Investigating Complement Mediated Interference in Class I HLA-Specific Antibodies Following Renal Transplantation

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Highlights

- 1.5% of bead reactions were affected by prozone
- Using ROC curve analysis, EDTA has >90% sensitivity and ~100% specificity in overriding the prozone effect due to complement mediated interference (CMI)
- Twenty percent of an unselected sensitised post-transplant cohort display CMI in class I HLA-specific antibody testing

Abstract

INTRODUCTION: Single antigen bead (SAB) testing for HLA-specific antibody enables efficient organ allocation and aids in the diagnosis of antibody mediated rejection. In this retrospective cohort study, a population of kidney transplant recipients possessing HLA Class I antibodies was used to evaluate the best method for resolving complement interference, the so called "prozone" effect. The aim was to compare the use of EDTA versus a Biotin-Streptavidin Complex (BSC) as methodological approaches for abating the prozone effect using a fixed 1 in 10 dilution as validation.

METHODS: One hundred and seventeen patients transplanted in our centre between 2009-2014 were identified as having class I HLA-specific antibody(-ies) using a Labscreen® Mixed assay. Positive sera underwent class I HLA-specific SAB testing; for comparison a standard SAB with and without EDTA, BSC and dilution (1 in 10) modifications were utilized. Samples were processed on the Luminex platform generating 11349 bead reactions for analysis.

RESULTS: We identified sera from 23 patients giving rise to 170 bead reactions showing complement interference. Using linear modelling, we observed slightly higher MFIs on average in both EDTA and BSC modifications when compared to the standard assay, allowing the nominal threshold MFI of 2000 in the standard assay to be adjusted to 2097 and 2033 in the EDTA and BSC assays respectively. We calculated 99% prediction intervals (PI) to establish outlier bead reactions for each assay. The 1 in 10 dilution was used as a crosscheck for determining which prozone reactions were overcome by EDTA and BSC. Using ROC curve analysis, EDTA was found to be ~90% sensitive and 100% specific compared to BSC which was ~60% sensitive and 100% specific in ameliorating prozone positive reactions at the thresholds defined by linear models.

DISCUSSION: Our data indicates that both EDTA and BSC are suitable assays in overcoming CMI. We recommend that all clinical laboratories adopt a validated assay designed specifically to abrogate CMI for all potential renal transplant recipients, as the standard assay is inhibited in nearly 20% of a post-transplant cohort.

Keywords

SAB; prozone; EDTA; biotin-streptavidin; renal transplant

Abbreviations

AbMR	Antibody Mediated Rejection
BSC	Biotin-Streptavidin Complex
Da/kDa	Dalton/Kilo-dalton
DART	Dual Antibody Rapid Test
CMI	Complement Mediated Inhibition
cPRA	Calculated Panel Reactive Antibodies
EDTA	Ethylenediamine Tetra-acetic Acid
FNR	False Negative Rate
FPR	False Positive Rate
HLA	Human Leukocyte Antigen
MFI	Mean Fluorescence Intensity
PE	Phycoerythrin
SAB	Single Antigen Bead
PBS	Phosphate Buffer Solution
PI	Prediction Interval
QC	Quality Control
ROC	Receiver Operator Characteristic
VXM	Virtual Cross Match

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Figures in Print

Colour artwork is required for all figures going to print and for the supplemental figures.

1. Introduction

Single antigen bead (SAB) assays for identifying HLA-specific antibody have been a major asset to the renal transplant community, enabling sensitive and specific determination of pre-transplant sensitisation to class I and class II Human Leukocyte Antigens (HLA) and in the investigation of antibody mediated rejection (AbMR) post-transplant. SAB assays have helped to establish more efficient peri-transplant protocols by allowing patients to proceed to transplant without performing a prospective physical crossmatch. Thorough evaluation of patient sensitisation using SAB assays resulted in acceptance of the so-called virtual crossmatch (VXM) to become widely utilised world-wide. This is particularly significant in deceased donor transplantation where opportunities to reduce cold ischaemia are valuable.

The basic premise of SAB testing is that each bead represents a single HLA molecule. A patient's serum is applied to the beads, and any HLA-specific antibody(-ies) in the serum will bind to beads expressing the corresponding epitopes. A fluorescently labelled anti-human IgG antibody is then applied, generating a signal referred to as mean fluorescence intensity (MFI). Any beads displaying an MFI value above a pre-determined threshold indicate the patient has HLA-specific antibody(-ies) to the antigen(s) on those beads

Various limitations to the SAB assays have been reported both in terms of false negativity and false positivity [1]. The focus of this paper is primarily on false negativity. The mechanisms leading to false negative results are believed to be attributable to two linked phenomena, namely steric hindrance and the so called "prozone effect". Simply put, steric hindrance, is overcrowding, where a sufficiently high concentration of patient's antibody prevents binding of the HLA-specific antibody to its corresponding bead, giving a lower signal on the SAB test than would normally be expected [2], [3]. The prozone effect also occurs when there is high concentration of anti-HLA antibody. Prozone is reliant on the close spatial relationship of anti-HLA antibodies which results in the formation of immune complexes which interfere with the binding of the detection antibody; these include complement mediated processes [4] and IgM [5]. Johannes F.M Jacobs *et al* demonstrated these effects; Figure 1 has been redrawn on the basis of their work. In particular, c and d show steric hindrance and complement fixation leading to immune complex formation which impedes binding of the detection molecule (we refer to this effect as the prozone effect) [6].

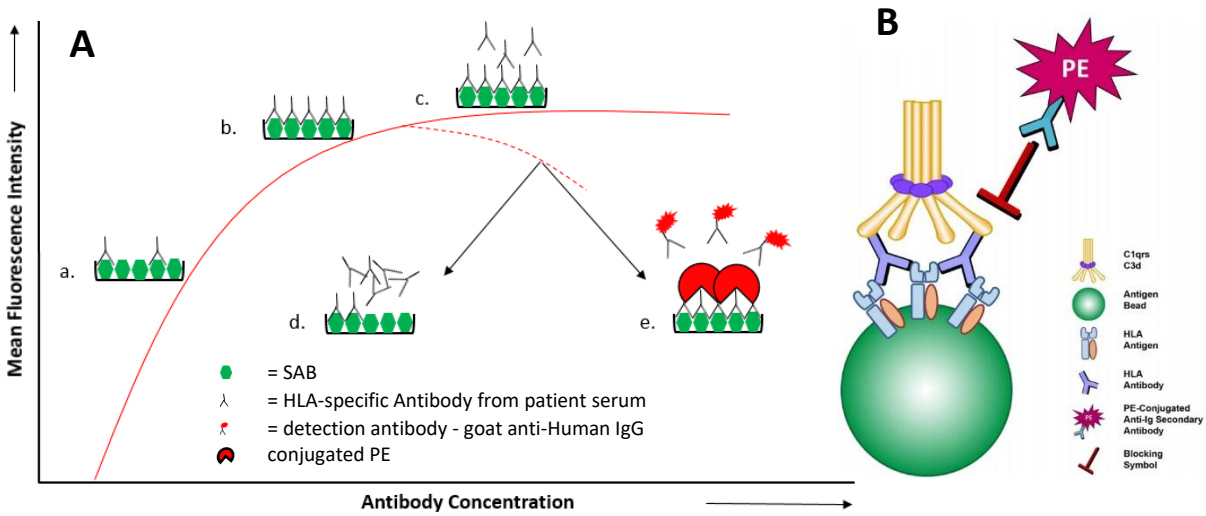

Figure 1 A): a) A non-saturating concentration of HLA specific antibody binds to SABs with increasing MFI; b) the assay is saturated with high concentration antibody providing maximum signal; c) There is redundancy in the assay with excess antibody that cannot bind, but in the absence of inhibitory influences the maximum signal is still achieved; d) very high concentration of antibody causes overcrowding which impedes access of antibody to its binding sites on the SABs, thereby reducing MFI; e) The prozone effect is caused by complement fixing antibodies bound to SABs triggering the formation of complement mediated immune complexes which inhibit binding of the secondary detection antibody and so MFI is reduced (following Johannes F.M Jacobs *et al*); **B)**: A detailed representation of complement-mediated interference (a.k.a. ‘‘prozone” effect). The large C1qrs and C3d complex, bound to primary HLA antibody and residing on the surface of the bead, are thought to sterically block the binding of PE-conjugated anti-Ig secondary antibody. Reproduced with permission from *H Gebel* and *R Bray* [7].

A suggested mechanism for EDTA’s ability to mitigate the prozone is its disruption of the binding of complement component C1 to the Fc portion of IgG1 and IgG3 which is a calcium dependent process [4], [8]. EDTA is inexpensive, widely available and its use incorporated as standard practice in many laboratories worldwide. However, there are concerns that EDTA has potential limitations as its chelation of heavy metal ions is non-specific. Chemically altering the serum may have effects on the SAB assay through mechanisms not yet understood [9].

An experimental modification to the standard SAB test has been suggested using a Biotin-Streptavidin complex (BSC). Work by Bray and Gebel has shown that using a BSC approach can mitigate the prozone effect [7], [10]–[12]. This assay overcomes prozone and steric hindrance by using an additional binding step in which a Biotin conjugated secondary antibody binds with high avidity to the primary antibody, followed by a tertiary step wherein streptavidin (which has a high affinity for Biotin) conjugated to phycoerythrin (PE), becomes the reporter probe. The postulated mechanism of action for this assay is the relationship between steric hindrance and prozone. PE is a large molecule with a mass of approximately 240kDa compared to the filamentous Biotin whose mass is 0.24kDa (x1000 smaller) meaning that the biotin-conjugated secondary antibody can negotiate overcrowding and/or the rigid structure of immune complexes and bind to primary antibodies on the single antigen beads, whereas the bulky PE-conjugate cannot do so directly. The Biotin molecule is attached to the immunoglobulin via a 6-atom spacer thereby extending the biotin molecule away from the antibody surface and facilitating the binding of the avidin-PE conjugate at a distance from the immunoglobulin [7], [10], [12].

Previous studies have inconsistently defined the prozone effect; methods range from stratifying MFI increases with EDTA treatment into categories of magnitude of prozone effect [9], [13] to a doubling of MFI values between the standard assay and the peak MFI in the assay modifications adopted [14]. An additional confounder is that sera are often collected from highly sensitised patients [13], [14] which is independently associated with prozone and hence potentially introducing bias into evaluations of the assay.

One study, using sera from an unselected population of pre- and post-transplant, patients reported a prevalence of complement interference (prozone) in the SAB assay of 29.5% of patients involving class I HLA antibodies and 45.9% involving class II antibodies with HLA-A and HLA-DQ antibodies the most prevalent [15]. In contrast, studies using sera from highly sensitised subjects (cPRA >95%), show a higher prevalence of class I HLA-specific antibodies demonstrating prozone (>70%) [13], [14]. Improving detection of and understanding the prozone effect will help to optimise the utilisation of SABs in transplantation whilst ensuring maximum peri-transplant efficiency is retained.

2. Objective

In this cohort study, we evaluated sera collected from a population of kidney transplant recipients from the Royal Liverpool Hospital. Patients received a renal transplant between 2009 and 2014 and had post-transplant sera stored and available for testing. The aims were threefold: to compare EDTA and BSC assays to the standard assay in order to observe the overall relationship between MFIs among the tests to allow for adjustment of the nominal MFI threshold for the assay modifications in data analysis; to compare EDTA and BSC tests with regard to abrogating/mitigating complement interference of class I HLA-specific antibodies; and to formally define prozone numerically.

3. Materials & Methods

Ethical approval was granted from the Health Research Authority National Research Ethics Service, study number 11/NW/0279 and the local Research and Development Department, who acted as sponsors, reference number 4049. Patients had received a kidney transplant at the Royal Liverpool Hospital between 2009 and 2014.

Following informed consent, all samples available for each patient at 2 weeks, 1 month, 3 months, 6 months, 9 months, 12 months and then yearly following transplant underwent a Labscreen® Mixed screening assay (LSM12, Lot 17) to broadly determine the presence or absence of Class I or Class II HLA-specific antibodies. Briefly, screening assays were performed as per manufacturers protocol (Labscreen® negative control, One Lambda). A locally validated modification utilised a vacuum suction to dry the plates between washes, which has been shown to have no adverse effect on MFIs generated [16][17].

The plate was then placed in the Luminex machine (Lx-200) to determine the Mean Fluorescent Intensity (MFI) for class I and class II HLA-specific antibodies for each sample. A normalised MFI was generated (raw MFI minus negative control value for the serum), with a local policy defining a positive result as a normalised MFI of ≥500. Results were interpreted using HLA Fusion 3.0 software and raw data processed using Microsoft Excel.

Samples with a positive MFI for class I were retained for single antigen bead testing. For each patient, only the first positive sample for class I was tested. The technique for testing with single antigen beads was the same as that used for the Labscreen® Mixed assay, however, the threshold for a positive result was locally set at a normalised MFI of ≥2000 and

the beads were specific for single Class I: HLA Labscreen® Single Antigen HLA Class I – Combi (lot numbers 9 and 10).

The single antigen bead assay was performed for the EDTA and 1 in 10 dilution modified assays in the same manner as for the standard detection assay with the following differences:

- For EDTA treatment, 95µL of serum was mixed with 5µL of pre-prepared 6% EDTA (weight/volume) and put on the roller for 5 minutes prior to use.
- For 1 in 10 dilutions, 10µL of patient serum was mixed with 90µL PBS and put on the roller for 5 minutes prior to use. A fixed dilution was used for convenience and has been reported in the literature as an acceptable alternative to serial dilutions [14][18].
- For the BSC assay, the standard Luminex assay was performed as described previously, until the addition of the secondary antibody. At this point, 95uL of a 1:250 dilution (locally established protocol based on manufacturers range of recommended titrations) of an anti-Human IgG goat antibody conjugated to Biotin (Biotin-SP®, Lot 120783) was added to each well and incubated at room temperature for 30 minutes. Then, four additional wash steps were performed, and 95uL of a 1:100 concentration of phycoerythrin-conjugated streptavidin (Streptavidin, R-Phycoerythrin Conjugate (SAPE), Lot 1755577) was added to each of the dry wells. The plate was incubated for 15 minutes followed by four additional wash steps. The beads were resuspended in 80uL of PBS as per the standard Luminex protocol.

For data analysis, the initial MFI values were normalised, following standard lab practice (the total raw MFI minus the negative control). This gave a range of MFIs values covering five orders of magnitude. Data covering such a large range of values generates a disproportionate variability in the low MFI range, thus, to determine global relationships without undue interference from this effect, data were transformed by taking natural logarithms. Linear regression analysis was performed on log (normalised) MFI values, both with and without outlier removal. Linear regression without outlier removal was used to demonstrate the global linear relationship between different test types, as indicated by the r^2 value which is described in the supplemental information (Supplementary File – S. Figure

1). The global relationship permits the development of prediction intervals, based only on values that show standard method-to-method variation, beyond which candidates for suspected prozone / steric hindrance effects are identified.

In the data analysis shown, the creation of the linear model is performed *with* outlier removal to avoid any interference from reactions at i) the upper register - containing the majority of prozone reactions, which would introduce pre-test bias; ii) the lower register - a high level of variation is observed even following log transformation, including values of 0 (an artifact of the normalisation process) – see Figure 2. Thus, the range of MFI values used for creating the linear regression was log MFI >5 (MFI >148) and log MFI <9.5 (MFI <13359.7) for EDTA and BSC and log MFI > 2.5 (MFI > 12) and log MFI < 9.5 (MFI <13360) for the dilution test, where MFI values are lower in non-prozone affected reactions.

Following development of the linear models (outliers removed), the trend line was mapped onto the whole dataset *without outlier removal*. Prediction intervals (PIs) were calculated at 99% from the linear models, to indicate that 99% of values would be expected to fall within this region and mapped onto the whole dataset. Any values beyond the prediction intervals could thus be classified as unusual results, for example indicative of prozone effect. As noted in the *Results*, different subsets of data were defined for analysis of outliers from PIs, false positive analysis and false negative analysis.

To determine the sensitivity and specificity of the EDTA and BSC modifications, the standard assay versus fixed 1 in 10 dilution regression analysis was used to facilitate identification of prozone positive, prozone negative and prozone indeterminate candidates (Figure 2D). These were defined, as a “gold standard” against which EDTA and Biotin results were compared for their MFI recovery of prozone positive reactions.

To be identified as a prozone positive candidate, the dilution series value was higher than the upper limit for the 99% PI for standard versus dilution. In addition, prozone positive candidates had log MFI of the dilution sample >6.98 (MFI 1074.9). This threshold was calculated as the equivalent prediction from linear modelling the dilution series against the standard test, and taking the upper limit of the 75% PI for a log MFI of 7.6 (typical threshold of 2000). The rationale for this threshold was to include only data that was considered to be certainly positive at a clinically relevant threshold i.e. the test results would certainly pass

the typical MFI > 2000 threshold used elsewhere. Thus, the prozone positive set was 170 data points (Figure 3A), 1.5% of the bead reactions examined.

To be defined as prozone negative, we calculated a 90% prediction interval for the dilution series versus the standard test. Prozone negative was thus defined as any value less than the upper prediction interval in this regression analysis i.e. there was no evidence from the regression that these values were abnormally higher on the dilution series compared to the standard test, giving 10849 data points (Figure 4B). This left 330 data points in the prozone indeterminate (or unclassified) group.

Data analysis code was written in R version 3.6.2.

4. Results

From a cohort of 460 post-transplant patients, 168 were positive for the Labscreen® Mixed assay of which 142 were positive for class I HLA-specific antibody. Five of these either did not have sufficient serum to undergo further iterations of testing or had failed to achieve adequate controls (a positive control of ≥2000 and a negative control value of ≤1500). Furthermore, 11 samples had insufficient bead counts, thus 126 were eligible for further testing with the assay modifications (Supplementary File – S. Figure 2).

In order to minimise the confounding effect of spurious samples (mislabelling, operator error, unknown phenomena), a quality control (QC) correlation analysis was performed to identify completely un-associated samples (non-significant p-values from linear modelling between different test results from the same sample). The analysis used only log MFIs <9.5 to eliminate the influence of beads affected by CMI. Nine patients who did not show sufficient association were excluded from the analysis, based on the QC step. Thus, a single serum sample from 117 patients were analysed with the assay modifications amounting to a total of individual 11349 bead reactions. The reasons for sample exclusion are details in the Supplementary File (Table S1).

4.1 Linear modelling analysis

Linear regression analyses (outliers removed) were performed between the standard test and EDTA, BSC and 1 in 10 dilution modifications to determine trend lines and derive 99% prediction intervals (see Figure 2), which were mapped onto the whole dataset (no outlier

removal). Those values beyond the prediction intervals can be thought of as outliers (i.e. not expected by the natural variation of the assays) and these will be analysed in greater detail in the following section.

The linear models were used to suggest a nominal equivalent to the locally used standard assay MFI threshold of 2000 (log MFI = 7.6) adopted in this analysis for the EDTA and BSC modifications. The trend lines predict an average EDTA equivalent at MFI = 2097.4 (log MFI = 7.65) and BSC MFI = 2032.9 (log MFI = 7.62). The minor differences in MFI cut off do not relate to the assays' performance, but acknowledge a slightly higher average MFI in both EDTA and BSC compared to the standard assay, which is adjusted for in the onward analysis.

As expected, there is heterogeneity in the following areas: i) the lower MFIs despite logarithmic transformation; ii) a cluster of data points at ~log MFI 10 on the y axis (representing the assay permutations) but lower values (log MFI from 5 to 8.5) on the x axis (representing the standard assay) - the putative prozone effect/steric hindrance cohort. With these exceptions noted, the data have a distinct linear relationship on each plot, and can be considered to reveal an association between the two assay types (assuming no additional biological phenomena).

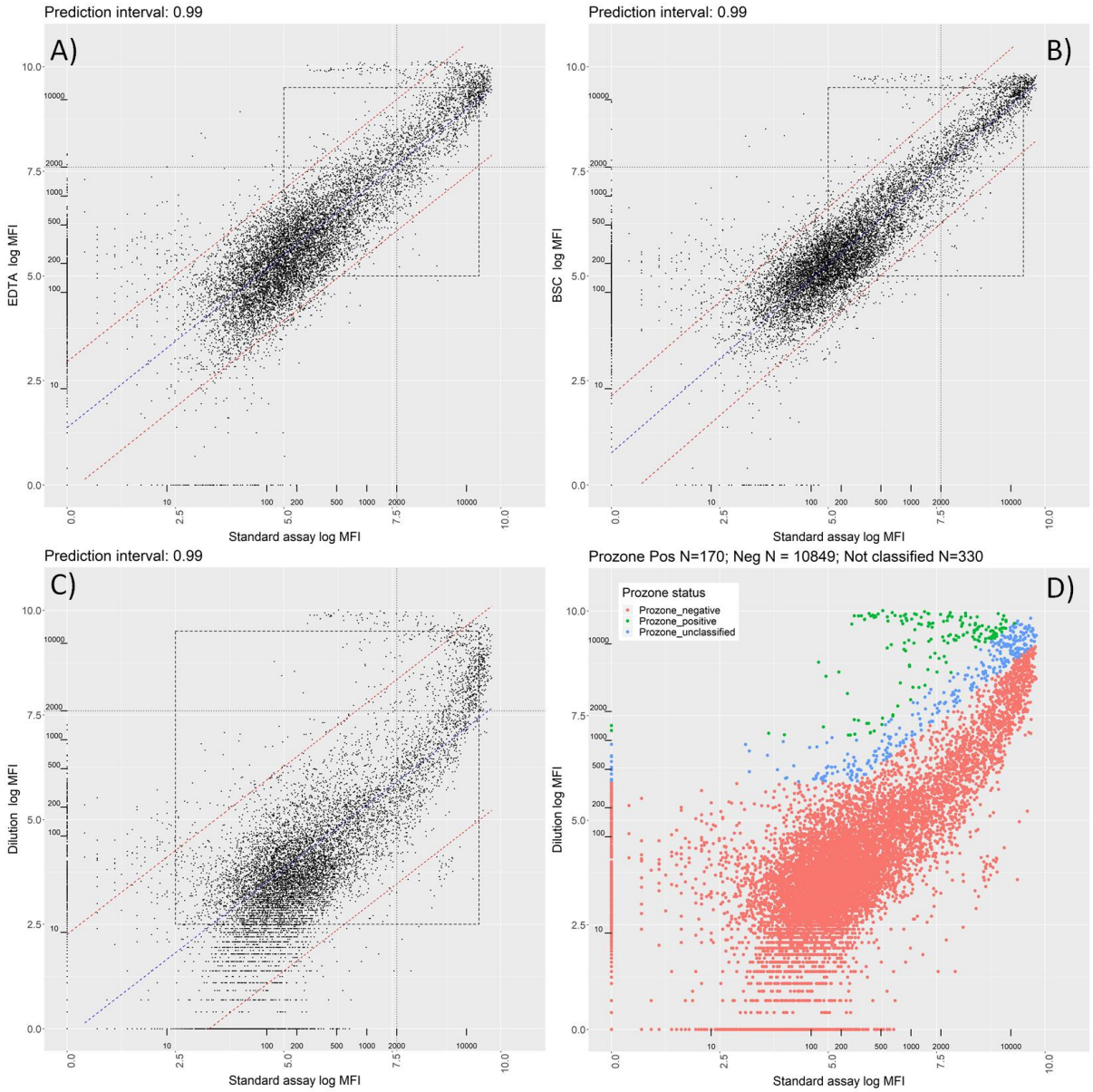

Figure 2: Log normalised MFI values for A) Standard (x axis) and EDTA (y axis); B) Standard (x axis) and BSC (y axis); C) Standard (x axis) and 1:10 Dilution (y axis); D As in C but with colour coding to delineate prozone positive (green), prozone indeterminate (blue) and prozone negative (pink). The linear model (blue line) was fitted on the values within the rectangular boxed region to exclude outliers in A-C. The 99% prediction intervals from the model are displayed as blue dashed lines in A-C. Horizontal lines are displayed for MFI = log (2000), to indicate the typical threshold used in the laboratory for a positive test result in A-C. Inset axis labels display equivalent MFI values on the linear scale.

4.2 Outlier Analysis

Using the 99% prediction interval technique 1200 (10.6%) (standard versus EDTA), 1227 (10.8%) (standard versus BSC) and 683 (6%) (EDTA versus BSC) outlier beads were identified. To ensure that obviously negative reactions were not included in the outlier analysis , at least one of the log MFIs had to be ≥7.6 (log 2000) normalised MFI. There were 142 (standard versus EDTA) and 121 (standard versus BSC) outlier beads identified using this inclusion criterion (see Figure 3).

The patterns in Figure 3A and 3B are very similar, again demonstrating the cluster of beads around the log MFI 9.5-10 on the y axis which are clear examples of CMI. A second prominent feature is that most of the beads have a log MFI above the 99% PI i.e. higher than expected for the alternative test compared to standard: 135 / 143 (95%) in the EDTA versus standard comparison and 96/119 (81%) in the BSC versus standard comparison. Those beads were candidates to be considered as CMI reactions, recovered by EDTA or BSC assay variants.

Figure 3C and zoomed panel show the EDTA and BSC comparison, with several clusters marked (i – v). Cluster i) has outliers (both data sets) with log MFI > 9 on both EDTA and BSC tests, beyond the upper 99% prediction interval from the standard test indicating prozone recovery on both tests. Cluster ii) contains ~15 SAB results with log MFI of around 10 for EDTA, but a range of values for BSC, log MFI 7 to 8, indicating a high signal from the EDTA test but a mid-range signal from the BSC test indicating that EDTA is more efficient in mitigating CMI in these instances. Cluster iii) contains SAB results mostly flagged as outliers on either EDTA testing only or both tests, with a range of MFI values and an apparent linear relationship; potentially indicating a weak prozone effect recovered by both tests or natural variation within the test. Cluster iv) contains 14 SAB results where the signal for BSC is considerably higher than EDTA. They have been classified as outliers in some cases since the EDTA signal was lower than expected (blue colour), BSC was higher than expected (red colour) or EDTA was unusually low and BSC was unusually high (“Both”, coloured red). Six of these results came from one sample (15.6506188 – see Supplementary File 1 for source data), in which EDTA, standard test and dilution assay all find all SAB results to be negative, but BSC testing has seven positive (>2000 MFI) results. The origin of this disparity is unclear, but there is no supporting evidence (e.g. from dilution assay) that these are CMI cases

discovered by BSC testing, but missed by EDTA testing. Cluster v) shows 15 results were EDTA has a higher log MFI (7.5-9) than the BSC assay log MFI (5.5-7) – classed as low outliers on the BSC test i.e. the EDTA values are concordant with the standard test, but BSC signal was outside the lower range of normal. The results overall in Figure 3 point towards EDTA having an improved ability to abrogate prozone.

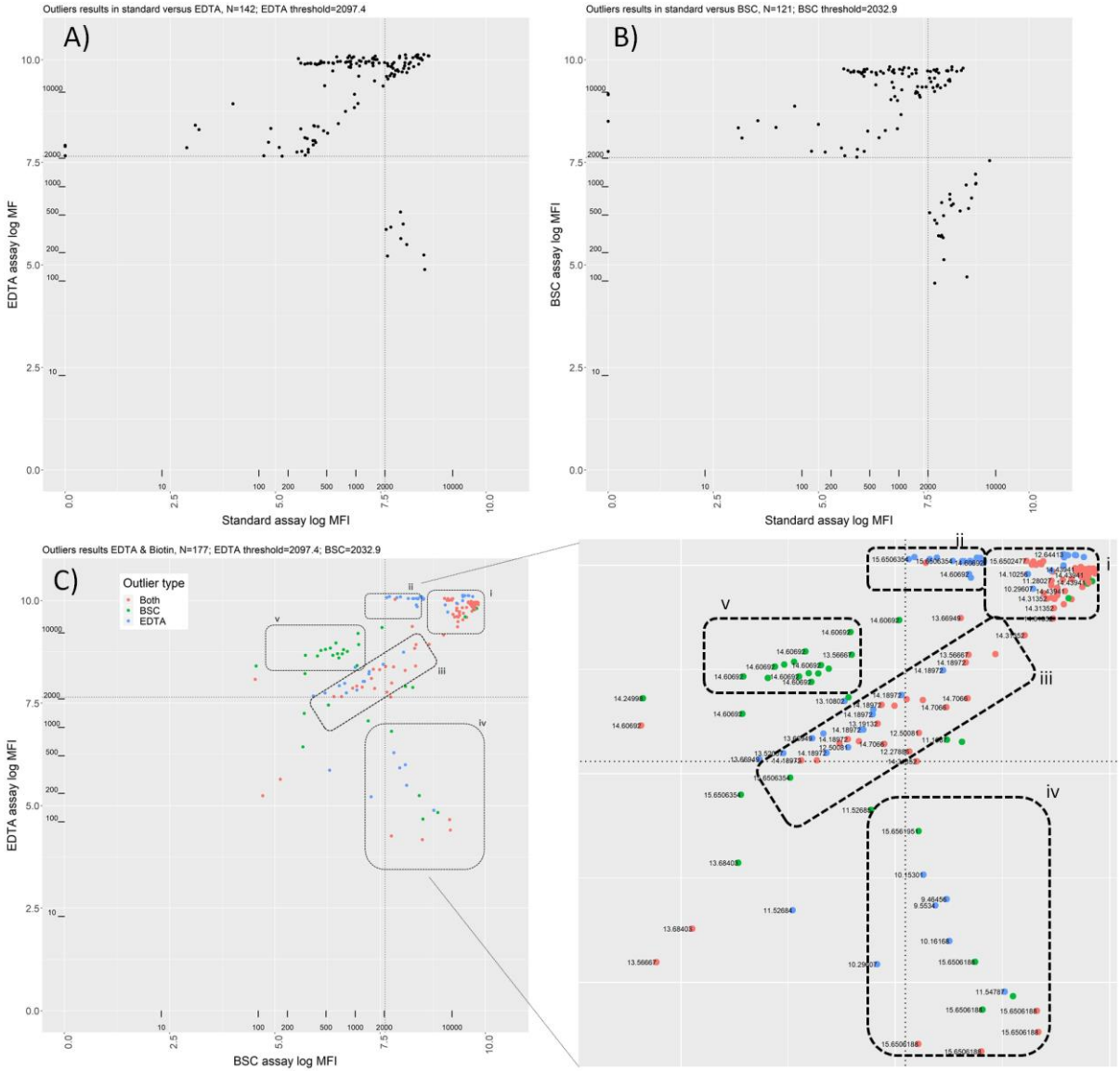

Figure 3: Outlier bead natural log MFIs for a) standard (x axis) versus EDTA (y axis); b) standard (x axis) versus BSC (y axis); c) All results in A or B, plotted for BSC (x axis) versus EDTA (y axis), colours indicating from which data set they originated) Clusters i-v indicate outlier beads defined based on falling outside the 99% prediction interval and at least one value having MFI > log 2000 (or the equivalent predicted thresholds for EDTA and BSC testing). Zoomed region is annotated with the sample identifiers, where they do not

overlap. The supplementary information contains a version of the plot annotated also with the antigen type of the bead, and all samples annotated.

4.3 Defining and Detecting Prozone

To corroborate the findings of the EDTA and BSC assays, a fixed 1 in 10 dilution SAB was performed for each sample in the analysis. A dilutional assay permutation can simultaneously provide information about prozone affected samples and the effect of steric hindrance. A simple regression analysis was performed for the standard assay versus the 1 in 10 dilution in the same way as for the EDTA and BSC assays. A subset regression analysis of the log MFIs 5-9.5 was calculated and the trend line and 99% PI mapped onto the original dataset (see Figure 2C).

The pattern observed for EDTA and BSC when plotted against the standard assay is seen again for standard versus 1 in 10 dilution with the cluster of MFIs around 9.5-10 on the y axis indicating significantly higher MFIs with dilution.

The results of the linear regression between the standard assay and fixed 1 in 10 dilution have enabled the following analysis, which aims to establish the sensitivity and specificity of EDTA and BSC assay permutations in abrogating prozone effect.

4.4 Determining Sensitivity & Specificity

As described in the Materials and Methods section, the sensitivity and specificity of the EDTA and BSC modifications, were determined using the standard versus fixed 1 in 10 dilution assays to facilitate identification of prozone positive, prozone negative and prozone indeterminate candidates (Figure 2D). One hundred and seventy prozone positive reactions, 330 prozone indeterminate and 10849 prozone negative reactions were identified.

Figure 4A shows the box plots for the standard assay MFIs in prozone positive and prozone negative groups. As expected the MFIs are appreciably higher for the prozone positive cohort, but with a median MFI of log 7.5, just under the positive threshold. From Figure 4B we can observe two clusters. Cluster i) contains results with a range of values from the standard test (log MFI 5 – 9) and the three alternative tests giving similarly high values (log MFI > 9), with no clear linear relationships between the standard test and the alternatives.

Cluster ii) contains a range of values on both axes indicating linear relationships between the standard test and alternative tests remain largely intact. Figure 4C shows the “prozone negative set” in which there is a clear underlying linear relationship between all the alternative tests and the standard test; the dilution series as expected has significantly lower MFI values than other tests. BSC and EDTA have a similar range of values.

Figure 4D shows a ROC plot (true positive rate versus false positive rate), testing the ability of EDTA and BSC to differentiate prozone positive versus prozone negative cases. The input data was ordered by the MFI value on the EDTA test or BSC respectively. For this analysis, a subset of data, whereby all data points (prozone positive and negative) with MFI < 1000 (conservative negative) on the standard assay were assessed. Under ideal conditions, only (and all) the prozone positive samples would have MFI > threshold defined above (2097.4 EDTA and 2032.9 BSC).

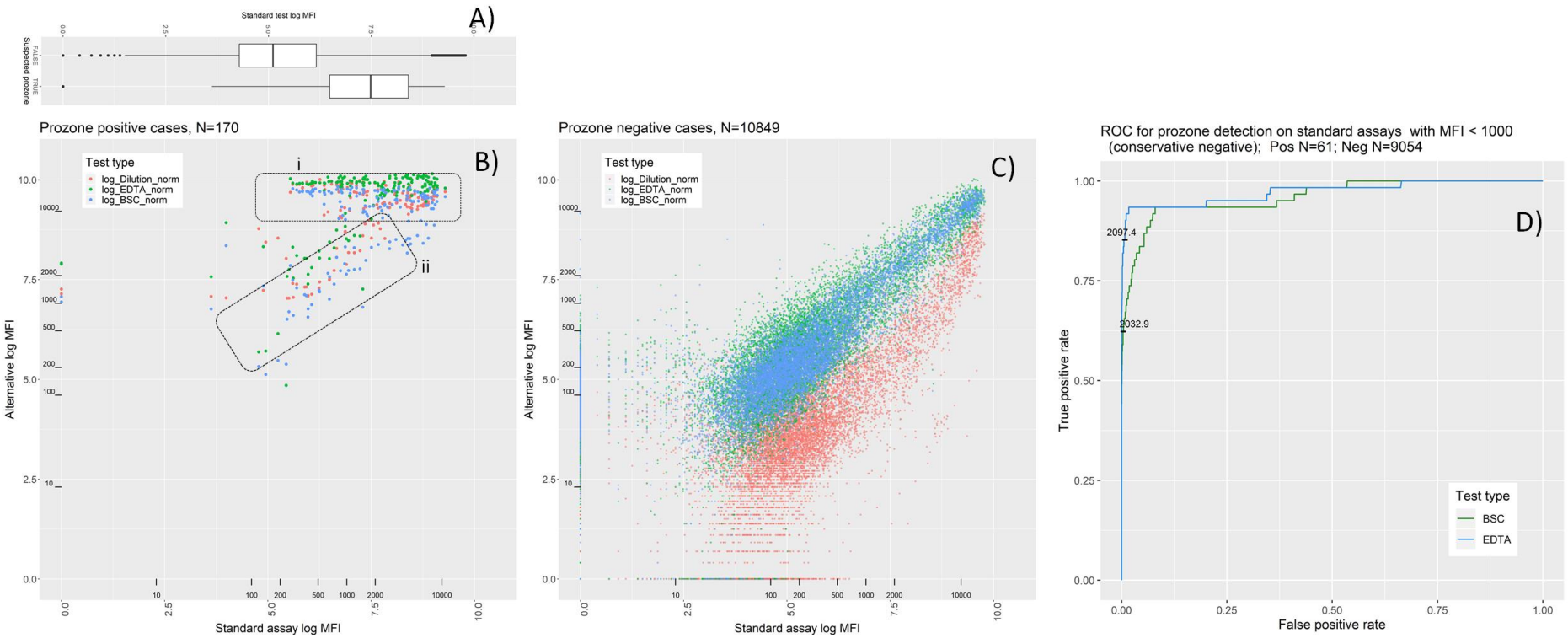

Figure 4 We have defined two sets of SAB results – as suspected "prozone positive" and "prozone negative" (see Methods). A) Boxplots for the two sets, showing the log MFI values from the standard test. SAB results (log MFI) from the EDTA, BSC and Dilution tests (y-axis) versus the standard test (x-axis) for B) suspected prozone positive cases with two clusters determined visually (further discussed in the text), and C) prozone negative cases. D) A ROC plot showing the ability of EDTA and BSC tests to detect prozone positive versus prozone negative, with the threshold MFI indicated at the point of sensitivity (MFI 2097.4 for EDTA and 2032.9 for BSC). A subset of data was used for the ROC analysis, whereby all data points (prozone positive and negative) had MFI < 1000 (conservative negative) on the standard assay.

4.5 False positive analysis

The data demonstrate that both EDTA and BSC testing can abrogate the prozone effect. However, it is also important to consider whether EDTA and BSC testing can lead to false positive results. This facet is particularly challenging to test, since there is no "gold standard" to define a true negative.

To create a data set which we were reasonably confident was composed only of true negatives, more conservative thresholds were applied. Only SABs giving results from the standard assay MFI < 1000 and the dilution series MFI < 208 (predicted linear equivalent from Figure 2C) are called negative. We then plotted the results of this subset of data, and classified whether they were deemed positive by EDTA or BSC tests, at the equivalent predicted thresholds (compared to >2000 for the standard assay), as shown in Figure 5. For this set of confident negative reactions, there are only a few instances of EDTA or BSC testing giving positive results, using the suggested thresholds from linear modelling, with false positive rates of 0.004 and 0.002 respectively.

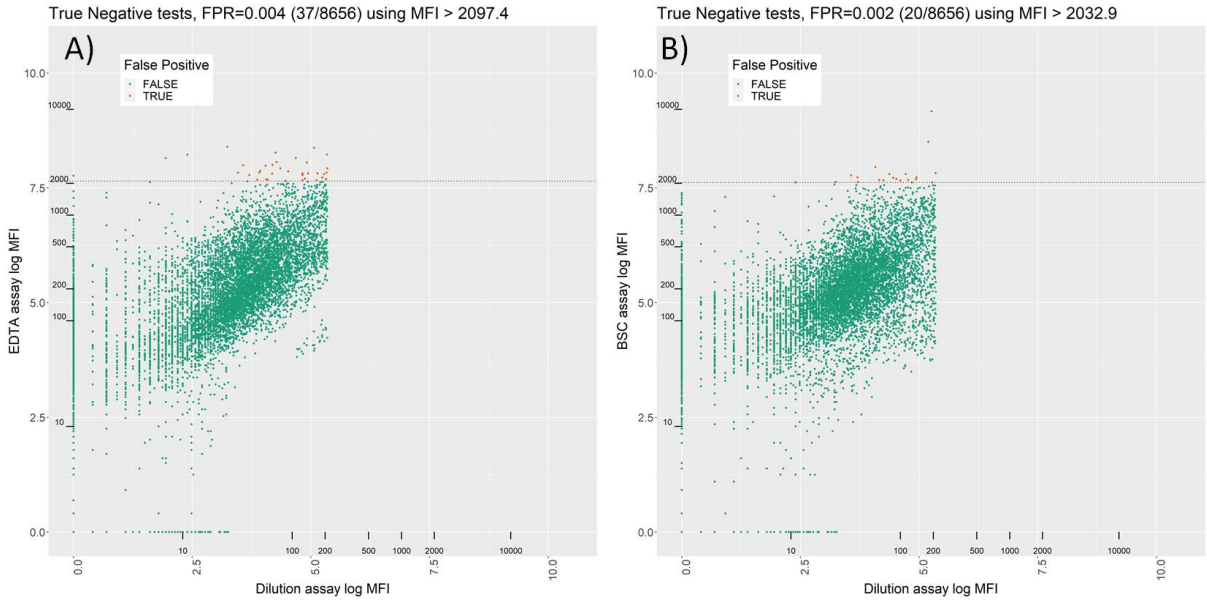

Figure 5 Scatter plots of the log MFI values for "true negative" result set (see main text for definition) for A) EDTA testing (y-axis) versus dilution assay; and B) BSC assay versus dilution assay. The horizontal line shows the threshold for a positive result: log MFI > log (2124) for EDTA and log MFI > log (2031) for BSC testing. FPR=false positive rate.

4.6 False Negative Detection

A set of SAB results were defined as true positives where the standard test MFI > 4000 and the dilution assay was > 640.7 (the equivalent threshold to 4000 determined by the linear fit of dilution to standard) – giving 936 test results. The rationale for these thresholds is that using an MFI close to the threshold of 2000 could easily fall below it on re-testing. A value of 4000 on the standard assay, and "confirmed" on the dilution assay with a comparatively high value is unlikely to be a true negative result. Using the previously defined positive thresholds for EDTA and BSC tests, results were classified as false negatives if they fell below these positive thresholds (2097 for EDTA and 2033 for BSC). As shown in Figure 6, only one false negative was detected by EDTA testing (FNR = 0.0011) and 13 false negatives were detected for BSC testing (FNR = 0.0139). Notably, 10 out of 13 test results were all derived from the same BSC sample (14.60692 – see Supplementary File 1 for raw data), described in section 4.2.

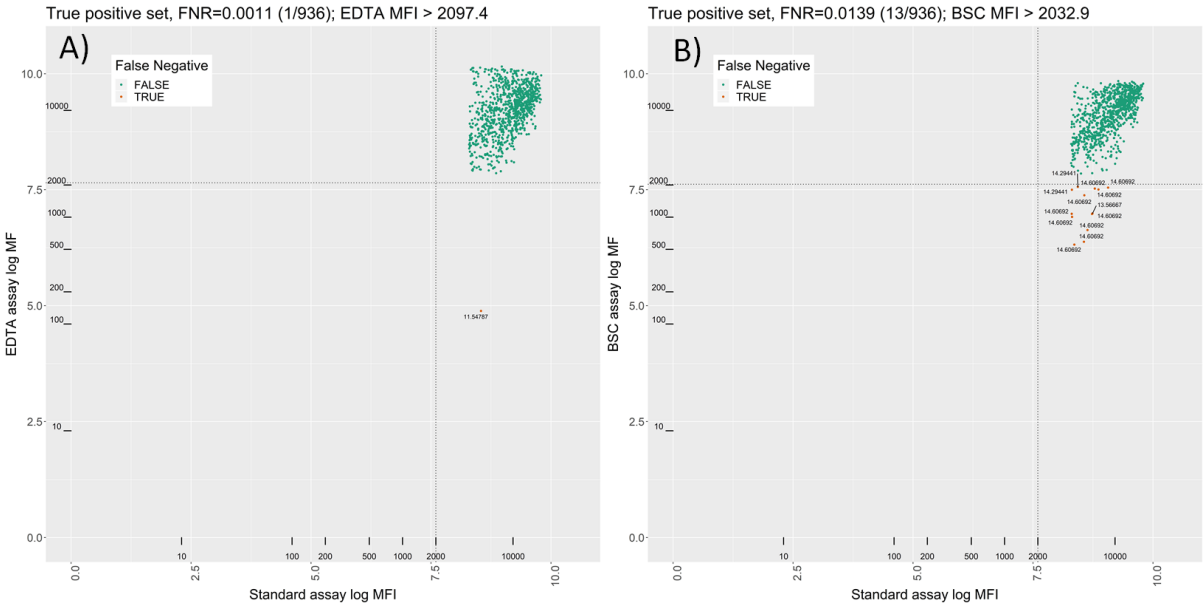

Figure 6 Scatterplot showing log MFI values for the "true positive" set n =885 (see main text for definition) for A) EDTA versus standard assay and B) BSC versus standard assay. FNR=false negative rate.

4.7 Population Analysis

In our population analysis, we identified that all 170 prozone positive reactions were derived from sera from 23/117 (19.7%) patients tested with standard SAB and modifications. Using our new tool to define prozone positivity, these values are considerably lower than those described by others in both population and highly sensitised groups[9], [13]–[15]. Of the 170 prozone positive beads, 80 (47.1%) were HLA-A specificities, 78 (45.8%) HLA-B and 12 HLA-Cw (7.1%) representing 52% (HLA-A), 65% (HLA-B) and 26% (HLA-Cw) of the samples. Examining the whole population of reactions at the standard assay threshold MFI of 2000 reveals 1565 positive results of which 564 (36%) were directed against HLA-A, 902 (55.5%) against HLA-B and 141 (8.4%) against HLA-Cw.

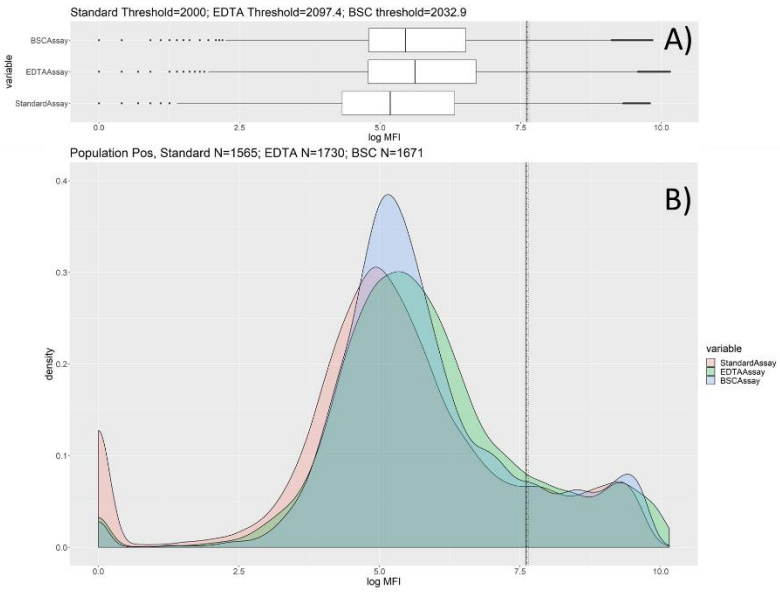

Figure 7 Full population analysis, plotting the log MFI for the three test types as A) boxplots; B) density plot; using the linear model predicted thresholds for EDTA and BSC, equivalent to standard test MFI > 2000.

The whole population analysis is shown in figure 7 which enabled determination of the relationship between MFI distributions in the standard, EDTA and BSC assays. We have referred to this at multiple points during the paper and it is this large data series that has enabled our estimation of different threshold MFIs for EDTA (2097) and BSC (2033) for determining positivity based on a single point cut-off. Using these thresholds, 1730 SABs gave a positive test for EDTA, 1671 for BSC and 1565 for the standard assay. While this is a substantial number of additional positives from EDTA testing (165 more) – as demonstrated

above, almost all of these were explained by the prozone effect, and we do not see evidence of false positive results in the EDTA set.

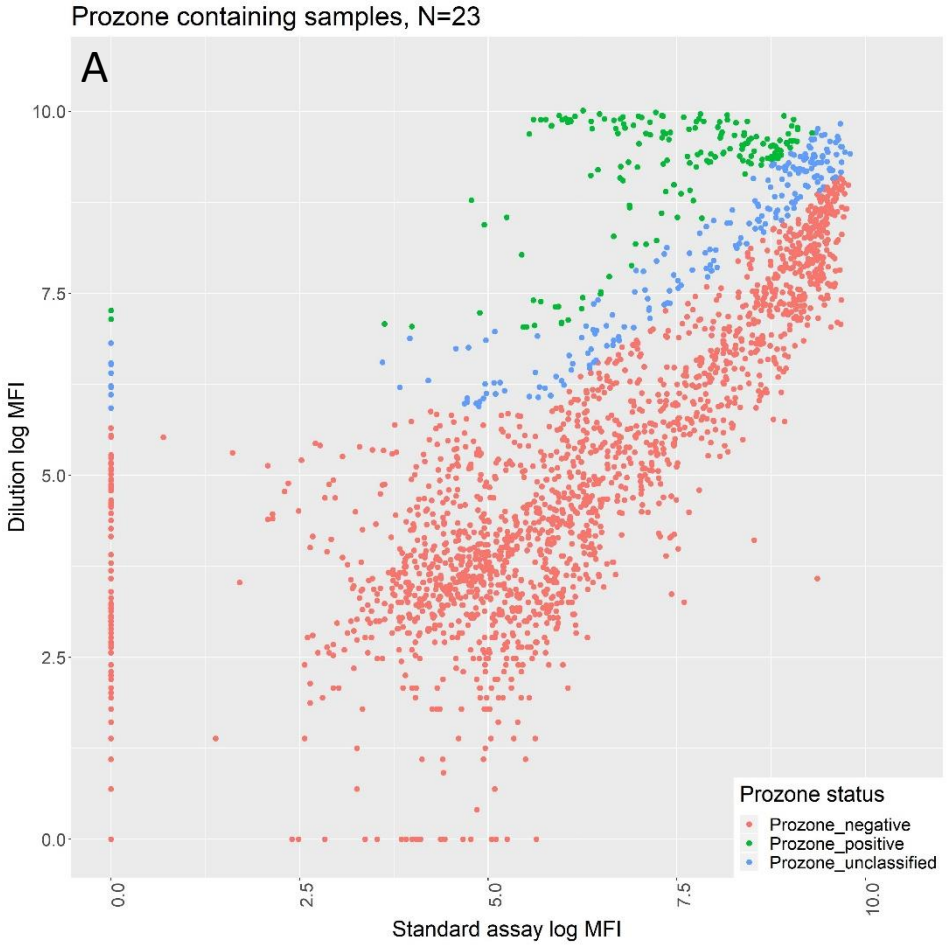

Figure 8: A) All bead results for prozone positive samples (N=23) showing prozone positive beads (green), prozone negative beads (pink) and prozone indeterminate (blue).

Finally, our prozone positive population data allows for a more detailed inspection of the prozone indeterminate data points (n=330), which seems an acceptable unassigned rate of 2.9%. Figure 8 shows all data points for the 23 samples deemed to have at least one prozone positive bead. Two hundred and thirteen of the 330 (64.5%) prozone unclassified data points belong to the 23 prozone positive samples (19.7% of the total samples tested). This group of indeterminate results may belong to the prozone positive dataset as these patients were unequivocally prozone positive at other specificities.

5. Discussion

To our knowledge, this is the first population-based post-transplant cohort study to examine the prozone phenomenon. It is also the first description of a large study examining the ability of the BSC to ameliorate prozone when compared to a more thoroughly investigated alternative assay, EDTA, and a natural validation test, a 1 in 10 dilution. Previous studies investigating BSC have used serum from small numbers of patients (10 for class I HLA specific antibody), which have been investigated because of suspicion of prozone either with prior EDTA testing or discrepancies with cross-matching[10], [12].

Modifications to the standard SAB assay have been described previously which abrogate/reduce the prozone effect. These include serial dilutions[14], use of the C1q assay[14], [19], EDTA treatment[9], [14], [20], [21] and the dual antibody rapid test (DART) which combines the standard SAB test with a C3d assay; the latter assay looks for C3d complement cleavage products[4], [9], [20], [22]–[25]. To date, there is no consensus about which modification offers the most effective strategy to tackle the prozone effect.

We described the EDTA and BSC assays and how they ameliorate prozone compared to a fixed 1 in 10 dilution approach as a "natural" assay validation. We observed that both EDTA and BSC are effective in mitigating prozone. Using ROC curve analysis, we observed that BSC and EDTA have very low false positive (0.002 and 0.004 respectively) and false negative (0.0139 and 0.006 respectively) detection rates. The false positive reactions in Figure 5 have been defined on the basis that they have not been confirmed as prozone positive reactions by a high MFI in the dilution assay. We do acknowledge that there are a small number of reactions which demonstrate such high levels of CMI that they require titres up to 1:1024 and are therefore not revealed by a lower titre of 1:10[14].

As described, most of the false negative reactions in the BSC analysis are derived from 1 sample and we, thus, cannot conclude it would have a higher false negative rate than EDTA if more widely adopted. The ROC plot shows EDTA has a sensitivity ~0.9 at close to zero FPR (and best performance achieved close to the 2097 threshold). BSC performance was still good, with ~0.6 sensitivity at 0 FPR. This means however that around 40% of prozone positive cases were not recovered by BSC testing, but only 10% by EDTA. The EDTA assay is cheaper and less time consuming as it does not require a tertiary step as in the BSC assay,

although it does require the sera to be pre-treated and may result in a slight dilutional effect (EDTA occupies 5% of the treated serum volume) which may account for the wider prediction interval seen for EDTA compared to BSC in Figures 2A and B.

Using prediction intervals, we described how the EDTA and BSC assays can be related to the standard assay in terms of MFI. Specifically, we described suggested thresholds for these assays in a clinical laboratory setting (using a positive MFI threshold in the standard assay of 2000 consistent with our local practice) in order to avoid classifying unacceptable antigens where true positivity does not exist. We do, however, acknowledge that there are many cases of genuine antibody positivity at MFI <2000, but report our findings on the basis of local laboratory practice. Of particular concern are the public epitopes such as Bw4 / Bw6 [26] and those described by El-Awar et al. such as epitopes 422 (shared by 6 HLA-As) and 21 (shared by 10 HLA-Bs) [27]which are expressed on many of the single antigen beads and whose significance can be disproportionately under-estimated by individual MFI values. Studies indicate that such public epitopes are frequently observed following kidney transplant (seen in >90% of recipients with class I HLA-specific antibody post-transplant) [28].

Concerns regarding over-estimation of EDTA MFIs resulting in assignment of additional positive specificities which may not be truly positive and may be a barrier to transplantation are not warranted for two reasons. Firstly, using our derivation (based solely on the linearly associated MFI range, which excludes the prozone positive reactions), there is only a 4.9% gain in MFI cut-off with EDTA which is substantially less than the intrinsic variation of the standard assay (which can be up to 62% coefficient of variation in a non-standardised setting over a full range of MFIs)[14], [29]. Secondly, we have now provided a new cut-off which is applicable locally, providing a framework for other laboratories considering adopting an adjusted threshold for EDTA treatment using the same principle to ensure that no additional specificities are assigned positive.

Outliers were statistically defined with PIs, affording the opportunity of their detailed analysis. Outliers were classified as three different types: a) putative prozone; b) those following the natural assay trend but at its very outer limits and c) potential assay failures. The small proportion of beads falling below the 99% PI in 3A and 3B (5% and 19% respectively) show a significantly higher log MFI with the standard assay in comparison to

either EDTA or BSC. The majority of these values cluster close to the cut-off for the (99%) prediction interval, indicating that they may represent "natural" variation in the tests. In both Figure 3A and B, there are fewer than 10 values that have a considerably higher log MFI value on the standard test compared to the alternative i.e. not close to the prediction interval, which might be indicative of random test failures for EDTA or Biotin, or an undescribed scientific phenomenon which compromises the detection of CMI by EDTA or BSC.

The supplementary material contains frequency tables of the number of times each particular bead falls into the outlier category and prozone positive subset (Supplementary Figures S3/4). We did not detect any particular trend indicating certain beads were outliers more frequently than others. We have not examined outliers which have negative MFIs on both assays of which there are 1058 in the standard / EDTA analysis and 1106 in the standard / BSC analysis. It is possible that there is CMI in these reactions (most of the values are above the prediction intervals in Figures 2A and B), but given that they have low MFIs on standard and EDTA/BSC assays and the dilution assay has not identified any unexplained high MFI in the false negative analysis, any potential effect is unlikely to be significant.

We classified >97% of beads as prozone positive or negative with a reasonable degree of certainty. Using our definition, 1.5% (170/11349) of bead reactions demonstrate prozone; *Tambur* et al. previously described 0.5% of bead reactions demonstrating prozone in a selected population examining both class I and class II HLA-specific antibody[14]. In the prozone positive cohort, 47% of reactions were directed against HLA-A, 46% against HLA-B and 7% against HLA-Cw, representing 52%, 65% and 26% of the prozone positive samples (n=23) respectively. The positive reactions in the total population are richer in HLA-B (56%) compared to HLA-A (36%). Guidicelli *et. al* also describe a predominance of HLA-A reactivity, but in their prozone positive cohort 71% of samples had HLA-A interference. The difference probably relates to different methodology used in prozone quantification [15]. HLA-Cw appears consistently lower both in overall terms of antibody prevalence and susceptibility to prozone in our study and in the published literature [13], [15].

Although further interrogation of the prozone indeterminate cohort is outside the scope of the paper, the overall pattern indicates that with larger cohorts and more specific dilutional titrations, it may be possible to further refine prozone positive, negative and indeterminate

definitions. The possibility of prozone in the confidently negative samples i.e. those not reaching a positive result in any of our assay permutations has not been explored but has been described in the literature for antibodies with an exceptionally high titre (≥256) where EDTA fails to abrogate prozone[14]. The Flexmap 3D® instrument may offer an advantage in detecting high level of antibody[11]. In addition, we acknowledge that the samples which were excluded on the QC analysis may represent interesting biology and true antibody activity due to undescribed interactions between the beads and sera. Elucidating any such phenomena is beyond the scope of this paper to establish.

We have not examined the low titre antibodies which give a high MFI signal on the standard test i.e those which are seen to dilute to negligible MFIs on a fixed 1 in 10 dilution. The former subset of beads is difficult to characterise because many of the MFIs fall outside of the range observed to show a linear trend. Such a high level of heterogeneity between assays at these lower MFIs makes simple deductions such as what qualifies as an outlier challenging enough without moving onto higher order scientific phenomena like prozone. The latter relies on precise dilutional titration and is outside the scope of this study.

Another point for future consideration is any effect of prozone on the Labscreen® Mixed assay. Prozone may affect this assay similarly (which is the benchmark assay used to establish candidate samples positive for HLA-specific antibody). Small cohort studies from Cambridge[30] and Los Angeles (in respect of MICA antibodies)[31] suggest the prozone effect is not strongly observed in the screening assay. However, a validation study is needed to establish the effects of prozone on the Labscreen® Mixed assay.

5.1 Conclusion

We recommend both EDTA and BSC as suitable assays to overcome prozone. We believe that clinical laboratories should adopt a validated assay modification to abrogate prozone as routine in determining sensitisation for all potential renal transplant recipients, as the standard assay is susceptible to prozone in nearly 20% of a sensitised post-transplant cohort.

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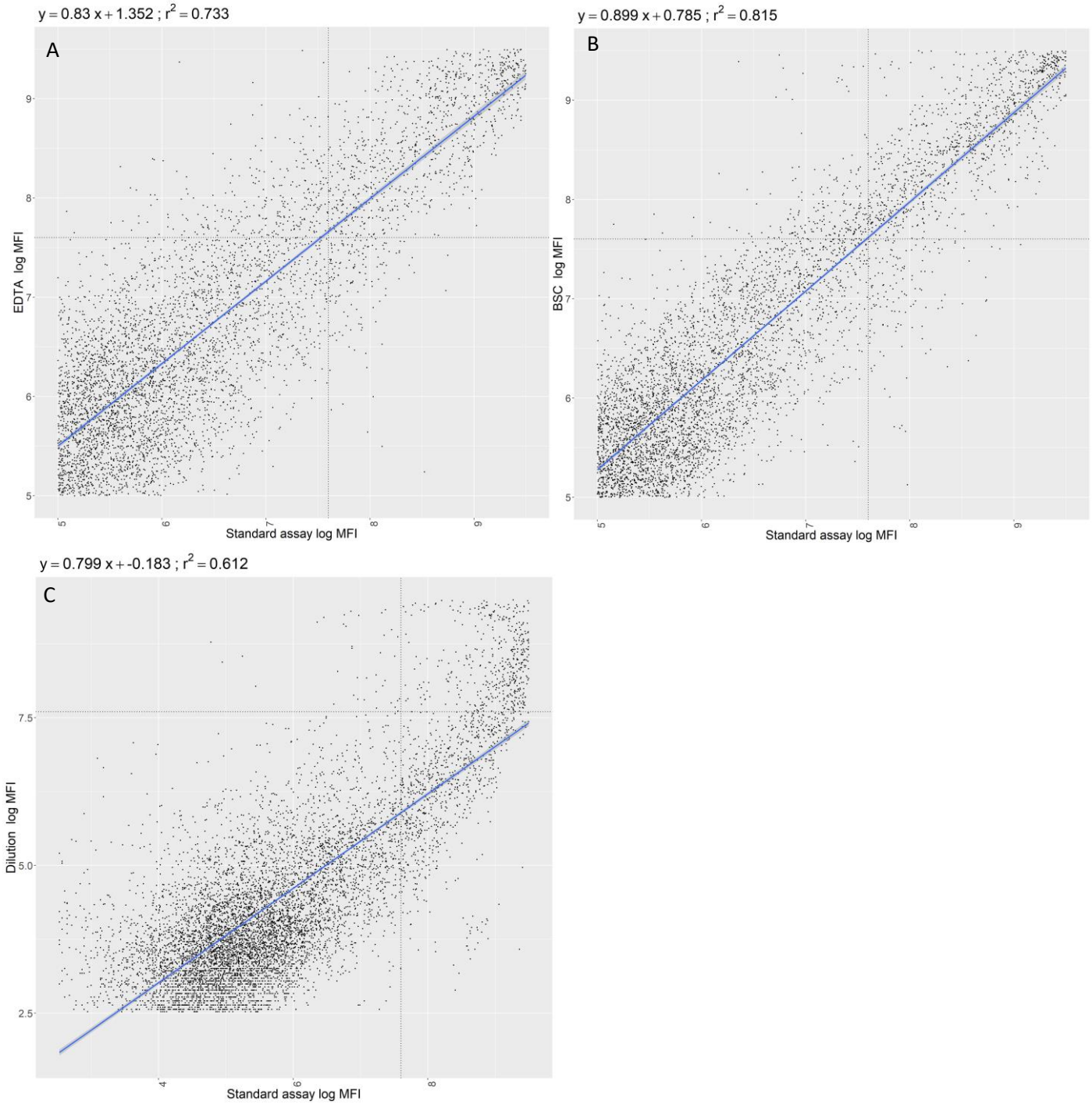
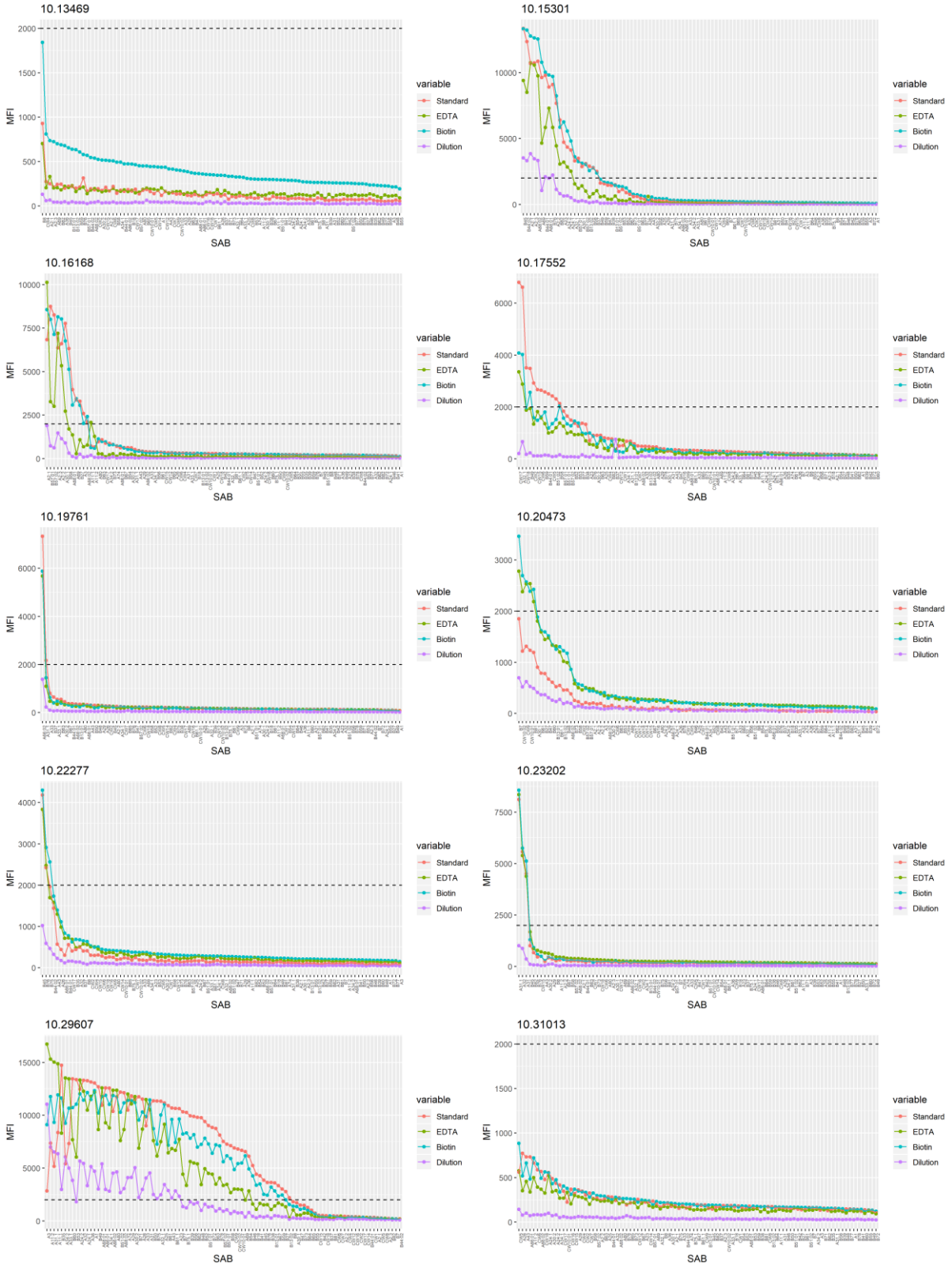


Supplementary Figure 1: Linear regression for natural log transformed MFIs showing R^2 values with outliers removed for a) standard (x axis) and EDTA (y axis); b) standard (x axis) and BSC (y axis); c) standard (x axis) and 1 in 10 dilution (y axis).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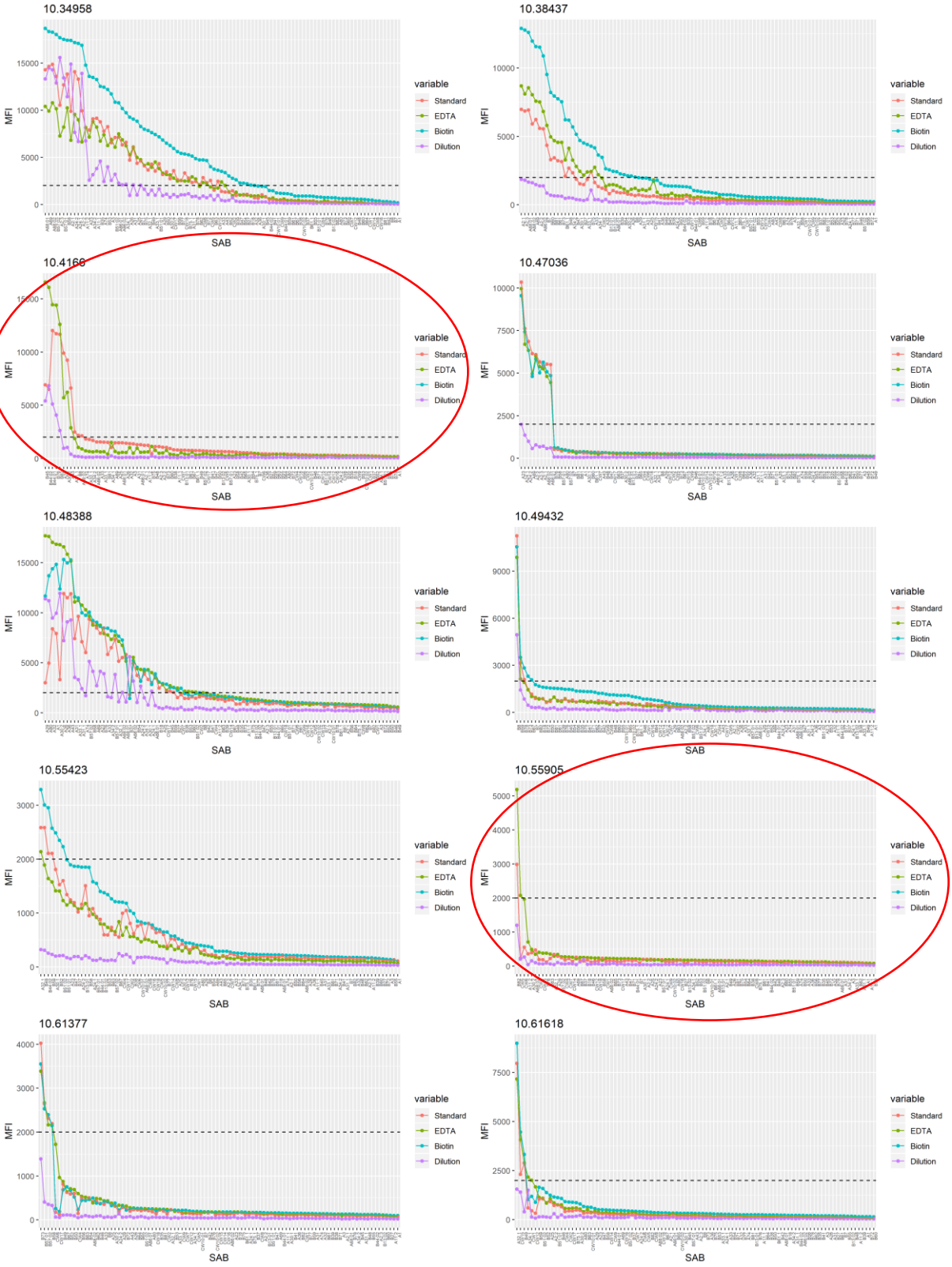
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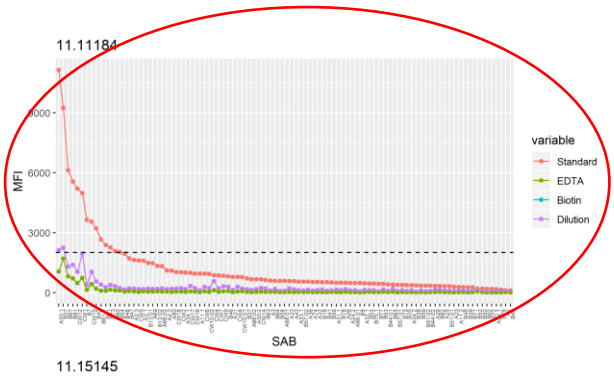
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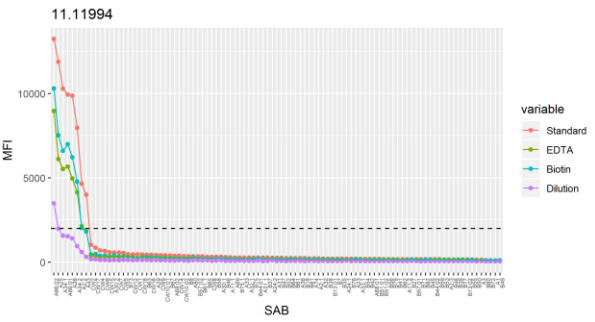
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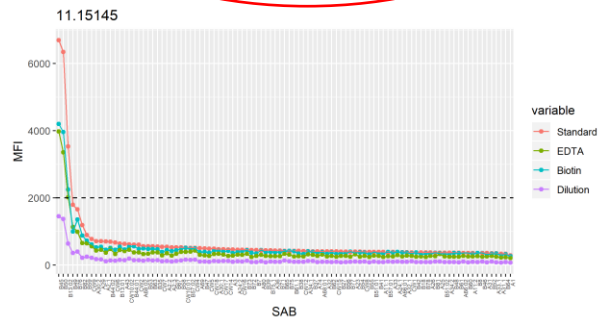
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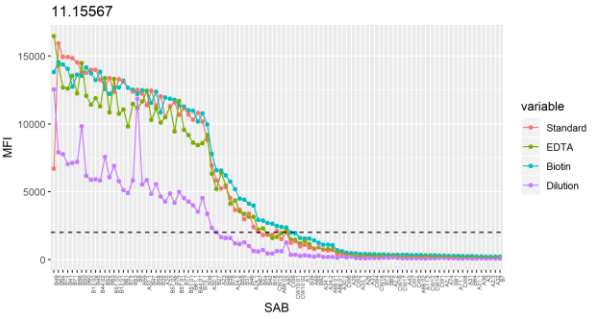
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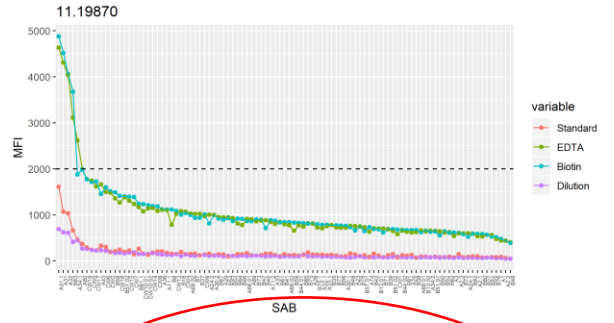
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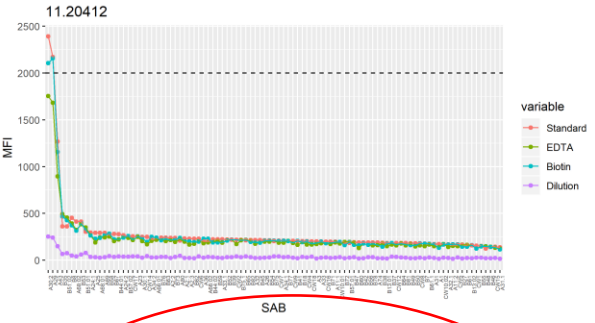
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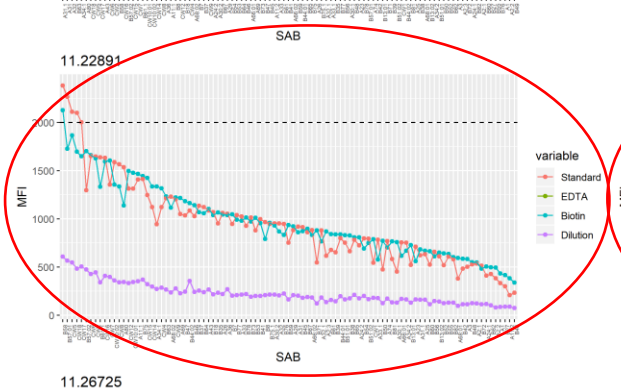
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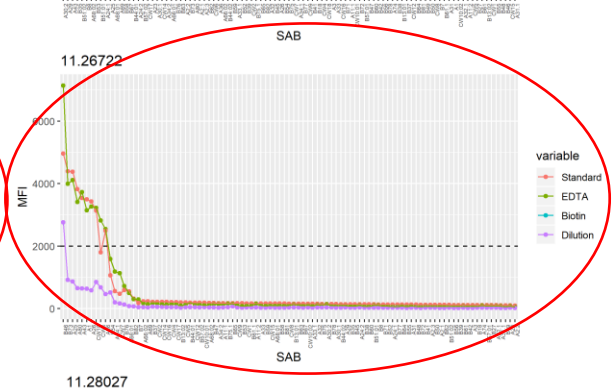
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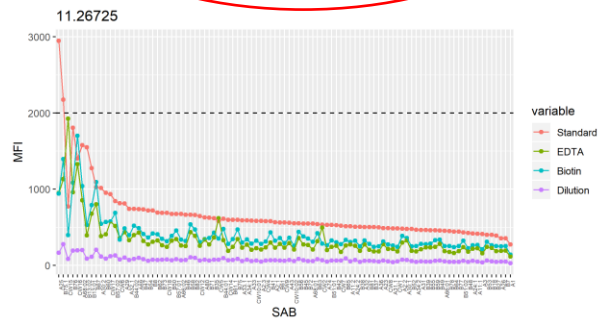
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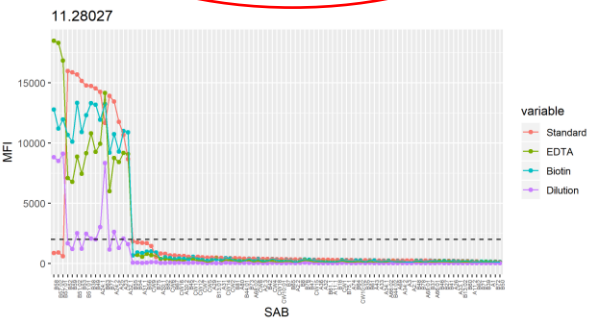
Biotin
Dilution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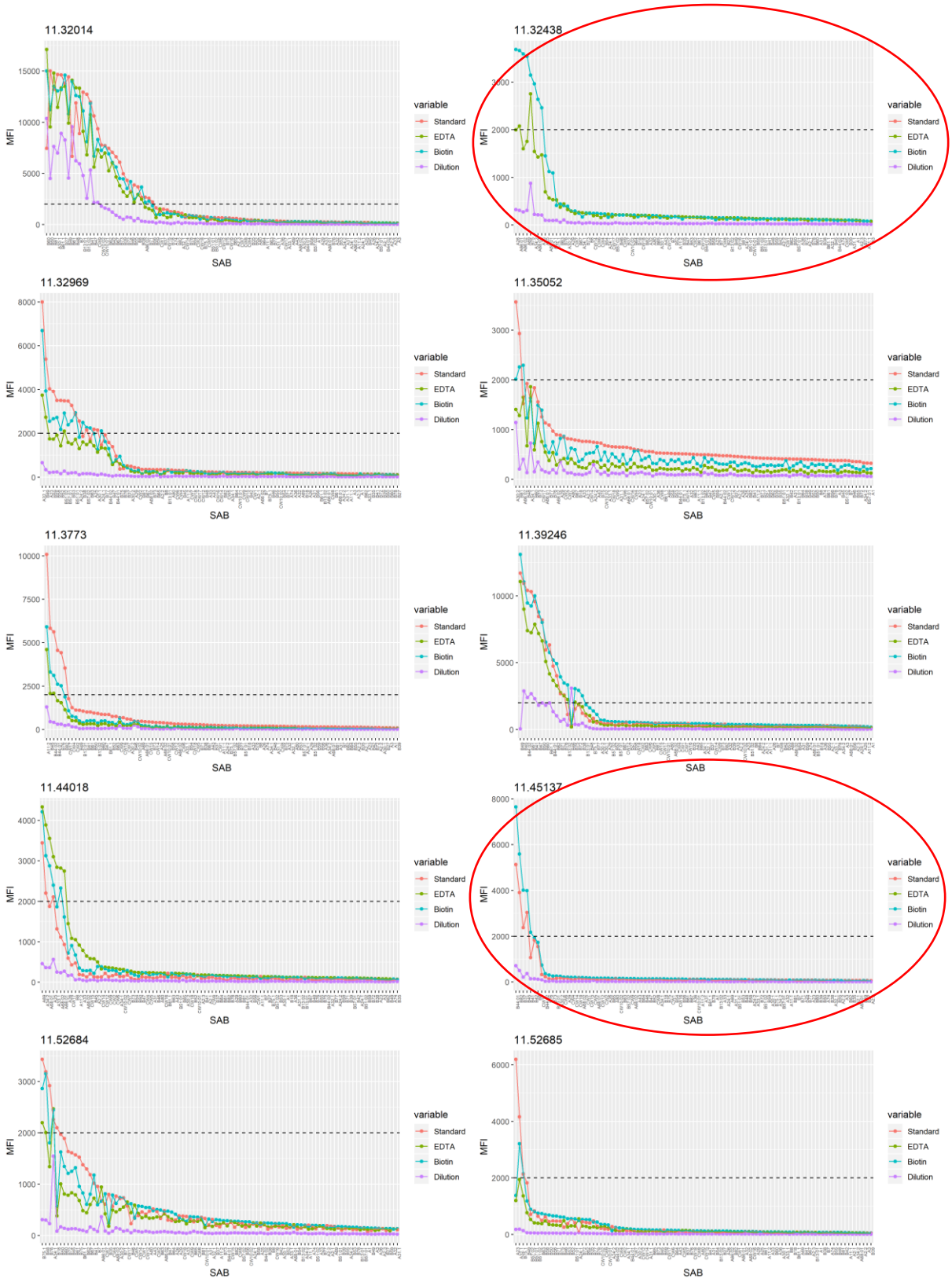
Biotin
Dilution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11.32969
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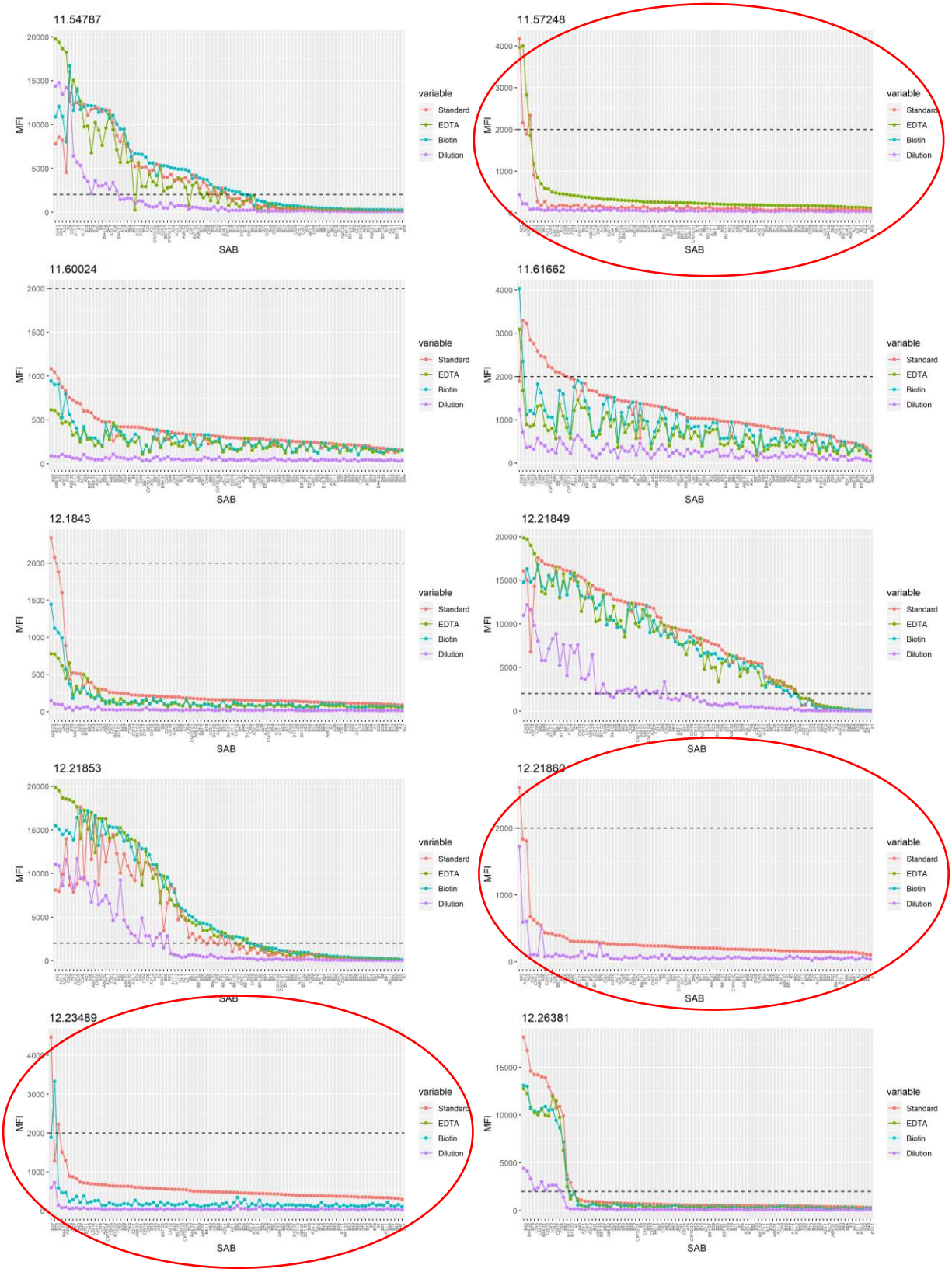
SAB
variable
Standard
EDTA
Biotin
Dilution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11.57248
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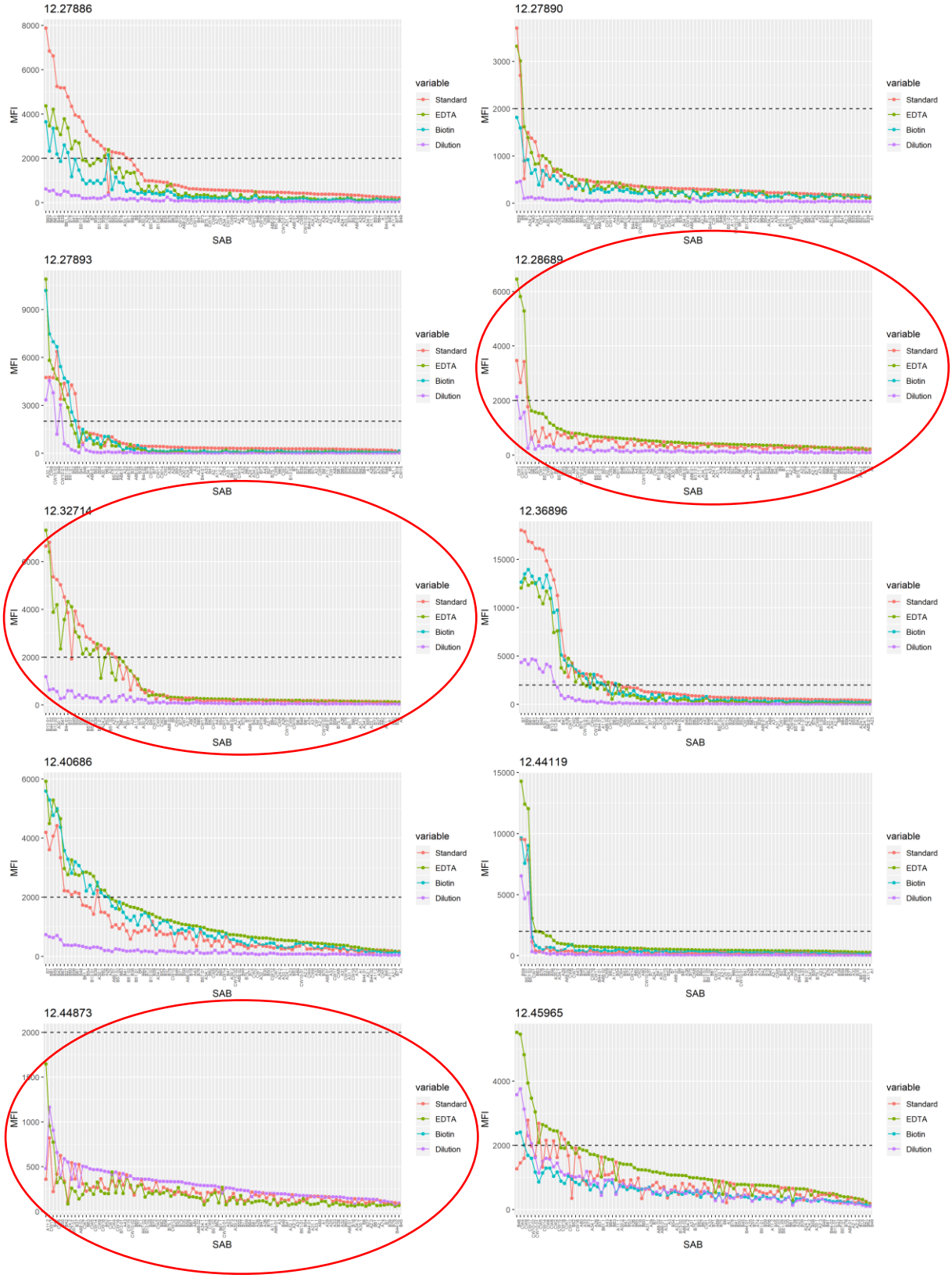
SAB
variable
Standard
EDTA
Biotin
Dilution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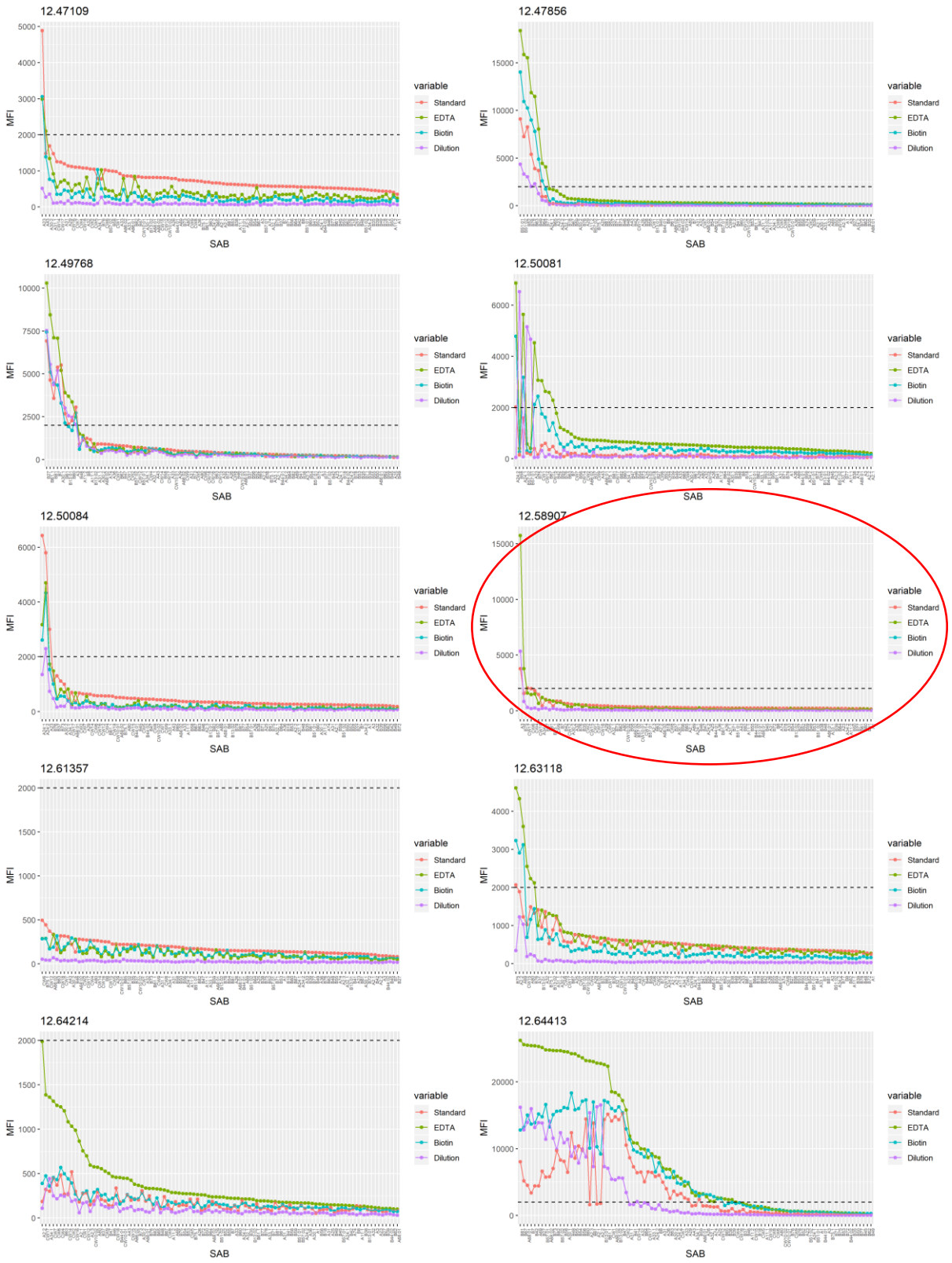
Biotin
Dilution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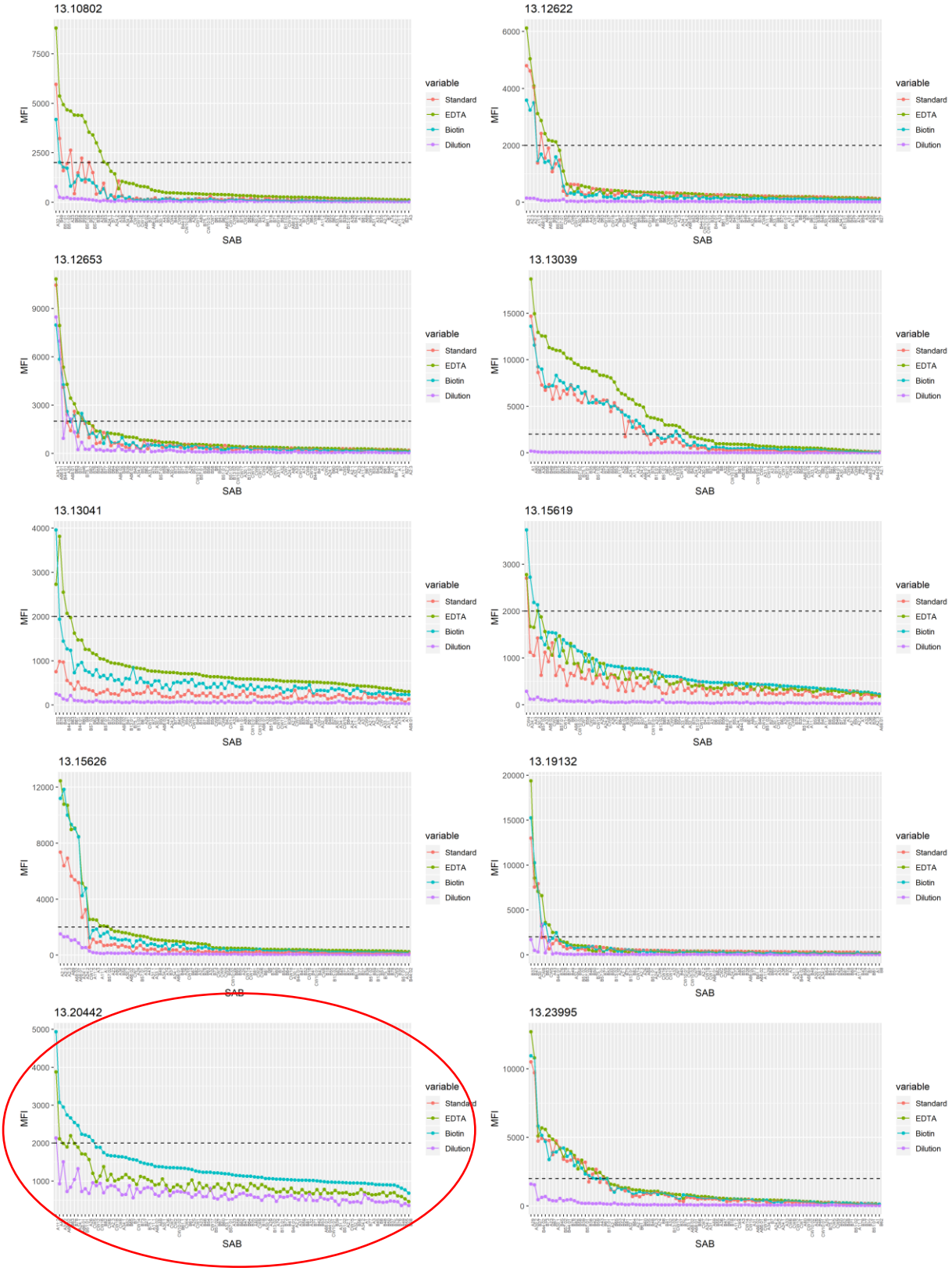
variable
Standard
EDTA
Biotin
Dilution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13.12653
13.13039
13.13041
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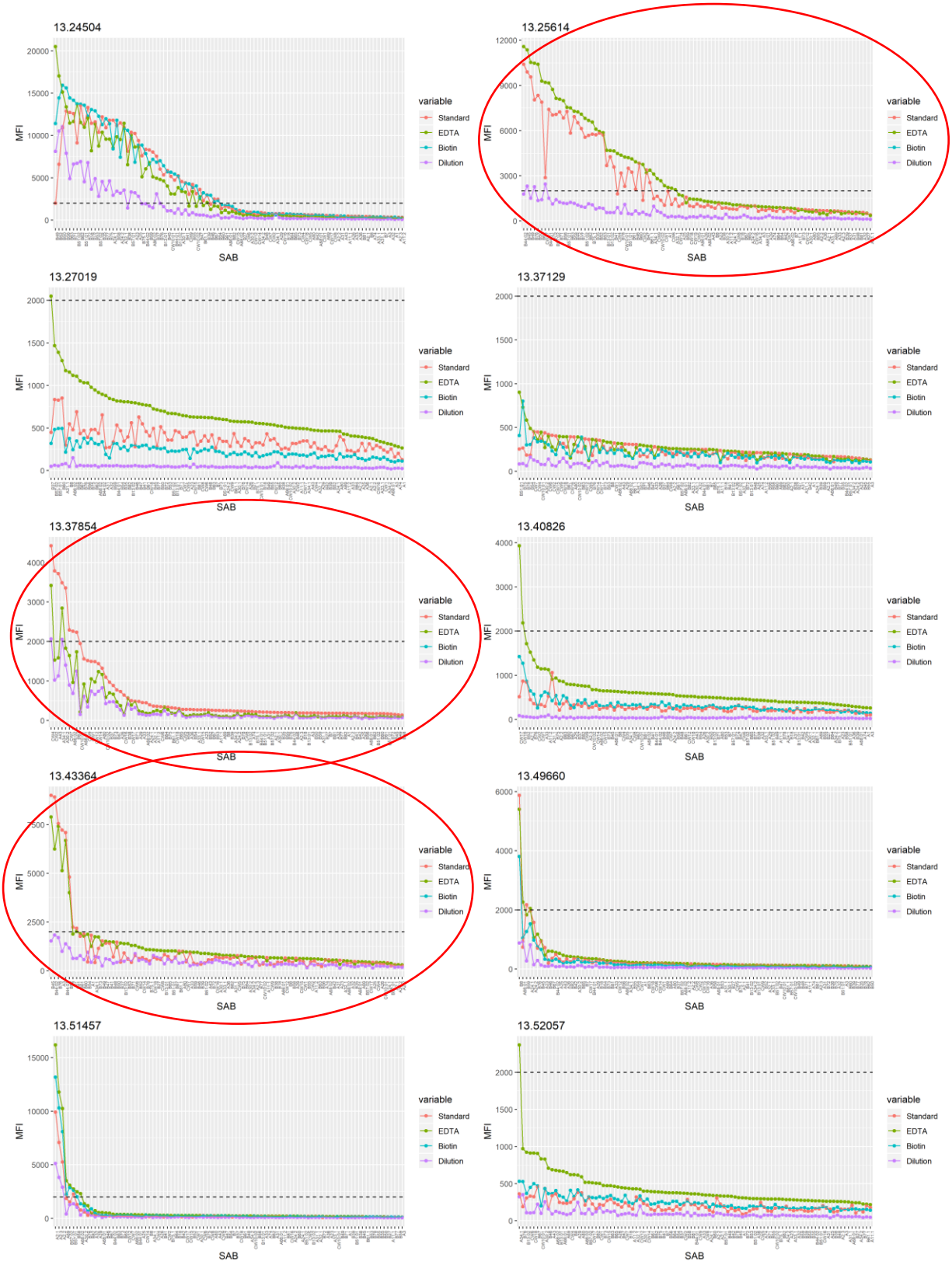
variable
Standard
EDTA
Biotin
Dilution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13.25614
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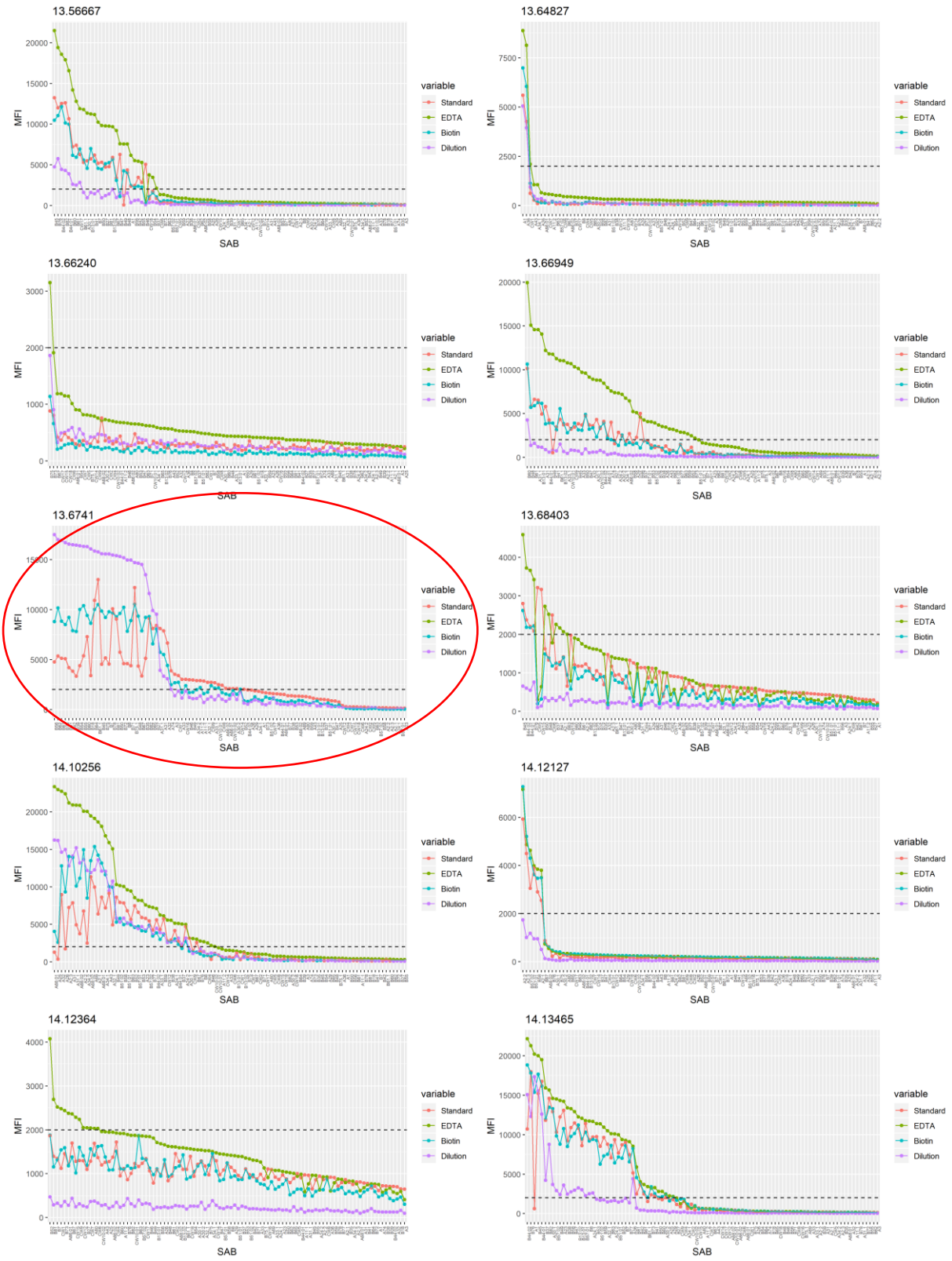
Biotin
Dilution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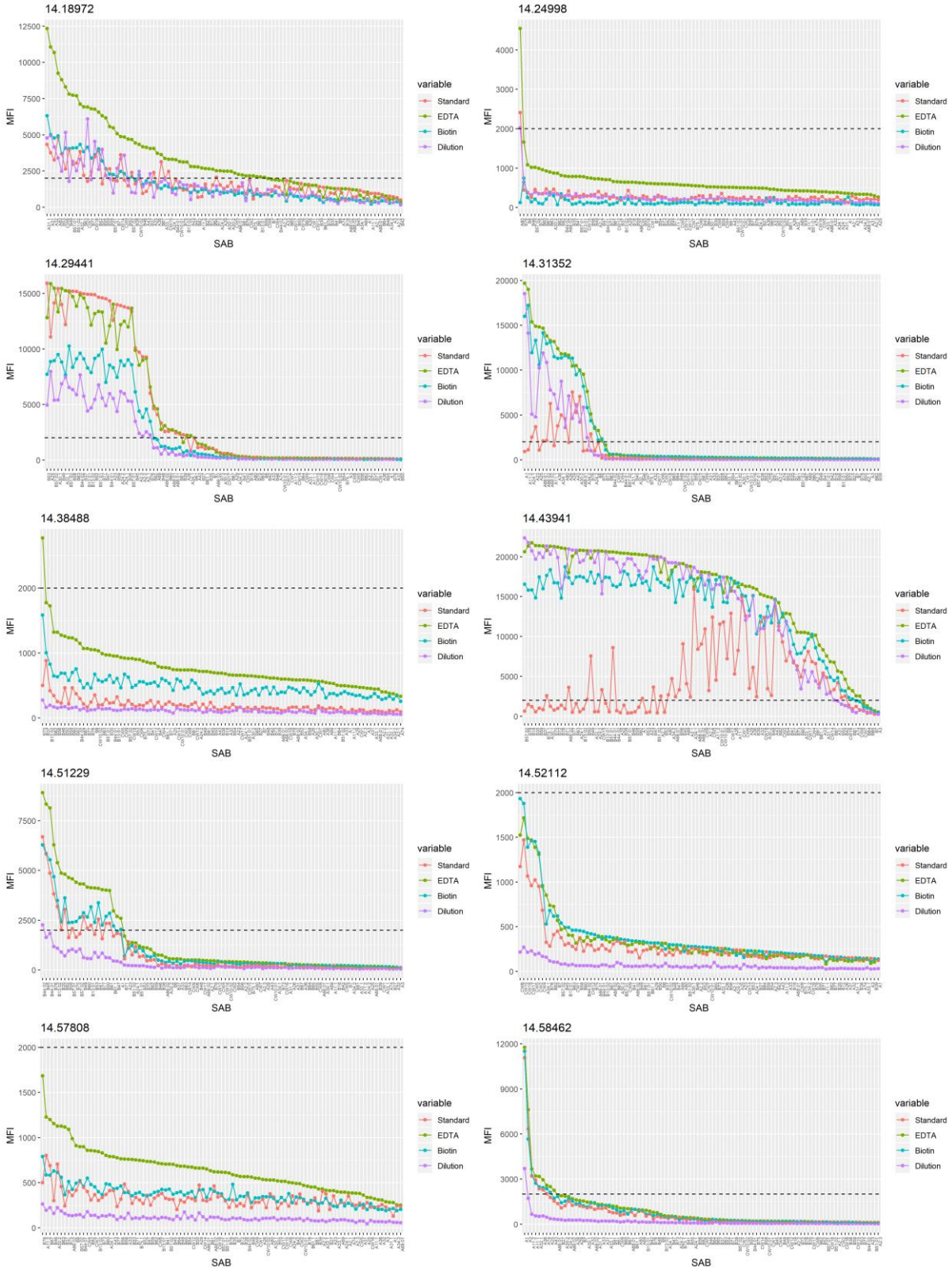
Dilution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14.38488
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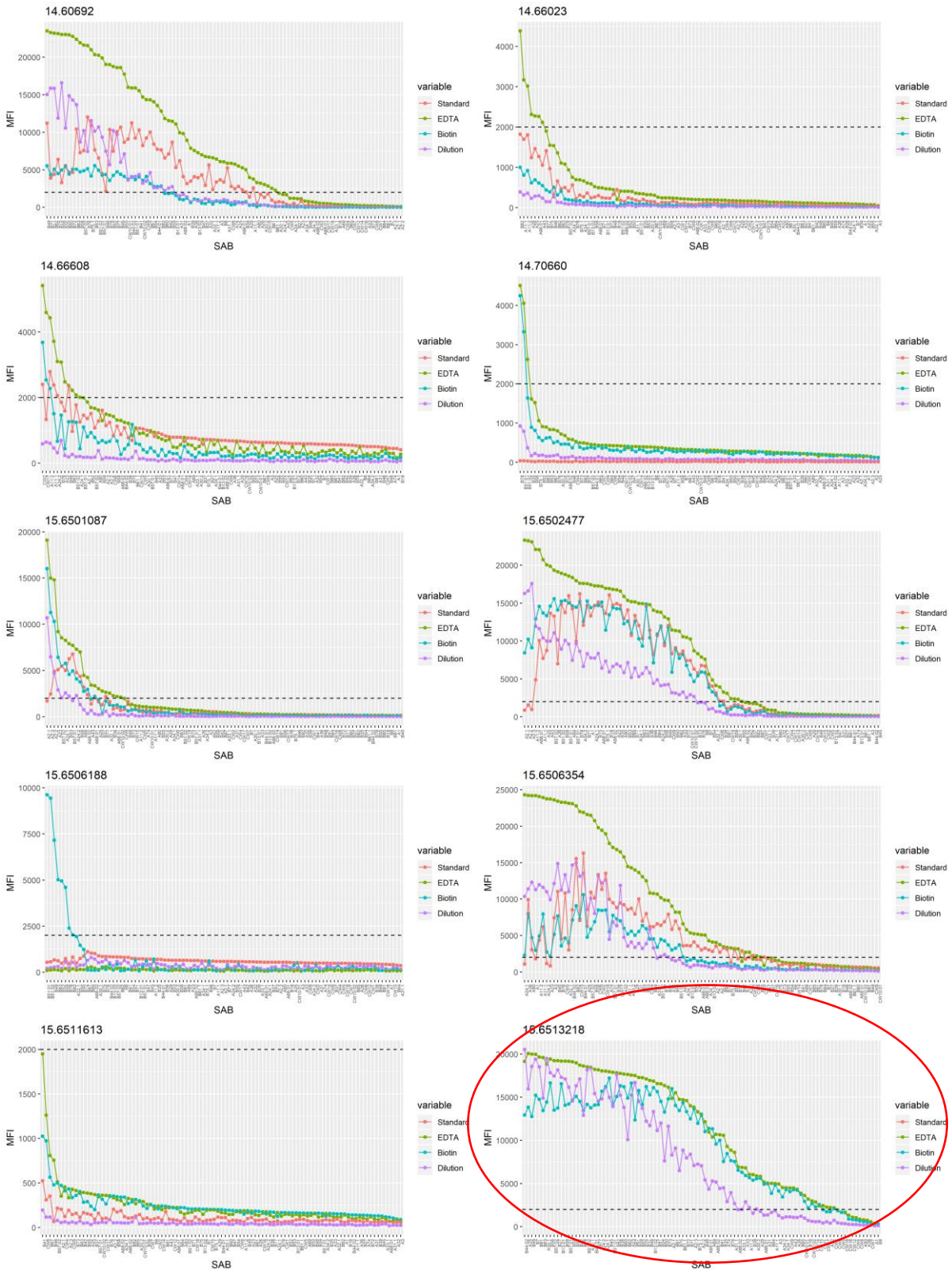
SAB
variable
Standard
EDTA
Biotin
Dilution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14.66023
14.66608
14.70660
15.6501087
15.6502477
15.6506188
15.6506354
15.6511613
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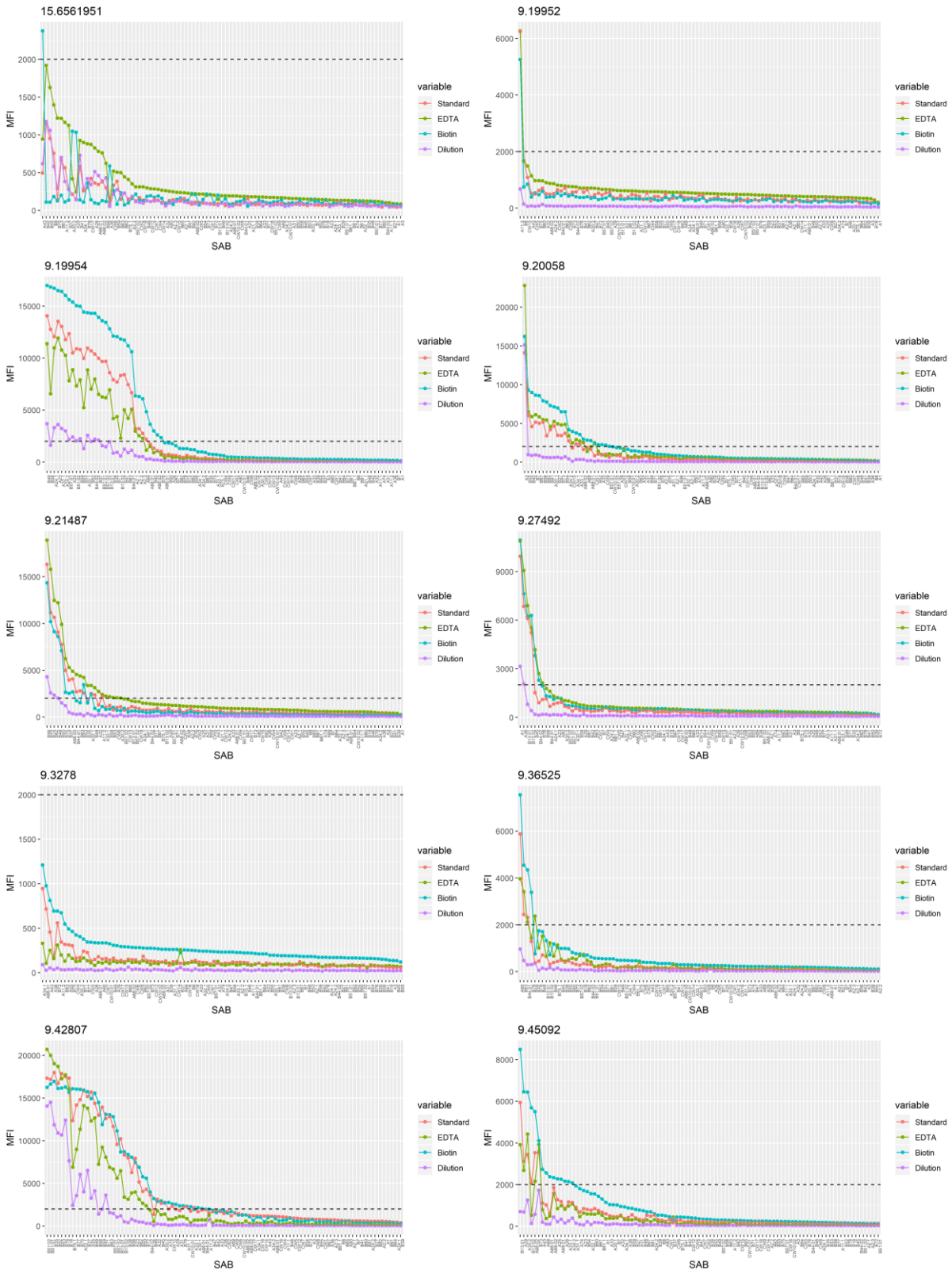
MFI
SAB
variable
Standard
EDTA
Biotin
Dilution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9.19952
9.19954
9.20058
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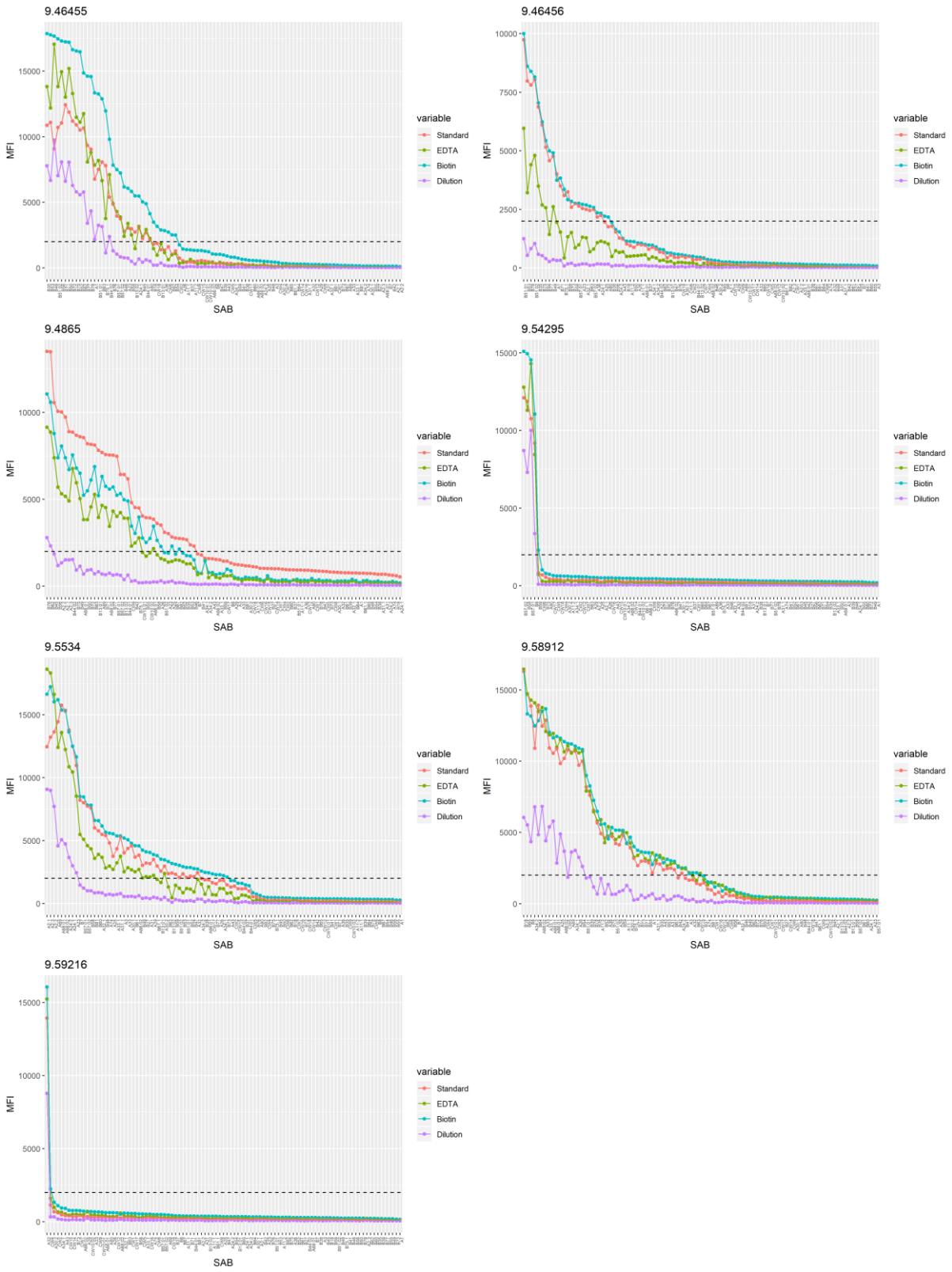
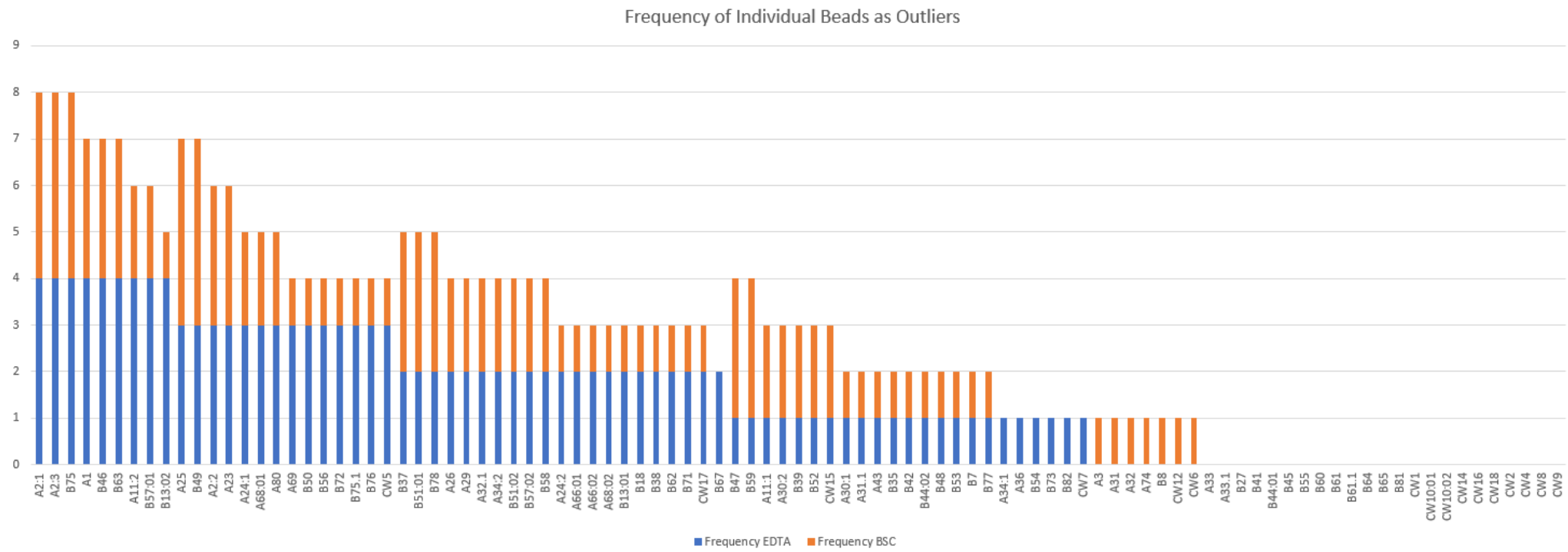
SAB
variable
Standard
EDTA
Biotin
Dilution



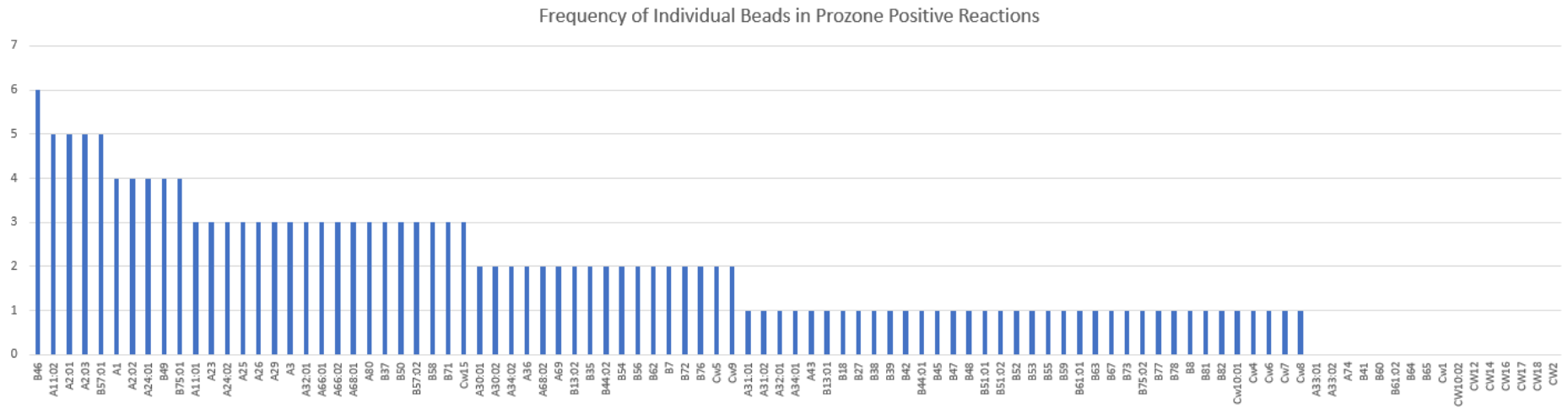
Supplementary Figure 2: Running over 14 pages, the plots present the MFI intensity for the standard test, EDTA, Biotin and Dilution series for 137 samples tested. The 20 samples indicated did not meet quality control thresholds, and were excluded.

Supplementary Table 1: Table to show reasons for sample exclusion

Reason for Exclusion	Samples Affected
Failed Bead Count (n=11)	10.55905 11.11184 11.22891 11.26722 11.32438 11.45137 12.23489 11.57248 12.32714 12.28689 12.44873
Failed Quality Control Analysis (n=9)	13.43364 13.37854 13.20442 13.6741 15.6513218 12.58907 10.4166 13.25614 12.21860



Supplementary Figure 3: Frequency table to show the number of times each bead falls into the outlier category: EDTA=blue bars; BSC=orange bars.



Supplementary Figure 4: Frequency table to show the number of times each bead falls into the prozone positive category