point as resolved.

D. Appropriate use of statistics and treatment of uncertainties: The accuracy of the DAR numbers seems to be higher than the variations among experiment. I understand that the complexity of the experiment does not allow triplicates and therefore standard deviations cannot be provided. However, I guess that a repletion of the experiment would result in a deviation bigger than the double digit given after the comma.

We thank the reviewer for the comment. As the reviewer correctly pointed out, performing mass spectrometry analysis of independently generated triplicate molecules would add excess complexity to this study and is beyond the scope of our work, which describes a screening approach for lead identification. The mass spectrometry measurements presented in this study are intended to provide the DAR for comparison between constructs. We understand that the two significant digits after the decimal point may convey a greater sense of certainty in the DAR than we intended. We have therefore updated Table 1 to display only one significant digit. We have also now included exemplary MS spectra that give more insight into how the DAR was calculated (Figure S6).

The authors address my criticism with reasonable arguments and present detailed exemplary data to support their initial date presented. I regard this point as resolved.

E. Conclusions: robustness, validity, reliability: The data generated and the example given makes it hard to estimate how reliable and robust the pair-ForCE technology is. However, it becomes clear that the method does tolerate a wide variety of conjugations and therefore is quite versatile in its application. It was not demonstrated, though, which properties of the payloads or conjugated proteins influence the efficiency of the chain-exchange have. It can be assumed that small molecule conjugates may not be able to alter the overall properties of the Fc, while protein conjugates are unproblematic as long as they exhibit sufficient stability and have no tendency for aggregation. Yield, Tm measurements and aggregation propensity determination would give a hint. However, larger set of various conjugates would be needed to draw the right conclusion.

See also our response to ‘F’ below: we agree that Tm measurements and determination of aggregation propensities are valuable to further assess the pair-ForCE educts and the ADCs resulting from our technology. We have therefore tested the thermal stability of HER2-targeting binder-acceptor molecules, unconjugated and MMAE-conjugated Fc donor modules, and the resulting HER2-targeting, pair-ForCE-generated ADCs. Tm and Tagg were determined using nanoDSF and SLS, respectively (see page 12 and Figure S8). The data show that the majority of the pair-ForCE educts and ADC products have favorable thermal stability, and that chain-exchange does not impact the thermal stability. Please refer to page 12 in the main text for a more detailed explanation. The thermal stability measurements were performed and

analyzed by Verena Maier. For this reason, we've added her to the author list. We also now include analytical SEC and CE-SDS analysis showing the quality of these molecules. This data is now available as supplementary Figure S7. Regarding yields of pair-ForCE educts, we observe good expression yields of these constructs in transiently transfected HEK293 Expi cells. The yields are similar to standard IgGs, as published in our earlier paper describing the ForCE technology (Dengl S., et al 2020, Nat. Comm., https://doi.org/10.1038/s41467-020-18477-7). We have added this information to the "Expression and Purification" section of the materials and methods.

My argument made was well understood be the authors. Their additional measurements (Tm and Tagg) support their claims. The numbers in the table are given in an accuracy, which exceed the precision of a single or triple measurement of the used technology.

F. Suggested improvements: experiments, data for possible revision 1. The argument that "the generation of ideal ADCs is complex, because their functionality depends on many parameters" is valid. But the authors make the reader believe that many of these parameters cannot addressed independently, which does not hold. Indeed, some parameters like payload toxicity, linker stability and internalization rate are strongly depending on each other and therefore are ideally optimized simultaneously. In contrast, it has been shown that random conjugation of antibodies with small molecules does alter the binding properties only minimally in most cases (PMID: 22531451), while its electrostatic properties or thermostability can be affected (PMID: 23777335). I suggest a more detailed analysis of know effects of payload conjugations on the antibody properties and to highlight the properties, where simultaneous optimization is essential.

We agree with the reviewer that the most important parameters for optimal ADC design are dependent on each other, and thus need to be optimized simultaneously. This is indeed the primary reason for developing pair-ForCE, and the main advantage of pair-ForCE as an ADC optimization platform. However, as the reviewer correctly noted, some parameters such as conjugation method could be optimized individually. We now address these topics more thoroughly in the discussion section (see pages 19-20). While many antibodies tolerate random NHS conjugation without altering binding affinity (however not all antibodies, especially those with lambda light chains – see answer to question 2 below), using site-specific Fc-labeling as in this study is a good starting point, because it guarantees that the binding regions will not be affected. Indeed, we now show using SPR that not only are binding regions unaffected when using pair-ForCE, the thermal stability of the resulting products is also maintained and not negatively impacted by chain-exchange (see Figures S8-S9 and page 12 of the main text).

I agree with the answer of the authors. No additional comments from my side.

2. It is important to note that lysins highly underrepresented in CDRs (PMID: 19875695). As a result the majority of antibodies tolerate random conjugation using EDC/NHS chemistry. I suggest the authors take this into account in their argumentation, why side-specific conjugation is needed.

We thank the reviewer for the comment. We agree that a potential loss of binding due to conjugation of lysines in the CDRs is only problematic for select antibodies, as lysines are underrepresented in CDRs (with the exception of antibodies containing lambda light chains – see page 3 of the main text and PMID: 37876265). We now directly address this topic in the introduction, results, and discussion sections (see pages 3, 8 and 19). We now put more focus on the other important reasons for site-specific conjugation, namely for increased molecule homogeneity for an improved PK profile, better therapeutic index, and increased payload delivery (less clearance of high DAR antibodies, less competition against low DAR antibodies). Indeed, third-generation ADCs are increasingly focusing on site-specific conjugation for the above-mentioned reasons (see PMID: 37790810), reflecting the need for a site-specific labeling approach in an ADC screening platform like pair-ForCE.

I see this point as resolved.

3. As mentioned in the summary, it is unclear to which degree the molecule quality and the change of properties like thermostability affect the data from the proliferation assay. I recommend performing analytical SEC and Tm determination to underline the findings from the cellular assays.

We agree with the reviewer that this data would make a strong addition to the manuscript. Please also refer to our response to “C” above, where we also addressed this topic. Therefore, we tested the thermal stability of the HER2- targeting binder-acceptor molecules and pair-ForCE-generated ADCs, as well as unconjugated and conjugated Fc donor molecules (Figure S8). We also now include analytical SEC and CE-SDS data showing the quality of these molecules (Figure S7). The thermal stability data shows that chain-exchange does not destabilize the product molecules. Additionally, analytical SEC data shows that there are no aggregates in the product molecules and CESDS data shows that the product molecules are of high purity (see page 12).

I see this point as well addressed and therefore resolved.

4. Figure S2: I suggest to add two bands using an anti-huFc HRP conjugated antibody for the detection, to demonstrate that indeed the correct antibody was detected. Also, I recommend to run HRP separately, to demonstrate its functionality in the experiment.

We recognize that the request for additional controls in Fig. S2 may be caused by us cropping the blot instead of showing the full blot. We’ve therefore now included the full blot which shows a ‘clean’ unambiguous band for the HRP-conjugated pair-ForCE product without background bands or additional artefacts. We agree with the reviewer about the control experiment using an anti-huFc-HRP secondary antibody, and have thus added this control as an additional lane. This control shows that the band detected by the secondary antibody (anti-huFc) is the same size as the band detected by the HRP-conjugated primary antibody in the previous lane, demonstrating the specificity and functionality of the HRP-conjugated pair-ForCE product. Regarding running HRP separately as a control, we already demonstrate its functionality by the presence of an EGFR-specific band in both lanes. Furthermore, HRP is a

standard and established Western Blot reagent, and is very often conjugated to secondary antibodies for use in ECL detection experiments.

The authors addressed my criticism appropriately. No additional comment from my side.

G. References: appropriate credit to previous work? 5. I suggest to refer to 2-3 review articles as references when the authors explain the difficulty ADC development.

We agree with the reviewer and have included additional citations that explain the challenges in ADC development (see page 3 of the introduction).

I see this point as resolved.

H. Clarity and context: lucidity of abstract/summary, appropriateness of abstract, introduction and conclusions According to my understanding the authors demonstrate that the pair-ForCE technology can be used a) characterization of antibodies in different assays by conjugation with e.g. biotin, HRP, or fluorophores, or b) for the simultaneous optimization of binding properties (epitope, affinity), conjugation (site, number), and linkers (length, stability). Therefore, I recommend that these two applications are better worked out in the introduction.

We agree with the reviewer and have modified the abstract, introduction, and discussion to better guide the reader to our coverage of these parameters (page 2, page 5, pages 18-19).

I see this point as resolved.

Referee #3 (Remarks to the Author): 1. Brinkmann and coworkers apply in this manuscript a previously published strategy of Fc strand exchange to generate Fc chimera in a straightforward manner, which carry in one arm preloaded compounds such as biotin, dyes, fluorescent proteins and enzymes, or cytotoxic drugs. They claim that this strategy can be used to generate a matrix of molecule combinations that facilitates candidate format pre-screening. The authors indeed showed convincing experimental examples corroborating the notion that the pair-ForCE technology is an excellent tool for the generation of antibodies that can be simultaneously and easily modified in a defined manner with different payloads using pre-modified in stock Fc modules. As application examples, the authors showed that the method is useful for determination of uptake efficiency of antibodies that address different epitopes on the same target. They also showed that it facilitates the estimation of influence of the conjugation site on ADC potency. However, there also exist some limitations that are related to transferability and developability when moving from these surrogate molecules to normal antibody formats for ADC generation, which are not clearly outlined in the manuscript: When using nonspecific coupling of payloads to Fc for generation of Fab Fc heterodimers via pair-ForCE technology, this results in variants that are exclusively labelled at their FC part in one Fc monomer. Transfer to a normal full length antibody format makes a big difference since the payload will be conjugated to both Fc monomers and conjugation will not be restricted to the Fc part (upon lysine coupling). A similar restriction of transferability also holds for site-specific conjugation, where in a non-surrogate final antibody format used for further development the

theoretical DAR will double upon payload conjugation, which occurs then to both Fc monomers. Further format limitations become obvious in the internalization studies performed. A trastuzumab N+C Fab construct is used as a surrogate for a bivalent antibody format. It displays high internalization (as expected) but a normal bivalent N+N format cannot be produced with this method (at least not in a matrix format), which may behave different with respect to internalization and endosomal/lysosomal trafficking. Moreover, the technology can't be applied to ADC generation via interchain disulphide bond opening and modification, a nowadays broadly applied method for ADC generation. These potential restrictions should be clearly indicated. Nevertheless, the technology is of general value for example for rapid comparison of the potency of different cytotoxic payloads and other applications beyond ADC generation such as fluorescent protein or enzyme labelling of antibodies for epitope binning or imaging purposes etc. We thank the reviewer for correctly identifying our work as a technology that is designed to be applied in early screening phases to identify the best-performing binder-format-payload combinations. Admittedly, highthroughput screening technologies have limitations, which the reviewer correctly identified as differences compared to final ADC formats and incompatibility with reducible linkers and hinge cysteine conjugation. We now address and expand upon those limitations in more detail in the discussion section, and have added suggestions about how this method could be modified to potentially be compatible with reducible linkers and hinge cysteine conjugation (see page 20). 2. Typo: Li 273: Replace moeity by moiety We thank the reviewer for catching this typo. We corrected the typo and re-checked the whole manuscript for spelling and grammar

Referee #4 (Remarks to the Author): Review of **[redacted]**

In this article, the authors begin by describing the challenges of testing the many variable involved in the preparation of Antibody-Drug conjugates (ADCs). The combination of the two modalities – the antibody and the drug, must retain both antibody binding affinity and specificity and the payload functionalities. As a result, the generation of ideal ADCs is complex, because their functionality depends on many parameters including binder identity, binder format, linker composition, conjugation site, stoichiometry (DAR), and conjugation method. The authors wrote that, because the production of matrices that cover all possible parameters is laborious, the generation and identification of optimal ADCs from many possible combinations is a major challenge. To overcome this bottleneck, they adapted their chain-exchange technology originally designed to produce binderformat matrices of bispecific antibodies (ForCE, published in 2020, REF 24) towards the generation of binderformat- payload matrices (pair-ForCE). In pair-ForCE, antibody derivatives with an exchange-enabled Fc-heterodimer (enabled by “knobs into holes” combined with strategically-placed charged residues, are combined with complementary payload-coupled Fc donor modules. Upon gentle reduction with TCEP, spontaneous chain exchange transfers the payload to binder acceptor modules in different formats, and subsequent affinity-capture removes unreacted educts and undesired by-products. The ADC is recovered in high purity in the flow-through fraction of the column, resulting in matrices of homogenous antibody-payload combinations. The authors provide examples that demonstrate the robustness and versatility of this approach include random and site-directed attachment of small compounds such as dyes, labels, biotin, and cytotoxic payloads, as well as large molecules such as enhanced-GFP. The relevance of

assessing pair-ForCE-generated combination matrices and deriving rules for ADC optimization is shown exemplarily for HER2-binding, MMAE-containing ADCs. For these molecules, the authors demonstrate the application of pair-ForCE to assess the variables of binder selection, format, valency, internalization, conjugation positions, and DAR. The authors conclude that "The analysis of this ADC matrix reveals the 'format defines function' rule applies not only to bispecific antibodies, but also to ADCs." This study is well-designed, elegant, well-executed and well-written. I have a few comments which may be regarded as recommendation for a minor revision. I'm raising a few questions, most of which can be addressed in the discussion. 1) The authors evaluate monovalent antibodies where the antibody Fab is either N-terminal or C-terminal, and bivalent antibodies with Fabs at both ends. "Real life" ADCs are almost entirely based on full-size IgGs to which the drug is conjugated through a linker that may or may not be labile. ADCs do not look like the model ADCs assembled by "pair-ForCE". With the term 'format defines function' in mind, Do the authors believe that the lessons learned by the thorough evaluation of format, valency, internalization, conjugation positions, and DAR can be translated to IgG-based ADCs? We thank the reviewer for the comment, and we agree that pair-ForCE molecules may not match the final ADC format, which is usually based on a full IgG (see also our reply above to reviewer 3, comment 1). As a highthroughput screening approach, pair-ForCE allows for comparison of important parameters influencing ADC design, namely comparison of cytotoxic activity, binding, internalization, and biophysical properties between different payloads, conjugation sites, DAR, and formats. We do believe that most 'design rules' derived from pair- ForCE can be applied to final ADC compositions. One example is the finding (see comment from reviewer 1) that bivalency (even though achieved by a different format in pair-ForCE) increases internalization and thereby enhances activity when other variables are held constant. This is expected for HER2-binding constructs, as bivalent binding has been shown to lead to increased internalization compared to monovalent binding (PMID: 34253591). We've now addressed this topic in more detail in the discussion section (see page 20). 2) drug conjugations to the "Donor molecules" was demonstrated with random conjugation and with site-specific conjugation. A find the statement in the discussion beginning with "The advantage of pair-ForCE for generating ADC matrices compared to individual conjugations lies in the application of Fc donor stock reagents . . . very convincing. Still, the study did not pursue DAR>2 (probably for a concern of hydrophobicity and aggregation). Is it feasible to achieve higher DARs with "pair-ForCE"? Yes, a higher DAR can be achieved by pair-FORCE, e.g. by adding 3 transglutaminase recognition sites to one Fc. We have tried this in preliminary pilot experiments and the DAR is reproducible, but one of the recognition sequences proved to be somewhat unstable and thereby resulted in an average DAR of 2.5 instead of 3. Another way to increase the DAR is to use branched linkers to attach more than one payload to any given position. We've added this information to the discussion section (see page 20). 3) a related question: to generate high-DAR ADCs, the Donor molecules should be conjugated to a very high DAR – because only a half of the conjugated drug ends up in the ADC. This could perhaps be possible with very robust donor molecules. Indeed, on page 9, the authors write: "Such donor molecules can be expressed with similar yields compared to standard IgGs, and have favorable biophysical properties (data not shown)". I wish some data were shared with the readers to convince them how robust the donor molecules are (un terms of stability, solubility, Tm etc). It seems important to me since in "Pair FOrcE" the requirements from the donors is more

demanding compared to “FOrcE” – here they are required to tolerate and survive conjugation. We agree with the reviewer and have therefore added thermal stability data (Tm and Tagg) of MMAE-conjugated Fc donor molecules, HER2-targeting binder acceptor molecules, and the HER2-targeting pair-ForCE products to show their robustness (see page 12 and Figure S8). Overall, the Fc donor molecules showed reasonable thermal stability. The conjugated donors were slightly less stable than their unconjugated counterparts, which was expected. Since all binder-acceptor educts and corresponding pair-ForCE products showed good thermal stability, we believe the stability of the Fc donor molecules is sufficient for their purpose as transfer reagents. We now expand on this point on page 12 of the results section. Regarding the expression yields of Fc donor molecules: they are similar to standard IgGs, as published in our earlier paper describing the ForCE technology (Dengl S., et al 2020, Nat. Comm., https://doi.org/10.1038/s41467-020-18477-7). We have added this information to the “Expression and Purification” section of the materials and methods. As for the reviewing questions: Are the manuscript’s conclusions supported by the data? R: I believe they are. Have the authors included appropriate statistics and chemical and biological characterization? R: I believe they are. And I do feel that this work will have a significant impact on the field and be of broad interest to chemical biologists?