RESPONSE TO REVIEWERS FOR "ALLELE-SPECIFIC BINDING AND EXPRESSION: A UNIFORM SURVEY OVER THE 1000-GENOMES-PROJECT INDIVIDUALS"

RESPONSE LETTER

Reviewer #1

-- Ref1 – General positive comment --

Reviewer Comment	This reviewer did not have formal comments to the authors as s/he found the revised paper to be satisfactory and endorses publication.
Author Response	We thank the reviewer for his/her thorough examination of our manuscript and endorsing our paper for publication.

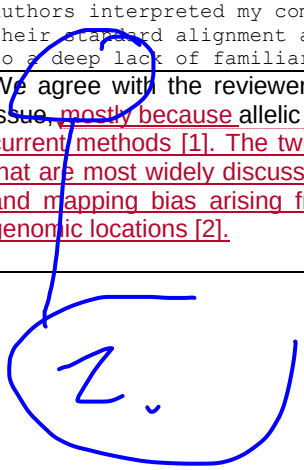
Reviewer #2

-- Ref2.1 – General comment --

Reviewer Comment	The authors did not adequately address my two major concerns.
Author Response	We thank the reviewer for the thorough examination of our manuscript. We have provided additional analyses and responses.

-- Ref2.2 – mapping to the personal diploid genome --

Reviewer Comment	My first comment was that mapping bias should be addressed. The authors replied by explaining that they excluded reads that map to more than one location. This is indeed a standard step in more alignment. Yet, the challenge when looking for ASE is not standard. Different alleles may have different mapping probabilities and this must be taken into account. Failing to do so results in a high number of falsely identified ASE. I must admit that it is a bit concerning to me that the authors interpreted my comment as a question regarding their standard alignment approach. In my mind, it points to a deep lack of familiarity with the ASE literature.
Author Response	We agree with the reviewer that allelic mapping bias is still an issue, mostly because allelic bias cannot be totally eradicated with current methods [1]. The two main types of allelic mapping bias that are most widely discussed in the field are the reference bias and mapping bias arising from sequence homology with other genomic locations [2].



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Reference bias has been widely regarded as the main source of mapping bias, since the more standard alignment procedure is, in fact, alignment of reads to the human reference genome, not to personal genomes [1,3,4,6]. A recent study by Panousis *et al.* found that the bias towards the reference allele contributes to the main bulk of the overall mapping bias in allele-specific expression [5]. Many publications have specifically cited the use of personal genomes as a rigorous but computationally intensive procedure to correct for reference bias [1,3,4,5,6]. Thus, we are acutely aware of this primary issue in mapping bias, and have chosen to focus specifically on rectifying the reference bias by aligning to a diploid personal genome. Nonetheless, we undertook this endeavor, to not only construct diploid personal genomes for all 382 individuals but also created tools for the personal genome construction.

While we expect the majority of the allelic bias to be accounted for, or at least alleviated, in the form of reference bias by the use of the personal genomes, we agree with the reviewer that a small proportion of the mapping bias still exists. This is especially the case in situations where short reads that carry one allele may map perfectly to a reference genome but reads with the other allele (multi) map to multiple loci (due to sequence homology in other regions) (Figure 1) as described also by previous studies [1,5,6]. Most studies have examined this allelic bias due to sequence homology in the context of the human reference genome. The primary solution to date has been the **removal of sites**, in which >5% of the total number of reads exhibit such allelic mapping bias [1,5,7,8,9,10]. However, we note that this can be overly stringent, because it potentially removes a considerable number of sites that might still be allele-specific even after **removing reads** with mapping bias, especially at sites with many reads.

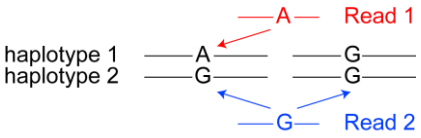

Figure 1. Adapted from van de Geijn et al. showing allelic mapping bias in a personal genome due to sequence homology in other locations. *Here, Read 1 uniquely maps to the haplotype 1, but Read 2 with the alternate allele maps to multiple locations in the other haplotype, and is therefore removed.*

We investigated the effect of the allelic mapping bias (due to sequence homology) and the two removing strategies on the detection of allele-specific SNVs, in the context of the diploid personal genome. Briefly, for each individual, we **(1)** first align the reads to the two reference haplotypes, each with their own sets

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of SNVs and indels. For each haplotype, we **(2)** retain only those reads that uniquely mapped to regions with heterozygous SNVs, and then artificially create the same reads but with a single allele change at the heterozygous SNV position. **(3)** We then map these simulated reads to the other haplotype. For those simulated reads that align to multiple loci in the other haplotype, **(4)** we filter their original reads from the read pool and conduct another remapping and counting with the beta-binomial test to detect allele-specific SNVs. At this juncture, we cautiously note that a read can map to more than one heterozygous SNV, and they can also affect allelic mapping bias. However, the number of simulated reads generated per original read increases exponentially with more SNVs that overlap. However, >90% of the reads typically map to a single heterozygous SNV; Table 1 shows an example from a ChIP-seq dataset of DNA-binding protein CTCF for the individual NA12878. Hence, given that we are able to capture almost all of the potential bias with reads that overlap a single heterozygous SNV, and also considering the fact that we do have to apply this on a large scale, we find it reasonable to trade a minor compromise in completeness for computational efficiency. The pipeline can be modified by the user to include all overlapping heterozygous SNVs, if required.

Number of heterozygous SNVs	Number of maternal reads overlapping this number of SNVs (%)	Number of paternal reads overlapping this number of SNVs (%)
1	197,532 (96.842%)	197,642 (96.859%)
2	6,282 (3.0800%)	6,299 (3.0870%)
3	156 (0.0765%)	107 (0.0524%)
4	2 (0.0010%)	4 (0.002%)

Table 1. *Table showing the number of uniquely mapped maternal (column 2) and paternal (column 3) reads of an NA12878 CTCF ChIP-seq dataset, which overlap a certain number of heterozygous SNVs (column 1). ~97% of reads that map uniquely to the maternal or paternal haplotype overlap only 1 heterozygous SNV.*

We chose two representative RNA-seq and two ChIP-seq datasets (from NA12878) for our allelic mapping bias analyses with personal genome alignments. In line with previous studies, we found that only a small proportion of SNVs (2-4%) associated with allele-specific expression (ASE) had an allelic bias >5%. On the other hand, there is a higher proportion of SNVs associated with allele-specific binding (ASB) that exhibit >5% allelic mapping bias (19-21%) (Table 2).

NA12878 dataset	Number of reads that map to multiple locations (% of input reads)	Number of allele (AS) SNVs with >5% allelic bias removed (% AS SNVs originally)	Number of allele-specific SNVs removed, after removing multi-mapping reads (% AS SNVs originally)
CTCF ChIP-seq dataset 1 (same dataset as in Table 1)	Maternal: 2,618 (1.34%) Paternal: 2,575 (1.32%)	4/19 (21%)	3/19 (15.8%)
CTCF ChIP-seq dataset 2	Maternal: 2,255 (1.48%) Paternal: 2,202 (1.44%)	11/58 (19%)	6/58 (10.3%)
RNA-seq dataset 1	Maternal: 7,653 (0.66%) Paternal: 8,359 (0.72%)	10/369 (2.7%)	6/369 (1.6%)
RNA-seq dataset 2	Maternal: 19,789 (0.93%) Paternal: 25,899 (1.24%)	21/607 (3.5%)	15/607 (2.5%)

Table 2. Summary results for four NA12878 datasets, after removing sites (column 3) or removing reads (column 4). We chose four datasets, two ChIP-seq and two RNA-seq datasets, to investigate how much allelic mapping bias might affect the detected allele-specific (AS) SNVs in ChIP-seq and RNA-seq datasets with personal genome alignments. Mapping bias seems to have a greater effect on ChIP-seq datasets. Between 10-21% of the detected AS SNVs are removed, depending on which bias removal strategy was adopted – removing reads that exhibit mapping bias is able to retain AS SNVs that are still allele-specific.

As discussed before, the removal of sites rather stringent. Thus, we further examined the set of SNVs that showed >5% allelic mapping bias and found that if we remove only the reads that exhibit allelic mapping bias, many of them are still detected as allele-specific under the beta-binomial test; for example, 5 out of 11 sites with >5% allelic bias (CTCF ChIP-seq dataset 2) and 4 out of 10 AS SNVs (RNA-seq dataset 1) were still considered allele-specific (Table 2).

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	As a result, we decided on only removing reads that exhibit such a bias from the original pool of reads and then re-align the filtered read pool to both haplotypes. This is computationally more expensive, but this strategy effectively removes potential false positives, and at the same time, retains those that are strongly allele-specific. Interestingly, while we were working on this submission, van de Geijn *et al.* published in *Nature Methods* a tool that also similarly removes reads, instead of sites [6]. Additionally, our approach is already conservative, with multiple filters in place, such as removing highly over-dispersed datasets and using the beta-binomial test with an FDR of 5% for RNA-seq and 10% for ChIP-seq datasets. The personal genome is also able to handle various mapping artefacts not easily handled by using only the reference genome. Particularly, with the ability to incorporate larger variants beyond single nucleotide variants (such as indels), the personal genome serves as a more representative genome, as demonstrated by a much better alignment of unique reads [11,12]. We also envision that this allelic mapping bias will be alleviated with longer reads being used in ChIP-seq and RNA-seq datasets in the near future. [1] Castel *et al.* (2015). *Genome Biol.*, 16(1):195 [2] Degner *et al.* (2009) *Bioinformatics.* 25(24) [3] Satya *et al.* (2012) *Nucleic Acids Res.* 40(16):e127 [4] Stevenson *et al.* (2013) *BMC Genomics*. 14:536 [5] Panousis *et al.* (2014). *Genome Biol.*, 15(9):467 [6] van de Geijn *et al.* (2015). *Nat Methods*, doi: 10.1038/nmeth.3582 [epub ahead of print] [7] Kilpinen *et al.* (2013). *Science*, 342(6159):744-7 [8] Lappalainen *et al.* (2013). *Nature*, 501(7468):506-11 [9] The GTEx Consortium (2015). *Science*, 348(6235):648-60 [10] Dixon *et al.* (2015). *Science*, 518(7539):331-6 [11] Rozowsky *et al.* (2011). *Mol Syst Biol.*, 7:522 [12] Sudmant *et al.* (2015). *Nature*, 526(7571):75:81 We have included new sections in the 'Results', 'Discussion' and 'Methods' section about our new addition on allelic mapping bias.
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-- Ref2.3 – Over-dispersion –

Reviewer Comment	My second major concern was regarding the binomial test to identify ASE. The authors begin their response by citing other papers that used such a test. I am not sure what it the argument presented here, especially since the authors proceed by acknowledging over-dispersion in their data.

	So, yes, other paper got it wrong in the past, but this is hardly a reason to perpetuate this mistake. As for their revised approach, estimating a global over-dispersion parameter is not effective. Removing some loci because of 'too much' over-dispersion is ad hoc and was not justified. But more importantly, there are at least 3 published methods now to identify ASE using models that estimate site-specific over-dispersion, account for mapping bias, and report p values based on permutation. Why not use one of those published methods?
Author Response	While we thank the reviewer for his/her comment, the purpose of the references is not to make any claims on the 'correctness' of the methods, but to point to the broader reality that there is currently a diversity of methods in the field, where there is no firm consensus on the 'right' approach. The fact that these publications are recent and peer-reviewed at influential journals indicates the plurality of the methods accepted by the community, each with their own advantages and limitations. For example, van de Geijn *et al.* [1] is a very recent publication in *Nature Methods* that presented a software, which performs alignment to the human reference genome, accounts for mapping bias and uses the beta-binomial test to account for an individual-specific (not site-specific) global over-dispersion. However, it is not able to take into account indels and larger structural variants, which can be accommodated by the construction of personal genomes. In particular, we have utilized our approach in the 1000 Genomes Structural Variant group, whose manuscript has recently been peer-reviewed and published by *Nature.* Moreover, the estimation of a global over-dispersion has also been employed extensively in many recent and peer-reviewed software that detect allele-specific expression [1-5]. Our revised approach estimates over-dispersion at two levels. An over-dispersion is estimated for each dataset to remove those that are deemed too over-dispersed and that might result in higher number of false positives. After which, for each sample (for RNA-seq and each sample and transcription factor, TF, for ChIP-seq experiments), we pool the datasets and estimate the individual-specific global over-dispersion (for each sample for RNA-seq and also each sample and transcription factor for ChIP-seq) and apply this estimation to the beta-binomial test for each site in that individual (or TF). Hence, in this manner, the estimation of the over-dispersion can accommodate user-defined site-specific estimation of over-dispersion if necessary. Our R code is provided on our website for modifications and more customized analyses by the user.

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	We further point out that our two-step serial procedure is novel and is introduced to homogenize the pooling of datasets, by removing datasets that are too over-dispersed at the outset. This fits very well into our pipeline as it facilitates the harmonization and uniform processing of large amounts of data and alleviates an ascertainment bias in which more positives might originate from these highly over-dispersed datasets if they are not removed. Hence, we have retained our estimation and use of a global over-dispersion for detecting allele-specific variants. [1] van de Geijn *et al.* (2015). *Nat Methods*, doi: 10.1038/nmeth.3582 [epub ahead of print] [2] Sun (20132). *Biometrics*. 68(1):1-11 [3] Mayba *et al.* (2014). *Genome Biology*. 15(8):405 [4] Crowley *et al.* (2015). *Nature Genetics*. 47(4):353-60 [5] Harvey *et al.* (2015). *Bioinformatics*. 31(8):1235-42
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Reviewer #3

-- Ref3.1 – General positive comment --

Reviewer Comment	The manuscript is much improved and the authors have sufficiently addressed the majority of my concerns. I have the following minor comments:
Author Response	We thank the reviewer for the thorough examination of the manuscript and we are pleased that the reviewer finds our improved manuscript satisfactory.

-- Ref3.2 – Include additional references --

Reviewer Comment	1) Imprinting discussion should reference recent imprinting paper from GTEx. Lappalainen in Genome Research. 2) Heritability analyses of ASE should reference Li, AJHG, 2014.
Author Response	We have included the references in the respective sections of the manuscript.
Excerpt From Revised Manuscript	Please refer to the 'Discussion' section and also the 'Results' section under "ASB and ASE Inheritance analyses using CEU trio". "It could also be a result of other epigenetic effects such as genomic imprinting where no variants are causal.[35]", where reference 35 is by the GTEx consortium and Baran *et al.* published in *Genome Research*.

	"The CEU trio is a well-studied family and with multiple ChIP-seq studies performed on different TFs. Previous studies have also presented allele-specific inheritance.[10,15,21]", where reference 21 is by Li *et al*. published in *American Journal of Human Genetics*.