

Methods that remove batch effects while retaining group differences may lead to exaggerated confidence in downstream analyses

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SUMMARY

Removal of, or adjustment for, batch effects or centre differences is generally required when such effects are present in data. In particular, when preparing microarray gene expression data from multiple cohorts, array platforms, or batches for later analyses, batch effects can have confounding effects. Many methods and tools exist for this purpose. One method, ComBat which is part of the R package *sva*, is particularly popular due to its ability to remove batch differences even when batches are small and heterogeneous. It also has the option of preserving the difference between study groups, estimated from a two-way ANOVA model, to avoid conflating batch effects and group differences during batch adjustments. Unfortunately, this frequently used and recommended approach may systematically induce incorrect group differences in downstream analyses when groups are distributed between the batches in an unbalanced manner. The scientific community seems to be largely unaware of this problem, which most likely has contributed to false discoveries being presented in the published literature.

Key words:

1. INTRODUCTION

Extraneous variables, if left unaccounted for, have the potential to lead an investigator into drawing wrong conclusions. In molecular biology, extraneous variables are often called "batch effects", probably due to the fact that reagents and other equipment, for instance microarray chips, are made in batches, and this is frequently observed as an effect in the measurements. See [Luo and others \(2010\)](#) for more examples.

For a typical experiment comparing group differences, the presence of batch effects will decrease the statistical power since it adds variation to the data. If the batch-group design is unbalanced, i.e if the study groups are not equally represented in all batches, batch effects may act as a confounder and induce false differences between groups ([Leek and others, 2010](#)).

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The standard way to handle an extraneous variable is to include it in the statistical model employed in the inquiry. However, many analysis tools for high throughput data do not cater for this option, and when available it could still be outside the competence of the investigator. Therefore, an alternative two step procedure has emerged. First the batch effects are estimated and removed, creating a “batch effect free” data set. In the next step, the statistical analyses are performed on the adjusted data without further consideration of batch effects. This appealing compartmentalization is also convenient for practical purposes, for example when data-processing and statistical analyses are performed by different personnel. Unfortunately, as we demonstrate in this paper, when the batch-group design is unbalanced, this approach may be unreliable.

A simple removal of batch effects can be achieved by subtracting the mean of the measurements in one batch from all measurements in that batch, i.e. mean adjustment or one-way ANOVA adjustment as implemented in the method `pamr.batchadjust` from the `pamr` package in R. When the batch-group design is balanced, mean-adjustment will remove most, but not necessarily all, variance attributed to batch and leave the between group variance, thus increasing the statistical power. However, when the batch-group design is unbalanced, batch differences will in part be influenced by group differences, and thus batch correction will reduce group differences and thereby reduce the statistical power. In very uneven group-batch designs with multiple groups, spurious group differences may even be induced in this way. Figure 1 illustrates both these effects.

To mitigate the above problems, one may simultaneously estimate batch effects and group differences, e.g. using a two-way ANOVA, and only remove the batch differences from the data. Effectively, group differences are estimated based on within batch comparisons, and applied to the batch adjusted data. In a balanced group-batch design, group differences and batch effects are independent, and this approach becomes identical to the above described zero-centering per batch. If the group-batch design is heavily unbalanced, estimation of group differences and batch effects are interdependent. However, in applying the estimated group differences across the entire data set, the uncertainties of these estimates are ignored. If the group-batch design is unbalanced and batch compositions ignored after batch adjustments have been made, later analyses will systematically underestimate the statistical uncertainties and exaggerate the confidence of group differences. Figure 1 illustrates how statistical uncertainties are deflated by this batch adjustment method by comparing them to the uncertainties from the original ANOVA.

The ComBat method described in [Johnson and others \(2007\)](#), and included in the `sva` package ([Leek and others, 2012](#)), can use either of the two above described approaches to estimate batch differences, but uses an empirical Bayes approach to avoid over-correction for batch effects for small batches. It has thus improved and popularised the two-way ANOVA procedure for retaining group differences when adjusting for batch effects, and the inclusion of group difference as a covariate when removing batch effects has been recommended in the `sva` tutorial and user fora. Based on actual use of ComBat by the authors and others, ourselves included, we suspect thus adjusted data are commonly treated as “batch effect free” in subsequent analyses. And as a consequence, confidence in group effects has been overestimated and false results reported.

Statisticians would most likely take extra precautions should the sample or batch sizes be very small. However, the effects of batch adjustment using two-way ANOVA or ComBat on unbalanced data sets remain even as sample and batch sizes increase. For example, group comparisons using one-way ANOVA on the batch adjusted data will essentially result in F statistics that are inflated by a fixed factor which depends on the unevenness of the design rather than the size of the sample or batches. The effect of this may be further exacerbated by running these analyses a large number of times, e.g. on thousands of genes, and use false discovery rate to determine significant cases: an approach that is particularly sensitive to inflated false positive rates.

2. METHODS FOR BATCH EFFECT CORRECTION

2.1 Model for data with batch effects

We will base our discussion on a simple model for data with batch effects:

$$Y_{ijr} = \alpha + \beta_j + \gamma_i + \epsilon_{ijr} \quad (2.1)$$

where $i = 1, \dots, m$ are the different batches, $j = 1, \dots, M$ are different study groups that we wish to compare, and $r = 1, \dots, n_{ij}$ are the different samples within batch i and group j .

When combining data from more diverse data sources, e.g. microarray data from different platforms, a more general model is required. One such model, used by [Johnson and others \(2007\)](#), is

$$Y_{ijgr} = \alpha_g + X_r \beta_g + \gamma_{ig} + \delta_{ig} \epsilon_{ijgr} \quad (2.2)$$

where $g = 1, \dots, G$ are different measurements, e.g. genes, performed for each sample, and X is the design matrix which in our case will indicate the study group. This permits independent rescaling of data from different batches. In addition, [Johnson and others \(2007\)](#) uses an empirical Bayes approach to estimate γ_{ig} and δ_{ig} to stabilise estimates, which is critical for use with small batches.

For simplicity, we consider the case with a single gene and constant scale, i.e. $\delta_{ig} = 1$. We will discuss the effect of empirical Bayes estimation of γ_{ig} later, but our main argument is more easily made in the simpler context. Empirical Bayes may reduce the amount of batch adjustment in cases where batch effects are small or cannot be accurately estimated, e.g. for small batches. However, in cases with large batches or substantial batch effects, it should differ little from the two-way ANOVA approach.

2.2 Standard batch correction methods

A common ambition of batch effect adjustments is to remove batch differences in such a way that downstream analyses of the adjusted data may be done without further corrections for batches.

The most common method for removing batch effects is to zero-centre each batch:

$$\Delta \tilde{Y}_{ijr} = Y_{ijr} - \bar{Y}_i \quad \text{where} \quad \bar{Y}_i = \frac{\sum_{j=1}^M \sum_{r=1}^{n_{ij}} Y_{ijr}}{\sum_{j=1}^M n_{ij}}. \quad (2.3)$$

An alternative is to centre each batch to the common average by adding the average value \bar{Y} across the entire data set: i.e. $\tilde{Y}_{ijr} = \Delta \tilde{Y}_{ijr} + \bar{Y}$. When comparing groups, the common value \bar{Y} has no effect, and so this is equivalent to zero-centring each batch. If the groups are unevenly represented in the different batches, the batch average \bar{Y}_i will tend to capture group differences as well as batch effects:

$$\Delta \tilde{Y}_{ijr} = \beta_j - \bar{\beta}_i + \epsilon_{ijr} - \bar{\epsilon}_i \quad \text{where} \quad \bar{\beta}_i = \frac{\sum_{j=1}^M n_{ij} \beta_j}{\sum_{j=1}^M n_{ij}}, \quad \bar{\epsilon}_i = \frac{\sum_{j=1}^M \sum_{r=1}^{n_{ij}} \epsilon_{ijr}}{\sum_{j=1}^M n_{ij}}. \quad (2.4)$$

Thus, batch centering will tend to reduce group differences in an unbalanced design, and thus reduce the power of downstream analyses. By reducing the differences between some groups, i.e. those found together in the same batches, it may also induce false differences between other groups.

Removing batch effects while retaining group differences can be done through a two-way ANOVA in which group effects, β_j , and batch effects, γ_i , are estimated simultaneously. Batch adjusted values may then be obtained by subtracting the estimated batch effects, $\hat{\gamma}_i$:

$$\tilde{Y}_{ijr} = Y_{ijr} - \hat{\beta}_j = \alpha + \beta_j + (\gamma_i - \hat{\gamma}_i) + \epsilon_{ijr}. \quad (2.5)$$

This will yield batch adjusted values where any systematic bias induced by the batch differences has been removed, while the group differences are retained.

The estimation error $\hat{\gamma}_i - \gamma_i$ affects all values within the same batch in the same manner. Thus, while the aim is to remove spurious dependencies within batches, it may also induce new dependencies. In a balanced group–batch design, this has the same effect on all groups and thus does not influence group comparisons. In an unbalanced design, however, the estimated batch effects $\hat{\gamma}_i$ will correlate with the estimated group effects $\hat{\beta}_j$. *Assuming the batch adjusted values are without further batch differences corresponds to assuming that $\gamma_i = \hat{\gamma}_i$, and ignoring the estimation uncertainty and its impact on the correlated $\hat{\beta}_j$. It results in over-confidence in the accuracy of the group differences, which can have severe impact on downstream analyses.* *[Denne forklaringen må jeg gjøre mye klarere.]*

3. RESULTS

3.1 A simple sanity check

The undesired consequences of preserving group effects when correcting for batch effect is readily illustrated with a sanity check using random numbers . The documentation accompanying the sva library has a runnable example demonstrating how to adjust a data set with ComBat followed by a F-test. Swapping the real data with random numbers from a normal distribution (mean=0, sd=1), but otherwise following the instructions, will generate the p-value distribution shown in Figure ?? *[Perhaps show Q-Q plot of F-statistics.]* The skewed distribution is an indication that this approach may have a unintentional adverse effect. *[Tror vi dropper permutasjonstesten.]*

If the number of samples is increased, the empirical Bayes estimates used by ComBat will shrink the batch effect estimates and thus moderate the batch adjustments. However, if random data with different means in each batch, but no difference between groups, are drawn, the problem remains. *[Bør dette komme her eller senere? Evt. understøttes?]*

3.2 Explanation for the simple two-group comparison

To explain more clearly what is happening, and quantify the size of the problem, we may consider the simple case of estimating the difference $\Delta\beta = \beta_A - \beta_B$ between two groups, A and B , when there are m batches with batch i containing n_{iA} and n_{iB} samples from each of the two groups for $i = 1, \dots, m$.

If we estimate the group difference within batch i , we get

$$\widehat{\Delta\beta}_i = \bar{Y}_{iA} - \bar{Y}_{iB} \sim N\left(\Delta\beta, \frac{\sigma^2}{\nu_i}\right) \quad \text{where} \quad \bar{Y}_{ij} = \frac{\sum_{r=1}^{n_{ij}} Y_{ijr}}{n_{ij}}, \quad \nu_i = \frac{1}{\frac{1}{n_{iA}} + \frac{1}{n_{iB}}}, \quad (3.6)$$

from which we may express the overall estimate of $\Delta\beta$

$$\widehat{\Delta\beta} = \frac{\sum_{i=1}^m \nu_i \widehat{\Delta\beta}_i}{\nu} \sim N\left(\Delta\beta, \frac{\sigma^2}{\nu}\right) \quad \text{where} \quad \nu = \sum_{i=1}^m \nu_i. \quad (3.7)$$

If batch and group effects are estimated using a two-way ANOVA, the estimate $\widehat{\Delta\beta}$ will be as stated above, and so the estimated group difference is unaffected. The batch effects are then removed, and the estimated group differences retained, leaving the estimated $\widehat{\Delta\beta}$ unchanged by the batch adjustment. However, if this batch adjusted data set is analysed without considering batch effects, the variance of $\widehat{\Delta\beta}$ will be computed under the assumption that it is derived from a comparison of $n_A = \sum_{i=1}^m n_{iA}$ versus $n_B = \sum_{i=1}^m n_{iB}$ samples, and thus satisfy

$$\widehat{\Delta\beta} \sim N\left(\Delta\beta, \sigma^2 \cdot \left(\frac{1}{n_A} + \frac{1}{n_B}\right)\right). \quad (3.8)$$

Using Jensen’s inequality, we may then derive

$$\frac{1}{\frac{1}{n_A} + \frac{1}{n_B}} = n \cdot \frac{n_A}{n} \cdot \frac{n_B}{n} \leq \nu = \sum_{i=1}^m \frac{1}{\frac{1}{n_{iA}} + \frac{1}{n_{iB}}} = \sum_{i=1}^m n_i \cdot \frac{n_{iA}}{n_i} \cdot \frac{n_{iB}}{n_i} \quad (3.9)$$

with equality if and only if the ratios $n_{iA} : n_{iB} = n_A : n_B$ for all batches $i = 1, \dots, m$.

3.3 Examples of undesired consequences

As the amount of false positive results when trying to retain group differences depends on the batch–group balance, we will show two examples with varying degree of unbalancedness.

3.3.1 Experiment 1 In the first experiment (Towfic and others, 2014), cells were treated with glatiramer acetate (a medicine for multiple sclerosis) or a generic and mRNA was measured using microarrays alongside control samples. A batch effect correlating to the chip (Illumina WG-6_V2, six samples per chip) was observed and adjusted for with ComBat, whereafter the data was tested for differentially expressed genes, yielding a list of 1000 genes (Table S5, Towfic and others, 2014). Unfortunately the batch–treatment design was unbalanced with several batches having only one of the main treatments of interest. When we re-analyzed their data without using ComBat, but instead blocked for batch effect in limma, only 9 genes were found ($\text{FDR} < 0.05$). *[Also run analysis using a mixed random effects model which assumes random batch effects much like in the empirical Bayes model.]* The above outlined sanity check with random numbers was also carried out. The distribution of p-values for different settings are shown in Figure ?? . Our conclusion is that most of the genes reported as differentially expressed in (Towfic and others, 2014) are false positives. This example is a sort of “worst case” scenario for applying ComBat, since it both has a very unbalanced batch–group design and a a priori assumption of no difference. The R-code for our analysis and a more extensive report can be downloaded from Github *[Update reference]*.

3.3.2 Experiment 2 The second example is taken from the supporting information for the original ComBat article (Johnson and others, 2007) where it is denoted “Data set 2”. Cells inhibited for the expression of the TAL1 gene were compared to controls on a microarray platform. The experiment was conducted on three different time points (used as batches) with a total of 30 samples and a fairly balanced batch–treatment set up (6-2, 3-4 and 9-6). ComBat was applied followed by a t-test in order to identify differentially expressed genes. First, we reproduced their analysis including the adjustment by ComBat, but using limma instead of the t-test, resulting in 1003 probes ($q < 0.05$). Then, we analysed their data without batch adjustment in ComBat, but blocking for batch in limma, resulting in 377 probes ($q < 0.05$). In addition, the sanity checks outlined above were performed. The distribution of P-values for different settings are shown in

Figure ??b. In contrast to the results obtained for [Towfic and others, 2014](#) (Figure ??a), the P-value distributions for the alternative analysis does not indicate a huge difference. Nevertheless, we believe that P-values are deflated for the ComBat adjusted analysis. The R-code for our analysis can be downloaded from [Github \[Update reference\]](#).

3.3.3 More? *[Her kan vi fylle paa med flere eksempler etter samme mal hvis det trengs. Feks en fersk artikkel fra Nature Genetic (Yoo and others, 2014). Der har de kombinert sekvenseringsdata (deres data) fra 9 lymfom pasienter med et (ekstern) microarray data set som har 6 lymfom proever og 5 kontroller, dvs kontroll gruppen er helt fravaerende hos dem. Dvs veldig ubalansert (9/0, 6/5), MEN skille mellom phenotypene er saa stor at de sikkert hadde funnet omtrent de samme genene signifikante med aa bare bruke de balanserte dataene (men det var jo ikke deres.) Dette er ikke en viktig del av paperet, men det er veldig feil selvom konsekvensene ikke blir store. Dog gir det et falskt inntrykk av at dataene deres ligner tidligere data, se heatmap i suppl.. Dessuten lager de GO analyser av dette som egentlig er 7 aar gamle andres data og ikke deres]*

4. DISCUSSION

Systematic underestimation of P-values combined with FDR is a particularly bad combination as FDR lends strength across tests.

4.1 Increased emphasis on preserving group difference

In the original ComBat article ([Johnson and others, 2007](#)) it is clear that the primary motivation behind ComBat was to employ an Empirical Bayes method for batch-effect removal to allow better handling of small batches. The feature of retaining group differences for unbalanced designs is optional and seems to be subordinate, only exemplified in the supplementary information. However, over the years this feature became more important judging from advice given on user fora. When ComBat was later incorporated in the sva package ([Leek and others, 2012](#)), [batch adjustment without covariates was no longer a default as specification of covariates became a requirement, although specification of a null model was still possible although not clearly documented in the sva documentation \[Is this correct?\]](#). Thus, we expect this usage to have grown more common.

4.2 Motivation for this warning

Our knowledge of the problem discussed in this article came through a typical use case when trying to adjust for batch effects in an unbalanced data set using ComBat. Upon realizing that the confidence on our group differences were exaggerated, the literature was searched for a better understanding of correct use and potential overseen limitations of ComBat. But the authors of ComBat and the sva package recommended our usage ([Johnson and others, 2007](#), [Leek and others, 2012](#)). In addition, other works looking into the problem of batch effects were mostly recommending ComBat without much concern ([Kupfer and others, 2012](#), [Kitchen and others, 2011](#)). A brief inquiry into some of the articles citing ComBat (574 Google Scholar) revealed few problems, and their method descriptions regarding ComBat were mostly sparse, limited to one or two sentences. A further indication of their carefree use of this potentially devastating procedure was the frequent omission of program parameters that were used, i.e batch labels or whether group labels were supplied as covariates. Often no effort was done in order to sub-

stantiate the existence of batch effects in their data, except for stating the presence of batches. The incorporation of the method into analysis pipelines ([16642009], TCGA) and other packages ([23452776], [21937664]) could make its usage even more trivial and parameters setting harder to perceive. Taken together we fear that many published results from data adjusted by ComBat are completely or partially false. We hope that our warning will enlighten the community and reduce this unfortunate combination of methods.

4.3 Practical advice

For an investigator facing an unbalanced data set with batch effects, our advice is to adjust for batch inside the statistical test and avoid the two step procedure outlined above. If this is not possible, then applying the method described in this article should only be done with great caution and downstream confidence estimates must be treated with suspicion.

Knowing that adjusting for batch effects while preserving the group difference may lead to varying degree of false results, to what extent can an investigator trust a result from a work applying such a method? Essentially, when the batch-group configuration is balanced, or group difference is ignored (i.e. group labels not given as parameters to ComBat), problems related to preserving group differences will not occur. For other cases, a re-analysis without using this approach is the most rigorous path. However, this thoroughness is not feasible if the downstream analysis can not adjust for batch effects by it self. To reach a reliable result, batch effects need to be handled in some way or another. To make matters worse, a re-analysis relies on the availability of the raw data and a description of processing and analysis steps taken in the original work. Even when this is available, the necessary bioinformatic skills and work hours could still be in short supply. For such situations, a superficial assessment can be performed, taking special note of batches were groups of interest are near missing and how likely a group difference is. In essence asking if the balanced parts (effective sample size?) of the data has enough power to detect the presumed effects and if this is the case (Johnson *and others*, 2007, [24584070]), treat the results more like an ordered list with the most likely true positives on top while de-emphasizing the somewhat deflated p-values. In contrast, if biological knowledge suggest that a group effect is unlikely ([24391845], [18414638]?, [21731603], [23630272]), an intermediate lack of batch-group balance could lead to a mostly false result.

5. SUPPLEMENTARY MATERIAL

Supplementary material is available online at <http://biostatistics.oxfordjournals.org>.

5.1 Reproducible research

The data and scripts used to generate the results in this work, i.e figures, are made available at <https://github.com/ous-uio-bioinfo-core/batch-adjust-warning-figures.git>. Additional analyses were also performed, but considered excessive for the current article. However, some readers might find this very interesting and we provide it as an extra resource at <https://github.com/ous-uio-bioinfo-core/batch-adjust-warning-reports.git>

*[vi boer klare aa tilfredstille kravene i <http://biostatistics.oxfordjournals.org/content/10/3/405.full>]
[For aa tilfredstille kravene skal data og script vare available. Jeg mener vi klarer dette. paa githuben er ikke data fra towfic, men det blir lastet av scriptet fra GEO. Ikke beskrevet her men i scriptet. Videre i forklaringen til biostatistics staar det at script og data skal bli lagt paa deres*

sider, som vi kan hvis de vil]

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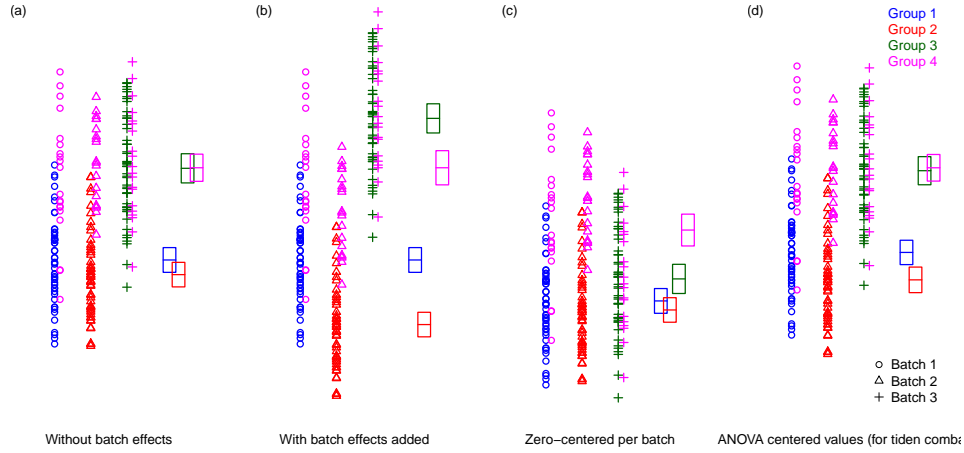


Figure 1. Simulated data from four study groups was generated where groups 1 and 2 have lower means than groups 3 and 4. These were placed in three different batches with batch effect added. Values and boxes showing mean and two standard errors of the mean are displayed for data without batch effects, after adding batch effects, after batch centering, and after ANOVA based batch centering. The last frame shows the least squares estimates of the group means from a two-way ANOVA analysis with 2 standard errors. This case, design and effects, was selected to illustrate the spurious effects that may arise.

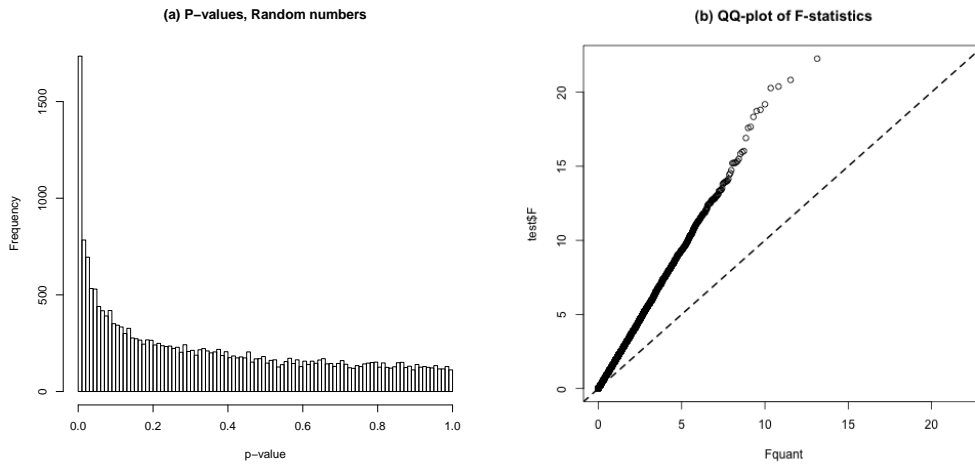


Figure 2. A sanity checks where the recommended use of ComBat fails. Adapted from the user guide in the sva package. Real data are substituted with random numbers from a normal distribution, but the batch-group design is retained. ComBat is applied followed by a F-test. a) P-value distribution b) QQ plot of the F-statistics

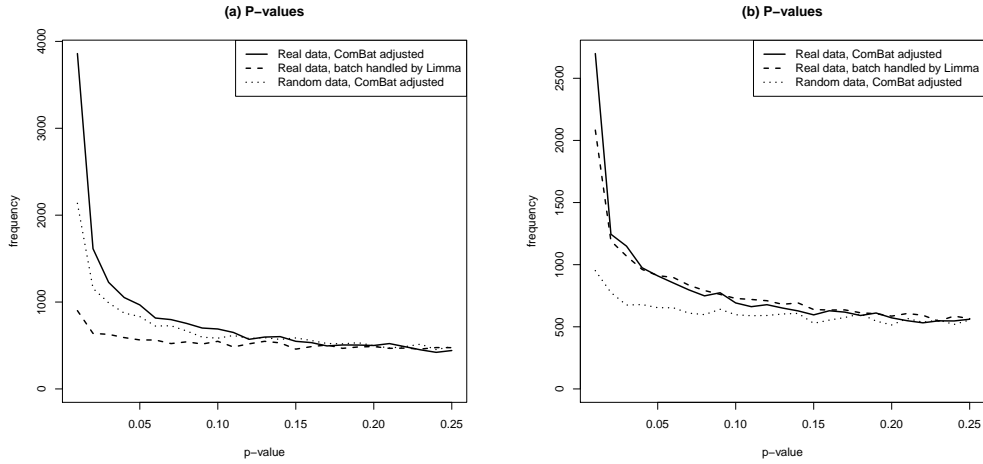


Figure 3. Three analyses of two works where batch effects were adjusted for with ComBat. First as described in the original works with ComBat on real data. Secondly, with ComBat but with random numbers instead of real data. Thirdly without ComBat and instead blocking batch with limma on real data. a) Re-analysis of [Towfic and others, 2014](#), glatiramer acetate vs. generic b) Re-analysis of "Data set 2" [Johnson and others, 2007](#), TAL1 inhibition vs. control