# BlackSheep: A Bioconductor and Bioconda package for differential extreme value analysis

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## Abstract

Unbiased assays such as shotgun proteomics and RNA-seq provide high-resolution molecular characterization of tumors. These assays measure molecules with highly varied distributions, making interpretation and hypothesis testing challenging. Samples with the most extreme measurements for a molecule can reveal the most interesting biological insights, yet are often excluded from analysis. Furthermore, rare disease subtypes are, by definition, underrepresented in cancer cohorts. To provide a strategy for identifying molecules aberrantly enriched in small sample cohorts, we present BlackSheep--a package for non-parametric description and differential analysis of genome-wide data, available at https://github.com/ruggleslab/blackSheep. BlackSheep is a complementary tool to other differential expression analysis methods that may be underpowered when analyzing small subgroups in a larger cohort.

## Introduction

Proteogenomic studies characterizing cancer have been completed by several groups, several of which also included proteome-wide phosphoproteome analysis (1–7). Outlier identification was used in a number of these studies to identify samples with aberrantly high levels of each phosphosite compared with the entire tumor cohort (1,8). In these studies, the outlier identification and subsequent subtype enrichment was used to highlight potential novel clinically relevant targets (1) or to identify candidate targets in a kinase inhibitor screen for sensitizers in drug-resistant cell lines (8). This method is of particular use for multi-omics studies as non-parametric approaches are more robust to the various sources of noise that are present in these data sets, which mainly come from sample collection and preparation procedures or variation in the analytical instrumentation.

Outlier values in a dataset are often assumed to be experimental artifacts and are discarded prior to downstream statistical analyses. However, sometimes recurrent outliers are the most meaningful values in the dataset, representing profound biological effects. In particular, when characterizing biological systems and identifying disease vulnerabilities, the largest changes in abundance are often the most revealing (9,10). Furthermore, many diseases, including cancer, are heterogeneous, with significant molecular variability requiring highly personalized approaches for successful treatment. Current strategies for identifying characteristic molecular patterns for groups of samples are underpowered for rare disease subtypes and use assumptions about the underlying distributions of the features in question, which are often inaccurate and/or discard extreme values with biological significance. We propose a

complementary strategy using the enrichment of outlier values within subtypes for characterizing disease subtypes, informing diagnostic panels, and potentially designing personalized therapeutic strategies for individual patients.

## Materials and Methods

BlackSheep is an easy-to-use package available on Bioconductor (https://github.com/ruggleslab/blacksheepr) and Bioconda (https://github.com/ruggleslab/blackSheep). It can be used in R, python or as a command line utility. BlackSheep has two major components: the 'DEVA' (Differential Extreme Value Analysis) module for calling outliers and differential analysis, and the 'run_simulations' module for assigning p-values to each outlier call. The input data is an expression matrix, structured as rows of features (genes, proteins, phosphosites, etc.) and sample columns, and a sample annotation file used to group samples for comparisons (**Supplementary Table 1A, 1B**). No prefiltering is necessary or recommended for DEVA. Normalization of the input matrix is strongly recommended; a function for this is provided. For normalized data, we suggest a sample coverage normalization followed by row $\log_2$ transformation.

### *Differential Extreme Value Analysis (DEVA)*

To call outliers, the median and interquartile range (IQR) for each row is calculated. The user specifies whether to call overly abundant (i.e. up) or depleted (i.e. down) values. Outliers are defined as any value more than a multiple of the IQR above or below the median, where the multiple of the IQR is user-specified, with a default of 1.5 (**Fig. 1A)**. After calling outliers, there is an optional aggregation step for collapsing rows containing different but related features into a single row (e.g. many phosphosites collapsed into a protein). Aggregation is achieved by counting outliers and non-outliers separately for each protein. The output is two tables, one with outlier and non-outlier counts per protein (**Supplementary Table 2A, 2B**), and the other containing the fraction of outliers in each sample, per row (**Supplementary Table 2C, 2D**).

### *Simulations and outlier p-values*

The second primary function in the package is 'run_simulations', which calculates a p-value for each sample for each gene. First, random numbers are generated to determine whether each value is present or missing in a simulated matrix based on the proportion of missing values in the input expression matrix. In the second step, there is a random resampling of the data, where a random value is pulled from the associated row in the expression matrix to represent the value

of that feature in the sample. This value is tested against the outlier threshold for that feature to determine outlier status. This is repeated for all rows, at which point a significance threshold is set at a user defined alpha (e.g. p<0.10). The output file (**Supplementary Table X**) contains a p-value for outlier status for each feature in each sample (**Fig. 1B**).

### *Cohort comparisons*

Groups of samples can be compared with DEVA to identify features with enrichment of outliers within a group. For every comparison in a user-supplied annotation table (**Supplementary Table 1B**), BlackSheep calculates enrichment of outliers for every group of samples identified in the annotation table. Analysis can be limited to a user-supplied list of genes, such as kinases (1).

To calculate enrichment, first a row-based filter is applied, removing rows where the average rate of outliers is lower in the group of interest than in the outgroup. Second, to ensure that results are not driven by a small subset of the group of interest, we only keep rows that have at least one outlier value in a user-defined proportion of samples in the group of interest; the proportion defaults to 0.3. Finally, DEVA performs a Fisher's exact test on counts from outlier and non-outlier values in the group of interest vs the outgroup. All p-values are then corrected for multiple hypothesis testing using the Benjamini-Hochberg procedure. Results can be output as a table of q-values for all comparisons (**Supplementary Table 2E, 2F**); a table with outlier counts, p-values, and q-values per comparison (**Supplementary Table 2G, 2H**); or a heatmap showing values per sample for rows with significant enrichments of outliers (**Fig. 2B**).

## Results

### *Application to breast cancer cohort*

To demonstrate the utility of BlackSheep, we applied it to a dataset from a proteogenomic breast cancer study (1) to find putatively over-active kinases, unique to each molecular subtype (1,11,12) with the idea that reproducible hyperphosphorylated kinases within a specific subtype or patient cohort will represent attractive targets for future drug development and repurposing (13–18). Here, we compare the results of BlackSheep to the commonly used rank-sum test to identify differentially abundant phosphosites in Her2-positive (Her2+) breast cancer samples vs all other samples (**Fig. 2A**). In this cohort, Her2+ is the smallest group, comprising 12 of the 76 samples. Using the full phosphosite expression matrix (63,130 phosphosites, 9881 proteins), results of BlackSheep and rank-sum test were corrected for multiple testing at equally stringency. At an FDR cutoff of 0.01, rank-sum calculated one enriched phosphosite in Her2+

samples, on the Her2 (ERBB2) protein: ERBB2-T1240 (**Fig. 2A**); the DEVA pipeline identified 10 additional phosphosites on ERBB2, as well as phosphosites on several other proteins, including established co-amplicons and modulators of Her2 signaling, such as GRB7 (19,20) (**Fig. 2A-B**). When applied to RNA abundance data from the same cohort, BlackSheep and rank-sum tests identify many of the same enriched genes at FDR < 0.01 (**Fig. 2C**, **2D**). In addition, rank-sum found genes that have high values in the group of interest, as well as some samples in the out group (**Fig. 2D, top**) while BlackSheep identified additional genes that are exclusively enriched or depleted in only the group of interest (**Fig. 2D, bottom**). BlackSheep will not be able to identify features that are enriched in large fractions of samples within a cohort – if there is a feature with consistently high values in a group that makes up a large fraction of the cohort, those values will increase the median and IQR, and will no longer be called as outliers. For understanding small groups within a cohort DEVA is able to identify enriched features (**Fig. 2A**).

## Conclusion

Several cancer types have patients that fall into rare subgroups with worse prognoses than the majority of patients (e.g. serous in endometrial cancer, basal-like in breast cancer). Due to the difficulty in acquiring sufficient numbers of samples, these patients are the hardest to study, yet they are the patients most in need of new therapies. While standard analysis techniques are useful for finding characteristics that are enriched in large subgroups of samples, these strategies often lack the power to find the same for small subgroups. BlackSheep provides a user-friendly method for the analysis of genome-wide measurements to delineate a characteristic enrichment pattern for a small group of samples within a cohort. BlackSheep's DEVA module can find enrichment of known markers for small groups of samples, such as ERBB2 and GRB7 in Her2+ breast cancer samples, which other commonly used analysis paradigms miss at the same FDR. BlackSheep is a flexible complement to other methods such DESeq2 and rank-sum tests; while the latter approaches work well for comparing larger subsets of samples, they lack power when searching for effects in small subgroups of samples, a task for which BlackSheep is perfectly suited. We anticipate that in the future BlackSheep-like strategies will be applied in the clinic to design and interpret diagnostic panels applied to single tumors to devise personalized treatments by repurposing drugs approved for other indications to targeting the observed outliers in the tumor.

## Figure Legends

**Figure 1. BlackSheep Workflow** (A) Outliers are identified for each feature (row) in the experimental dataset and (B) using simulations and data resampling significance value assigned for each sample/feature. (C) Cohort comparisons identify features with enriched outliers within a sample cohort of interest.

**Figure 2**. **Comparing BlackSheep and Rank-Sum Tests** (A, C) Signed $\log_{10}$ q-values from blacksheep.deva and rank-sum tests when comparing normalized values in Her2 vs all other samples in (A) phospho and (C) RNA data. Dotted lines indication FDR < 0.01. (B) Z-scores of relative $\log_2$ abundance of all phosphosites with FDR < 0.01 calculated by BlackSheep. * indicates ERBB2-T1240 had FDR < 0.01 using a rank-sum test. (D) Z-scores of log2 relative abundance of RNA with FDR < 0.01 calculated by rank-sum only (top) or blacksheep.deva only (bottom).

## Tables

**Supplementary Table 1. Example Input Files.** (A) Data expression matrix, structured as rows of features (genes, proteins, phosphosites, etc.) and sample columns, and (B) sample annotation file, containing comparison group labels for each sample.

**Supplementary Table 2. Example Output Files.** (A, B) Outliers output matrix containing the number of (A) up and (B) down outliers per row per sample, (C, D) the fracTable matrix containing the fraction of rows mapping to that parent molecule (e.g. gene) with (C) up and (D) down outliers per sample. (E, F) a significance output file containing a q-value for each row passing this adjustable percentile filter in any comparison for (E) up and (F) down outliers. (G, H) a table of outlier counts, p-values and q-values for the Her2 comparison in (G) up and (H) down outliers.

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*Conflict of Interest:* none declared.

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Figure 1

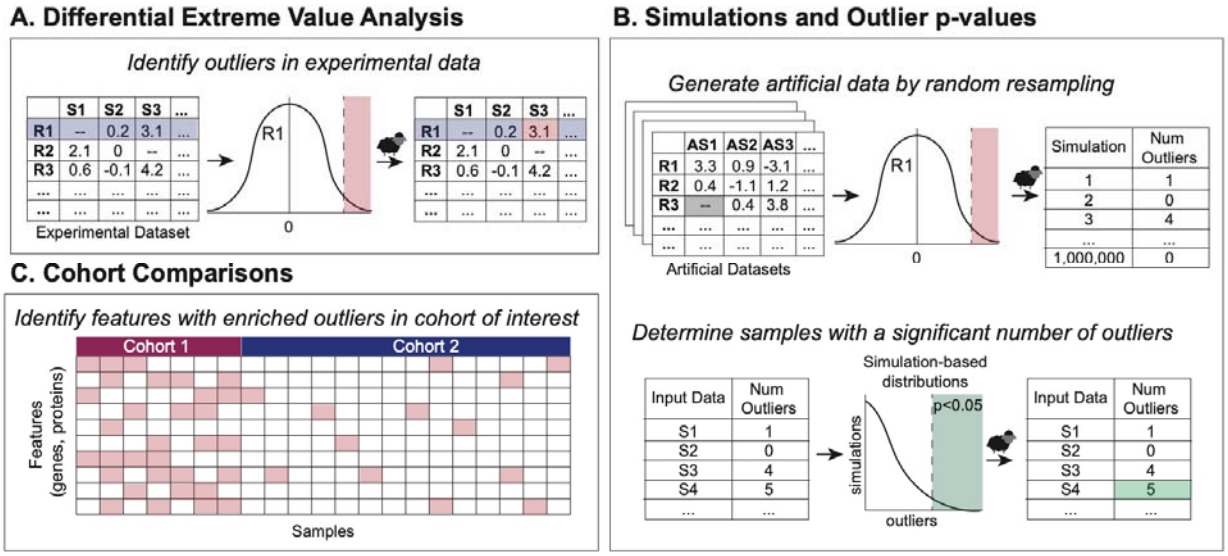


Figure 2