eSCAN: Scan Regulatory Regions for Aggregate
Association Testing using Whole Genome Sequencing Data

Yingxi Yang,1* Yuchen Yang,2,3,4* Le Huang,2 Jai G. Broome5,6, Adolfo Correa,7 Alexander Reiner,8,9 NHLBI Trans-Omics for Precision Medicine (TOPMed) Consortium, Laura M. Raffield2*, Yun Li2,10,11*†

1Department of Statistics, Sun Yat-Sen University, Guangzhou, Guangdong, 510275, China
2 Department of Genetics, University of North Carolina at Chapel Hill, Chapel Hill, NC, 27599, USA
3 Department of Pathology and Laboratory Medicine, University of North Carolina at Chapel Hill, Chapel Hill, NC, 27599, USA
4 McAllister Heart Institute, University of North Carolina at Chapel Hill, Chapel Hill, NC, 27599, USA
5 Department of Biostatistics, University of Washington, Seattle, WA 98195, USA
6 Department of Medicine, Division of Medical Genetics, University of Washington, Seattle, WA 98195, USA
7 Department of Medicine and Population Health Science, University of Mississippi Medical Center, Jackson, MS, 39216, USA
8 Department of Epidemiology, University of Washington, Seattle, WA, 98195, USA
9 Fred Hutchinson Cancer Research Center, University of Washington, Seattle, WA, 98195, USA
10 Department of Biostatistics, University of Washington, Seattle, WA 98195, USA
11 Department of Medicine, Division of Medical Genetics, University of Washington, Seattle, WA 98195, USA
12 Department of Medicine and Population Health Science, University of Mississippi Medical Center, Jackson, MS, 39216, USA
13 Department of Epidemiology, University of Washington, Seattle, WA, 98195, USA
14 Fred Hutchinson Cancer Research Center, University of Washington, Seattle, WA, 98195, USA
Abstract

With advances in whole genome sequencing (WGS) technology, multiple statistical methods for aggregate association testing have been developed. Many common approaches aggregate variants in a given genomic window of a fixed/varying size and are not reliant on existing knowledge to define appropriate test units, resulting in most identified regions not being clearly linked to genes, limiting biological understanding. Functional information from new technologies (such as Hi-C and its derivatives), which can help link enhancers to the genes they affect, can be leveraged to predefine variant sets for aggregate testing in WGS. Therefore, in this paper we propose the eSCAN (Scan the Enhancers) method for genome-wide assessment of enhancer regions in sequencing studies, combining the advantages of dynamic window selection in SCANG with the advantages of increased incorporation of genomic annotation. eSCAN searches biologically meaningful searching windows, increasing power and aiding biological interpretation, as demonstrated by simulation studies under a wide range of scenarios. We also apply eSCAN for association analysis of blood cell traits using TOPMed WGS data from Women’s Health Initiative (WHI) and Jackson Heart Study (JHS). Results from this real data example show that eSCAN is able to capture more
significant signals, and these signals are of shorter length and drive association of larger
regions detected by other methods.

**Main text**

In genome-wide association studies (GWAS), most significantly associated variants
are located outside coding regions of genes, making it difficult to interpret the biological
function of associated variants. Statistical power to detect rare variant associations in
noncoding regions, which is of increasing importance with the advent of large-scale whole
genome sequencing (WGS) studies, is also limited with a standard single variant GWAS
approach. Aggregate testing is necessary to increase statistical power to detect rare variant
associations; linking noncoding variants to their likely effector genes is necessary for
interpretation of identified aggregate signals. Many standard methods for aggregate analysis
of the noncoding genome are agnostic to regulatory and functional annotation (for example,
standard sliding window analysis, where all variants in a given location bin (for example a 5
kb or 10 kb window) are analyzed, followed by analysis of a subsequent partially overlapping
window, until each chromosome is assessed in full)\(^1\)-\(^3\). SCANG has recently been proposed as
an improvement on conventional sliding-window procedures, with the ability to detect the
existence and locations of association regions with increased statistical power \(^4\). SCANG
allows sliding-windows to have different sizes within a pre-specified range and then searches
all the possible windows across the genome, increasing statistical power. However, since
SCANG tests all possible windows, it can "randomly" identify some regions across the
genome regardless of their biological functions. Identified regions could often cross multiple
enhancer regions with distinct functions, thus impeding the identification of biologically
important enhancers and their target genes. This cross-boundary issue may also lead to a
higher false positive rate in a fine-mapping sense. The whole region/chromosome in which
the detected regions are located may not be a false positive, but locations of the detected
regions will not match the true association regions. Moreover, SCANG applies SKAT to all
candidate windows, but computing p-values in SKAT requires eigen decomposition. This
analysis method is therefore very time-consuming and has high computational costs, which
may not be feasible for increasingly large genome-wide studies.

In addition to sliding window approaches, many analyses of WGS data rely on
aggregate tests of predefined variant sets, attempting to link the most likely regulatory
variants (as defined by tissue specific histone marks, open chromatin data, sequence
conservation, etc) to genes prior to association testing, with variants assigned to genes based
on either physical proximity or chromatin conformation. There is increasing data available
to define these tissue specific regulatory regions, which are known to show enrichment for
GWAS identified noncoding variant signals. Recent biotechnological advances based on
Chromatin Conformation Capture (3C), such as promoter capture Hi-C data, can also better
link gene promoters to enhancers based on their physical interactions in 3D space. We here
propose an extension of SCANG which combines the advantages of both scanning and fixed
variant set methods (see Fig. 1 for illustration). Our eSCAN (or “scan the enhancers” with
“enhancers” as a shorthand for any potential regulatory regions in the genome) method can
integrate various types of functional information, including chromatin accessibility, histone
markers, and 3D chromatin conformation. There can be a significant distance between a gene
and its regulatory regions; simply expanding the size of the window to include kilobases of
genomic data around each gene will include too many non-causal SNPs, giving rise to power
loss, as well as difficulties in results interpretation. Our proposed framework can enhance
statistical power for identifying new regions of association in the noncoding genome. We
particularly focus on integration of 3D spatial information, which has not yet been fully
exploited in most WGS association testing studies. Our method allows users to input broadly
defined regulatory/enhancer regions and then select those which are most likely relevant to a given phenotype, in a statistically powerful framework.

Given our incomplete understanding of chromatin conformation and enhancer annotation, an annotation agnostic approach such as SCANG does have some advantages, in that no prior information is needed for rare variant testing. However, our simulations and the real data example presented here demonstrate the advantages of our eSCAN method, which can flexibly accommodate multiple types of annotation information and shows significant power gains over SCANG, as well as a lower false positive rate in different scenarios for both continuous and dichotomous traits. These advantages are demonstrated in our application of eSCAN to TOPMed WGS analyses of four blood cell traits in the Women’s Health Initiative (WHI) study, with replication in Jackson Heart Study (JHS).

The eSCAN procedure can be split into two steps: a *p*-value computing step and a decision-making step (using a *p*-value threshold). First, for each enhancer, set-based *p*-values are calculated by fastSKAT, which applies randomized singular value decomposition (SVD) to rapidly analyze much larger regions than standard SKAT, and then *p*-values are "averaged" by the Cauchy method via ACAT. Second, eSCAN calculates two types of significance threshold. The first is an empirical data-driven threshold computed by Monte Carlo simulation on the basis of a common distribution of *p*-values; the second is an analytical estimation by extreme value distribution. eSCAN then defines the enhancers with *p*-values below the threshold as significant. Further details are in the Supplemental Methods.

We next evaluated the performance of eSCAN using simulated data under the null model (more details in Supplemental Methods). On average, each simulated enhancer had a length of 4025 bp and contained 122 variants with MAF below 5%. For both continuous and dichotomous simulations, we applied eSCAN to 1,000 replicates with sample sizes of 2,500,
5,000 and 10,000, respectively, and set the genome-wide type I error rate at 0.05. Under all scenarios, our method has a well-controlled genome-wide type I error rate (Table 1).

To assess eSCAN under the alternative model, we applied eSCAN and two SCANGs, i.e. the default SCANG and enhancer based SCANG, to a wide range of simulated scenario to benchmark their performances in terms of power and false positive rate, using four metrics, namely causal-variant detection rate, causal-enhancer detection rate, variant false positive rate and enhancer false positive rate (more details in Supplemental Methods). For continuous traits, both the enhancer-based SCANG and our eSCAN analysis showed higher power than the default SCANG, at both the variant level and the enhancer level (Fig. 2a-b), for all tested sample sizes, suggesting the benefit of aggregating variants using enhancer information. Notably, the power gain between eSCAN and enhancer-based SCANG is much more pronounced than that between enhancer-based SCANG and default SCANG. eSCAN increases the variant-level power by 23.50%, 45.94% and 27.98% for the three tested sample sizes, respectively; and boosts the enhancer-level power by 17.60%, 45.47% and 24.14%, respectively. With respect to false positive rate, eSCAN showed a remarkably lower false positive rate than those from the two SCANG procedures, at both the variant-level and enhancer-level (Fig. 2c-d).

These results demonstrate eSCAN’s capabilities to powerfully and accurately detect causal enhancers. We further evaluated eSCAN in more simulation scenarios to verify eSCAN's robustness to dichotomous traits and the proportion of causal enhancers, as well as the proportion of causal variants within the causal enhancers. Results show that these gains are robust to choice of parameters (Fig. S1-3).

To assess the performance of eSCAN in real data, we compared eSCAN to both enhancer based SCANG and the default SCANG using WGS data in 10,727 discovery samples from the Women’s Health Initiative (WHI) and 1,970 replication samples from the
Jackson Heart Study (JHS) (Supplemental Methods and Table S1). We only considered variants with a minor allele frequency < 5% in each cohort. Windows with a total minor allele count (MAC) < 10 were excluded from the analysis. To achieve a fair comparison, we first applied eSCAN and enhancer-based SCANG for association analysis between putative enhancers and four blood cell traits measured at baseline in WHI, white blood cell count (WBC), hemoglobin (HGB), hematocrit (HCT) and platelets (PLT), with a genome-wide error rate at the level of 0.05 by Bonferroni correction in both methods. For eSCAN, enhancers were defined using promoter capture-Hi-C (PC-HiC) data in any tested white blood cell type (including neutrophils, monocytes, and lymphocytes) for WBC, erythroblasts for HGB and HCT and megakaryocytes for PLT\textsuperscript{11}, defining any noncoding region with statistically significant interactions with a gene promoter as an enhancer region. For enhancer-based SCANG, we analysed the subset of rare variants falling into any enhancer region as defined using PC-HiC annotation (more details in Supplemental Methods). For the default SCANG, due to the limited computational feasibility, we only performed the analysis for WBC.

Overall, eSCAN detected 19 significant regions associated with blood cell traits while enhancer-based SCANG only detected 7 regions (Table 2, Table S3 and Fig. S4A-D). Also, eSCAN showed consistently smaller p-values for top regions compared with enhancer-based SCANG (Fig. S4A-D and Table 2). Among the 19 genome-wide significant regions detected by eSCAN in the unconditional analysis, 4 were located within +/- 500kb of known GWAS loci and were still significant at the Bonferroni correction level of 0.05/4 after conditioning on known blood cell trait GWAS loci\textsuperscript{12-20} (Table 2 and Table S2). Also, of the significant regions, two were replicated at 0.05 level in replication samples. Note that the low replication rate is likely due in large part to the much smaller sample size of the JHS replication cohorts;}
we also note as a limitation that we did not correct for multiple testing in these replication analyses, due to this small sample size.

To more comprehensively compare the top regions of eSCAN and two SCANG procedures, enhancer-based SCANG and the default SCANG, we relaxed the significance level for WBC by using the empirical threshold (more details in Supplemental Methods). The detected regions by eSCAN are of shorter length and contains fewer variants and than those identified by the two SCANG variants (Fig. S5b). Also, each region identified by eSCAN contains a single regulatory element based on annotation from promoter capture Hi-C. By contrast, regions identified by SCANG can cross multiple regulatory regions (Fig. S5c), which indicates that, with the help of enhancer information, eSCAN can more effectively narrow down variants and/or regulatory regions associated with a trait of interest than SCANG. We further investigated a segment on chromosome 10 where two signals were detected by enhancer-based SCANG and four by eSCAN. The two regions from SCANG overlapped the four eSCAN signals. All four were smaller in size than the SCANG detected regions. We also note that each SCANG signal contains two eSCAN signals (Fig. 3a-c). We then removed the associated variants in the overlapped regions between eSCAN and SCANG (which are regions detected by eSCAN since in both cases, the eSCAN regions are subsets of the SCANG regions), and re-did SCANG analysis using the retained variants only. Both regions then became insignificant (p-values> 0.02) using SCANG (Fig. 3d), suggesting that the sub-regions detected by eSCAN were most likely the functional regions contributing to the original significant signal.

The computational complexity of eSCAN depends on the sample size, the number of considered enhancers along a certain chromosome, and the number of rare variants residing in enhancer regions. For JHS (n=1,970) and WHI (n=10,727) eSCAN takes an average of 3h and 26h, respectively, to examine all the sets of rare variants along one chromosome, using
our cluster computing platform with one computing node and 8Gb of memory (Fig. S6) while SCANG limited to enhancer regions takes an average of 2.6 days and 5.3 days respectively as more eigen-decomposition steps are performed.

We propose here eSCAN, a novel aggregation method for whole genome sequencing analysis, which can integrate various types of functional information to aggregate enhancers or putative regulatory regions from WGS data and test for association with phenotypes of interest. Our method has several important advantages: (1) it has higher power and lower false positive rate, enabling it to accurately detect more significant signals than other methods (Fig. 2 and S1-4); (2) the signals identified by eSCAN are of shorter sizes, which suggests eSCAN can more accurately locate the associated variants; (3) eSCAN boosts the biological interpretation of detected signals by incorporating functional annotation; (4) it is computationally efficient (Fig. S6).

eSCAN can be viewed as an extension of SCANG with respect to its use of dynamic searching windows and use of the $p$-value as its test statistic\(^4\). But it differs from SCANG in several key ways. SCANG restricts the size of searching windows within a pre-specified range and then tests all possible windows, "randomly" identifying some large regions across the genome regardless of their biological functions. eSCAN allows more flexible and biologically meaningful searching windows that mark putative enhancer(s) (Fig. 1). In addition, eSCAN builds on fastSKAT, a computationally efficient approach to approximate the null distribution of SKAT statistics\(^{21}\).

Based on our simulations in a variety of scenarios, eSCAN can be flexibly applied to different phenotypes, both quantitative and qualitative, and is able to detect more significant signals than competing methods with a better control over false positive rate than other WGS based methods (Fig. 2 and Fig. S1-3). Using WGS data from the JHS and WHI studies, we demonstrate an enrichment of association signals using eSCAN procedure. It can detect
reported signals which are not found by SCANG procedures, indicating that it is less likely to miss important regions. In addition, the regions detected by eSCAN are of shorter size than those of SCANG on average. By removing eSCAN signals from WGS data on chromosome 10 and re-running SCANG procedures, we verify that, at least for this segment, the signals detected by eSCAN drive the significant associations in larger regions identified by SCANG (Fig. 3; Fig. S5), a pattern we anticipate would be true for many associated regions.

Despite the modest sample size available for our blood cell trait analysis, interesting and biologically plausible rare and low frequency variant enhancer region signals were identified in our analyses from WHI. Of the genes regulated by replicated regions, BACH2 (regulated by a region on chromosome 6: 90,423,754-90,425,200) is a key immune cell regulatory factor and is crucial for the maintenance of regulatory T-cell function and B-cell maturation. Among other interesting genes CCL18 (regulated by a region on chromosome 17: 35,982,416-35,983,367, which was not replicated in JHS) was reported to stimulate the bone marrow overall, which could lead to increased platelets. These findings suggest that the associated enhancer regions identified by eSCAN may in fact play key regulatory relevant to the biological functions of blood cells, with eSCAN finding regions were not identified using the SCANG method. We do note, however, that these findings should be considered preliminary, given our modest sample size, and could be influenced by unadjusted for selection bias in WHI TOPMed sampling (enrichment for stroke and venous thromboembolism) and lack of adjustment for a genetic relationship matrix which could better capture cryptic relatedness and differential ancestry unadjusted for by PCs. However, these issues impact eSCAN and SCANG equally, and do not change our central methods comparison findings.

With respect to the weights in fastSKAT, we used two standard MAF-based weights: one is the Beta distribution with $a_1 = a_2 = 1$ reflecting that all the variants have equal effect
size, the other is $a_1 = 1, a_2 = 25$ upweighting rarer variants. One can also use external
measures by incorporating individual level functional annotations, such as FATHMM-XF$^{24}$
and STAAR$^{25}$, as the weight for each variant. Incorporation of functional evidence has
demonstrated its values in variant level association studies $^{26, 27}$. In addition, the eSCAN
framework is flexible regarding its unit aggregate tests. In our implementation, we use
fastSKAT because of its small computational cost, but other aggregate tests can also be used,
such as SMMAT, a recently proposed test which is an efficient variant set mixed model
association test $^{28}$.

Another attractive feature of eSCAN is its significance threshold. Since candidate
regions are highly likely to be correlated because of either physical overlapping or LD,
making the set-based $p$-values also correlated, the classic Bonferroni correction would be too
conservative. While we do use a classic Bonferroni correction in our real data example from
WHI, due to the small sample size available to us for replication, this is almost certainly over-
conservative. eSCAN provides two estimations of significance threshold, either empirically
or analytically, using the strategies from SCANG and WGScan respectively, which have
demonstrated significant enrichments of signals in Li et al.$^4$ and He et al.$^{10}$. In addition,
although our analyses focused on unrelated individuals, it can be readily extended to related
samples by replacing the generalized linear model (GLM) with the generalized linear mixed
model (GLMM) in the first step$^4$.

One potential limitation of eSCAN is the lack of base pair resolution in defining
regions important for gene regulation, due to the sparsity of reads with most Hi-C and
chromatin conformation assays (leading to resolution as broad as 40 kb when assessing
interactions between genomic regions). ATAC-seq data, albeit much finer resolution, still
results in open chromatin peak regions that usually contain multiple rare variants, particularly
as sample size increases, hurdles inference at the resolution of single base pair or single
variant. These limitations are intrinsic to the functional annotation data employed rather than to the eSCAN methodology. We anticipate that rapid technological improvements in the functional annotation datasets will continue mitigating these issues by providing increasingly finer resolution and more comprehensive data, which would render eSCAN even more valuable in the near future.
**Supplemental Data Description**

Supplemental Data include supplemental methods, six figures and three tables, which are included as an Excel file.

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The views expressed in this manuscript are those of the authors and do not necessarily represent the views of the National Heart, Lung, and Blood Institute; the National Institutes of Health; or the U.S. Department of Health and Human Services.

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Declaration of Interests

The authors have no conflicts of interest to declare.

Data and Code Availability

This paper did not generate any datasets. TOPMed data from the Women’s Health Initiative is available to approved researchers through dbGaP (phs001237), with phenotype data available at phs000200. TOPMed data from the Jackson Heart Study Data is also available to
approved researchers through dbGaP (phs000964), with phenotype data available at phs000286. Data is also available with an approved manuscript proposal through https://www.jacksonheartstudy.org/ (JHS) and https://www.whi.org/ (WHI).

**Web Resources**

We developed an R package for the eSCAN procedure. The package is available at https://github.com/yingxi-kaylee/eSCAN.

**REFERENCES**


**Figure titles and legends**

**Figure 1:** An illustration of eSCAN.
SCANG: scan the whole genome with all possible windows

Specify \( L_{\text{min}} \) and \( L_{\text{max}} \) for SCANG as the smallest window size and largest window size

Search all possible windows as following:

\[
\text{FOR size IN } L_{\text{min}} \text{ TO } L_{\text{max}} \\
\text{scan the whole genome with window of length 'size';}
\]

ENDFOR

OUR STRATEGY: aggregate enhancers across the genome

SCANG + OUR STRATEGY:
Scan the constellation of enhancers with all possible windows

Specify \( L_{\text{min}} \) and \( L_{\text{max}} \) for SCANG as the smallest window size and largest window size

Search all possible windows as following:

\[
\text{FOR size IN } L_{\text{min}} \text{ TO } L_{\text{max}} \\
\text{scan the constellation of enhancers with window of length 'size';}
\]

ENDFOR

OUR METHOD
eSCAN: scan the constellation of enhancers

Search all possible windows, and each window marks enhancer(s):
Figure 2: Power and false positive Rate Comparison of eSCAN and SCANG for Continuous Traits as Sample Size Changes.

We evaluated the performance of eSCAN for continuous traits analysis as sample size changes. The total sample sizes considered were 2,500, 5,000 and 10,000. For each configuration, we compared three methods: eSCAN and two SCANGs: enhancer-based SCANG (aggregating enhancers across the genome) and default SCANG (scan the whole genome). We evaluated power and false-positive performance at both variant-level and enhancer-level.

Panel a presents power at the variant-level (a.k.a. causal-variant detection rate). Panel b presents power at enhancer-level (a.k.a. causal-enhancer detection rate). Panel c presents results of the false positive rate at variant-level. Panel d presents the false positive rate at the enhancer-level.
Figure 3: A segment on chromosome 10 where two signals were detected by SCANG and four by eSCAN.

We further investigated a segment on chromosome 10 where two signals were detected by SCANG and four by eSCAN. The two regions from SCANG (chr10: 113,767,467-113,773,998 with p-value=2.66×10^(-7) and chr10: 113,774,365-113,779,787 with p-value=7.81×10^(-7)) overlapped the four eSCAN signals (a). All four is smaller in size than the SCANG detected regions (b). Specifically, eSCAN detected chr10: 113,770,735-113,773,147 with p-value=2.84×10^(-6); chr10: 113,773,148-113,774,046 with p-value=6.59×10^(-6); chr10: 113,774,047-113,775,910 with p-value=9.55×10^(-6) and chr10: 113,775,911-113,778,291 with p-value=1.92×10^(-5)). Each SCANG signal contains two eSCAN signals (c). We then removed the associated variants in the overlapped regions between eSCAN and SCANG (which are regions detected by eSCAN since in both cases, the eSCAN regions are subsets of the SCANG regions), and re-did SCANG analysis using the retained variants only. Both regions then became insignificant (p-values>0.02) using SCANG (d), suggesting that the sub-regions detected by eSCAN were most likely the functional regions contributing to the original significant signal.
Tables

Table 1: Genome-wide empirical type 1 error rates of eSCAN from simulation studies, shown for different sample sizes and trait distributions.

<table>
<thead>
<tr>
<th>Sample size</th>
<th>n=2,500</th>
<th>n=5,000</th>
<th>n=10,000</th>
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</thead>
<tbody>
<tr>
<td>Continuous Traits</td>
<td>0.003</td>
<td>0.002</td>
<td>0.001</td>
</tr>
<tr>
<td>Dichotomous Traits</td>
<td>0.001</td>
<td>0.001</td>
<td>0.002</td>
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</table>

Table 2: Significant results by eSCAN for blood cell traits in TOPMed whole genome sequencing data. Chromosome, start position (hg38), end position (hg38), p-value in discovery samples (Women's Health Initiative, WHI), p-value of nearest region tested by enhancer-based SCANG, known GWAS loci within +/- 500kb, associated trait (HGB, hemoglobin, PLT, platelet count, WBC, white blood cell count), p-value in the replication samples (Jackson Heart Study, JHS), gene(s) regulated by the detected region, total minor allele count (MAC) in the discovery samples, total MAC in the replication samples.
<table>
<thead>
<tr>
<th>Chr</th>
<th>Start</th>
<th>End</th>
<th>P-value</th>
<th>P-value by enSCANG (strat:end)</th>
<th>Known GWAS Trait</th>
<th>P-value (replication)</th>
<th>Regulated gene</th>
<th>MAC</th>
<th>MAC (replication)</th>
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</thead>
<tbody>
<tr>
<td>5</td>
<td>178,360,084</td>
<td>178,360,305</td>
<td>9.54E-10</td>
<td>5.82E-02 (178,342,330:178,367,547)</td>
<td>No HGB</td>
<td>0.56</td>
<td>COL23A1</td>
<td>22</td>
<td>30</td>
</tr>
<tr>
<td>8</td>
<td>98,026,570</td>
<td>98,027,915</td>
<td>1.01E-08</td>
<td>9.24E-04 (98,023,407:98,078,162)</td>
<td>No HGB</td>
<td>0.11</td>
<td>MTDH; LAPTMB4B; RNU7-177P; Y_RNA; MATN2; RPS23P1</td>
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<td>11</td>
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<tr>
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<td>32,397,109</td>
<td>32,404,847</td>
<td>2.52E-08</td>
<td>7.75E-03 (32,289,232:32,422,553)</td>
<td>Yes HGB</td>
<td>0.42</td>
<td>CAAP1; IFT74; RP11-337A23.3; IFNK; AL353671.1; AL353671.2; DDX58; TOPORS-AS1; TOPORS; NDUFB6; APTX; DNAJA1</td>
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<td>14,509,775</td>
<td>14,511,544</td>
<td>1.67E-08</td>
<td>1.78E-03 (14,479,136:14,781,283)</td>
<td>No HGB</td>
<td>0.76</td>
<td>ERCC4; RPS26P52; MIR193B; MIR365A</td>
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<td>16</td>
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<td>6.45E-07</td>
<td>1.04E-02 (53,483,984:53,535,907)</td>
<td>No HGB</td>
<td>0.80</td>
<td>CHD9; RP11-467J12.4; RBL2; AKTIP; CRNDE; IRX5; LPCAT2; MMP2; CNOT1</td>
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<td>1.46E-08</td>
<td>7.13E-04 (37,016,046:37,060,384)</td>
<td>No HGB</td>
<td>0.63</td>
<td>DHRSSI1; DHRSSI1; MRMI</td>
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