SciLifeLab RNA-seq course

Allele-specific expression, ASE

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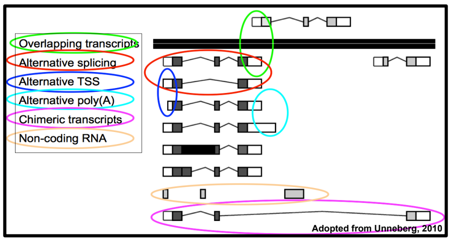
ASE: allele-specific expression

Outline

1. **Definition of ASE**
2. **Detecting ASE (introductory case)**
3. **Applications and prevalence of ASE**
4. **Important ASE considerations**
   (a) Variant calling
   (b) Mapping biasASE tools
   (c) Many variants in a gene
5. **ASE tools**
6. **GeneiASE – a tool to detect genes with ASE from RNA-seq data**

[1] Definition of allele-specific expression (ASE)

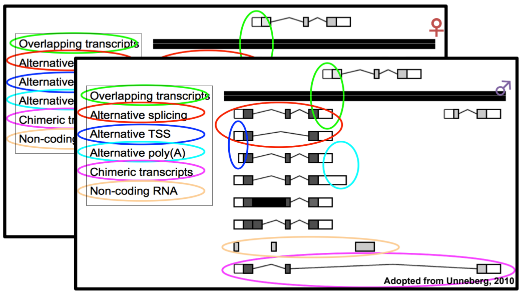
Adding another layer to transcriptome complexity...



Adopted from Unneberg, 2010

One gene can produce **many** different transcripts...

Adding another layer to transcriptome complexity...



Adopted from Unneberg, 2010

...and **each** gene is present on **two** chromosomes.

=> it has **two *alleles***

Allele, definition

An **allele** is the variant form of a given gene (or locus). Sometimes, different alleles can result in different observable phenotypic traits, such as different pigmentation.
/…/
If both alleles at a gene (or locus) on the homologous chromosomes are the same, they and the organism are **homozygous** with respect to that gene (or locus). If the alleles are different, they and the organism are **heterozygous** with respect to that gene (or locus).

https://en.wikipedia.org/wiki/Allele

Allele-specific expression, definition

An imbalance in transcription between the maternal and paternal alleles at a locus.

- *I.e.*, a **deviation from the expected 50/50 ratio** of transcription from the two alleles of a diploid organism.
- Can be assessed within a **single individual**

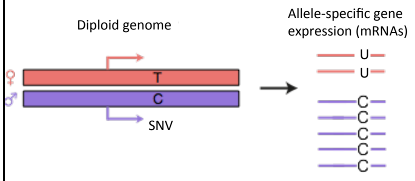
(Present also when ploidy >2, *e.g.*, plants)

Other events may also be "allele-specific", *e.g.*

- transcription factor binding
- DNA backbone methylation
- X-chromosome inactivation in female mammals

Allele-specific expression, definition

genomic DNA -> transcript (e.g. mRNA)



- SNV = single nucleotide variant
- The genomic SNV is reflected in the transcribed RNA (T is U in RNA).

[2] Detecting ASE

Detecting allele-specific expression

Wet lab technologies:

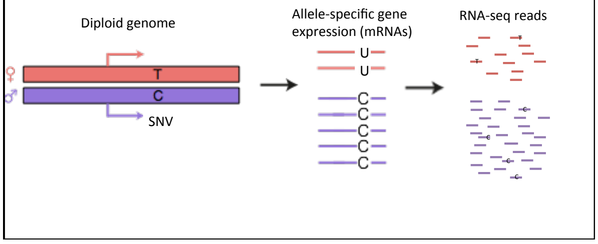
- microarrays (if designed properly)
- qRT-PCR + TaqMan
- pyrosequencing
- **RNA-seq**

N.B.: as these are sequence-based they will not provide any information in the case of a homozygous allele, although it may still be expressed predominantly from only one of the chromosomes.

eQTL – expression quantitative trait loci
Another approach!
Requires many subjects

Detecting allele-specific expression using RNA-seq data

- RNA-seq reads provide the sequence of a transcript
- ... which enables the determination of the allelic origin of the reads overlapping with the SNV



Detecting allele-specific expression using RNA-seq data

General outline:

1. Map the RNA-seq reads

2. Count the reads that map to either allele

3. Calculate effect size and p-value

Detecting allele-specific expression using RNA-seq data
1. Map the RNA-seq reads

Paternal allele (a) Maternal allele (A)
…AGTCTTCCAATTAGC… …AGTCTTCTAATTAGC…

Reads – 10x coverage of the locus
…AGTCTTCTAATTAGC…
…AGTCTTCTAATTAGC…
…AGTCTTCCAATTAGC…
…AGTCTTCTAATTAGC…
…AGTCTTCTAATTAGC…
…AGTCTTCTAATTAGC…
…AGTCTTCTAATTAGC…
…AGTCTTCTAATTAGC…
…AGTCTTCCAATTAGC…
…AGTCTTCCAATTAGC…

Detecting allele-specific expression using RNA-seq data
1. Map the RNA-seq reads

Paternal allele (a) Maternal allele (A)
…AGTCTTCCAATTAGC… …AGTCTTCTAATTAGC…

Mapped reads
…AGTCTTCTAATTAGC…
…AGTCTTCTAATTAGC…
…AGTCTTCCAATTAGC…
…AGTCTTCTAATTAGC…
…AGTCTTCTAATTAGC…
…AGTCTTCTAATTAGC…
…AGTCTTCTAATTAGC…
…AGTCTTCTAATTAGC…
…AGTCTTCCAATTAGC…
…AGTCTTCCAATTAGC…

Detecting allele-specific expression using RNA-seq data
2. Count the reads

Paternal allele (a) Maternal allele (A)
3x …AGTCTTCCAATTAGC… 7x …AGTCTTCTAATTAGC…

3 reads mapped to paternal allele
7 reads mapped to maternal allele

In total 10 reads mapped to the locus

Detecting allele-specific expression using RNA-seq data
3. Calculate effect size and p-value

Effect size: (other definitions possible)

$ASE_{effect} = c_{alt}/(c_{alt} + c_{ref}) – 0.5$

i.e., the fraction of counts mapped to alternative allele minus 0.5 =>

- if no ASE then $ASE_{effect}=0$
- range of ASE_{effect} is [-0.5, 0.5]

P-value: Use binomial with $p=0.5$ (assuming 50/50 transcription)

Our example from previous slide:

Effect size = $ASE_{effect} = c_{alt}/(c_{alt} + c_{ref}) – 0.5 = 3/(3+7) - 0.5 = –0.2$

P-value: binomial test for deviation from 50/50 distribution between alleles (in R):

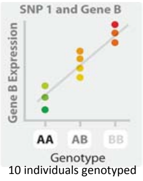
```
> pbinom(3, size=10, prob=0.5)
[1] 0.171875
```

⇒ Not significant in this particular example
⇒ If coverage was 30x (9+21 reads) instead of 10x (3+7), then p-value < 0.03

eQTL *vs.* ASE

eQTL

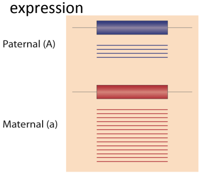
- Inter-individual differences in expression
- Modest effects
- Large number of SNP-gene combinations
- Many samples needed
- May use microarrays for gene expression
- Genotyping required



10 individuals genotyped

ASE

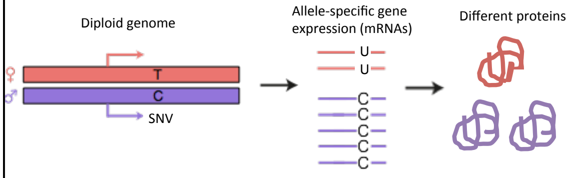
- Sufficient power with a single individual
- Identical cellular environment for the two chromosomes
- No association to regulatory region
- Must use RNA-seq for gene expression



[3] Applications and prevalence of ASE

Application of ASE

Find protein variants


Diploid genome

Allele-specific gene expression (mRNAs)

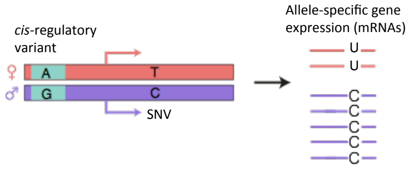
Different proteins

To infer a changed protein, the SNV must be

- in coding region
- non-synonymous

Application of ASE

Find *cis*-regulatory variant

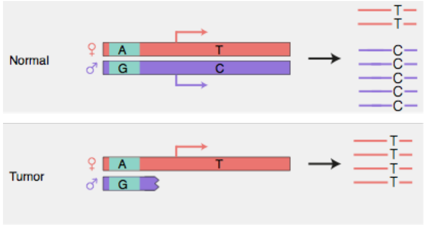

cis-regulatory variant

Allele-specific gene expression (mRNAs)

Possible to detect if you also have information about non-transcribed variants (e.g., from whole-genome DNA sequencing or SNP-array).

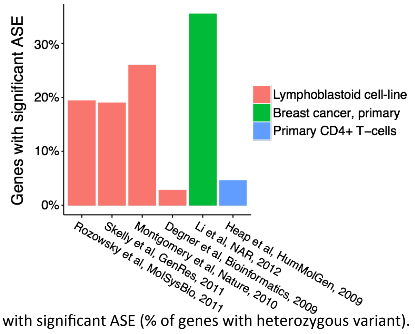
Application of ASE

Normal vs. tumor expression



Possible to detect if you have expression measured from both normal and tumor tissue (in the same individual).

Prevalence of ASE



Genes with significant ASE (% of genes with heterozygous variant).

[4] Important ASE considerations

Important ASE considerations

(a) Variant detection

(b) Mapping bias

(c) Many variants in a gene

[4] Important ASE considerations: (a) Variant detection

Variant detection

Variant = a position in the genome that is different from another genome.

- Homozygous variant: the two alleles are identical to each other
- Heterozygous variant: the two alleles are different
- “Ref.” = the allele is the same as for the reference genome
- “Alt.” = alternate = the allele is different from the reference genome
- SNV is one type of variant, others include insertion, deletion, ...

Variant detection = detecting what variants are present in a sample:

1. Variant calling – any position with evidence of an alternative base
2. Variant prioritization – define reliable variants with high confidence

Typically performed based on genomic DNA data, from

- Microarrays (*e.g.* Illumina Omni 2.5M)
- Sequencing (*e.g.* whole-genome re-sequencing or exome sequencing)

Variant detection from sequencing data

Start by map the reads.

Paternal allele (a) ...AGTCTTCCAATTAGC...

Maternal allele (A) ...AGTCTTCTAATTAGC...

Reads – 10x coverage of the locus

...AGTCTTCTAATTAGC...
...AGTCTTCTAATTAGC...
...AGTCTTCCAATTAGC...
...AGTCTTCTAATTAGC...
...AGTCTTCTAATTAGC...
...AGTCTTCTAATTAGC...
...AGTCTTCTAATTAGC...
...AGTCTTCTAATTAGC...
...AGTCTTCCAATTAGC...
...AGTCTTCCAATTAGC...

Variant detection from sequencing data

OK, piece of cake?

Paternal allele (a) ...AGTCTTCCAATTAGC...

Maternal allele (A) ...AGTCTTCTAATTAGC...

Mapped reads

...AGTCTTCTAATTAGC...
...AGTCTTCTAATTAGC...
...AGTCTTCCAATTAGC...
...AGTCTTCTAATTAGC...
...AGTCTTCTAATTAGC...
...AGTCTTCTAATTAGC...
...AGTCTTCTAATTAGC...
...AGTCTTCTAATTAGC...
...AGTCTTCCAATTAGC...
...AGTCTTCCAATTAGC...

Variant detection from sequencing data

This is what we *actually* have:

...AG ...C...

Reference sequence

...AGTCTTCTAATTAGC...

Mapped reads

...AGTCTTCTAATTAGC...
...AGTCTTCTAATTAGC...
...AGTCTTC*C*AATTAGC...
...AGTCTTCTAATTAGC...
...AGTCTTCTAATTAGC...
...AGTCTTCTAATTAGC...
...AGTCTTCTAATTAGC...
...AGTCTTCTAATTAGC...
...AGTCTTC*C*AATTAGC...
...AGTCTTC*C*AATTAGC...

=> need to detect the variant positions in the reference sequence

Variant detection from sequencing data

Standard: GATK (DePristo *et al.*, 2011) or Samtools – works on any mapped sequencing data.

GATK scores the SNVs by taking into account a number of characteristics, including:

- Sequencing depth (coverage)
- Mapping quality
- Position bias (base quality)

Specific RNA-seq based tools:

- Colib’read – Le Bras *et al.*, 2016
- RVboost – Wang *et al.*, 2014
- ACCUSA2 – Piechotta *et al.*, 2013

GATK the most widely used, even for RNA-seq.

Variant detection – VCF, Variant Call Format

VCF is a text file format (“flat text”). Example VCF output from GATK:

```
##fileformat=VCFv4.1
...
#CHROM POS   ID        REF ALT QUAL FILTER INFO FORMAT SAMPLE1_NA12878 [SAMPLE1_BLALBA] ...
1 873762 . T C 5231.78 PASS [ANNOTATIONS] GT:AD:DP:GQ:PL 0/1:173,141:282:99:255,0,255 ...
1 877664 rs3828047 A G 3931.66 PASS [ANNOTATIONS] GT:AD:DP:GQ:PL 1/1:0,105:94:99:255,255,0...
1 899282 rs28548431 C T 71.77 PASS [ANNOTATIONS] GT:AD:DP:GQ:PL 0/1:1,3:4:26:103,0,26 ...
1 974165 rs9442391 T C 29.84 LowQual [ANNOTATIONS] GT:AD:DP:GQ:PL 0/1:14,4:14:61:61,0,255...
...
```

GT: the genotype of this sample at this site (0/0, 0/1, 1/1, 1/2, ...). 0=ref., 1=alt.

AD: allele depths*, i.e.*, the number of reads that support each of the reported alleles

GQ: quality of assigned genotype (max=99)

Full specification of VCF file format: http://samtools.github.io/hts-specs/

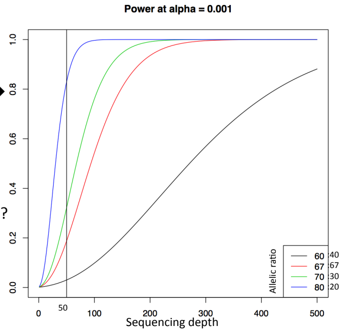
Variant detection – which variants to use (prioritization)?

Variants from RNA-seq

- What sequencing depth? influences the power, see ➔
- Heterozygous
- Other criteria

Filtering of known variants

- Keep only variants in dbSNP?



[4] Important ASE considerations: (b) Mapping bias

Mapping bias

Reference genome variants (“ref.”) have an advantage in the mapping.

```
Maternal allele: ...ATCGAATGAAGCTCATTGGATCAGAT...  (ref.)
Paternal allele: ...ATCGAATGAAGCTTATTGGATCAGAT...  (alt.)
Reference:       ...ATCGAATGAAGCTCATTGGATCAGAT...
```

<u>Mapping of reads</u>

```
Read from maternal allele:          AGCTCATT
                                    ::::::::
Reference:                 ATCGAATGAAGCTCATTGGATCAGAT
                                    :::: :::
Read from paternal allele:          AGCTTATT
```

The paternal allele read will map with a lower mapping quality.

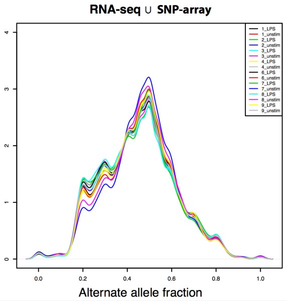
In case of sequencing error or poor base quality at another position, this might push the mapping quality of the paternal allele read below the threshold, and the read will be discarded.

Mapping bias – example in real data

Heterozygous variants (alt/ref) mapped to reference genome.

X-axis, alternate allele fraction [0, 1]
Y-axis, density

(Data from 16 RNA-seq experiments; Variants detected with RNA-seq data or SNP array).



Mapping bias – ways to get around it in ASE detection

<u>Masking variants ({A,C,G,T}=>N)</u>
You loose information.

<u>Construct all possible versions of the genome from existing variants</u>
Can soon generate a prohibitive amount of genome versions.

<u>Map reads to diploid genome (or transcriptome)</u>
Requires that you either have or construct the diploid genome (or transcriptome) of the individual.

<u>Modfiy the binomial probability p to reflect the mapping bias.</u>
Requires simulation to properly modify *p*.

Mapping bias – ways to get around it in ASE detection

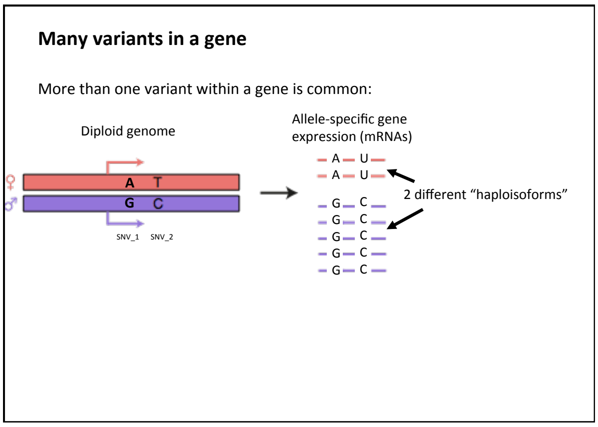
Masking variants ({A,C,G,T}=>N)
You loose information.

Construct all possible versions of the genome from existing variants
Can soon generate a prohibitive amount of genome versions.

Map reads to diploid genome (or transcriptome)
Requires that you either have or construct the diploid genome (or transcriptome) of the individual. *E.g.*, Turro *et al.* (2011) (transcriptome).

Modfiy the binomial probability p to reflect the mapping bias.
Requires simulation to properly modify *p*. *E.g.*, Montgomery *et al.* (2010).

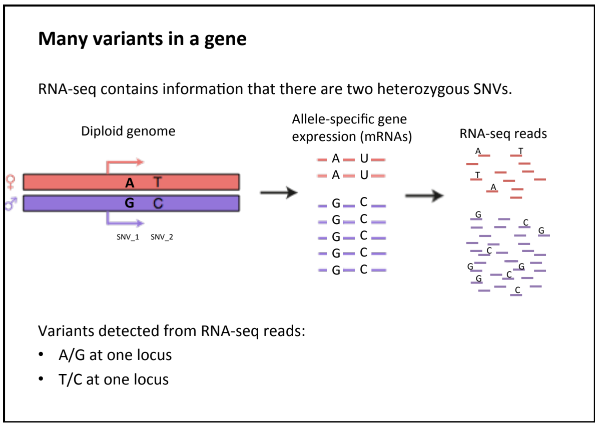
[4] Important ASE considerations: (c) Many variants in a gene



Many variants in a gene

More than one variant within a gene is common:

Diploid genome
♀
♂
A T
G C
SNV_1 SNV_2
Allele-specific gene expression (mRNAs)
A U
A U
G C
G C
G C
G C
G C
2 different “haploisoforms”

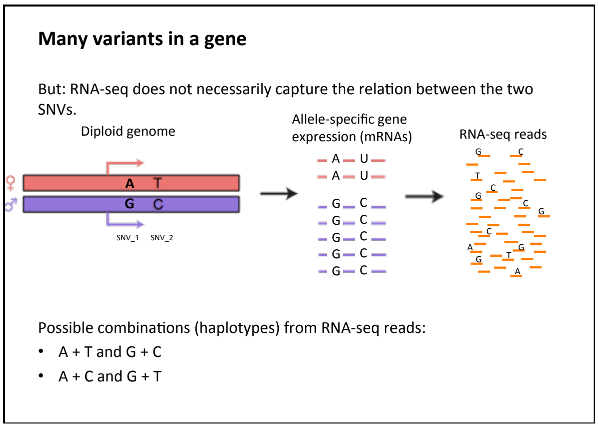


Many variants in a gene

RNA-seq contains information that there are two heterozygous SNVs.

Variants detected from RNA-seq reads:

- A/G at one locus
- T/C at one locus

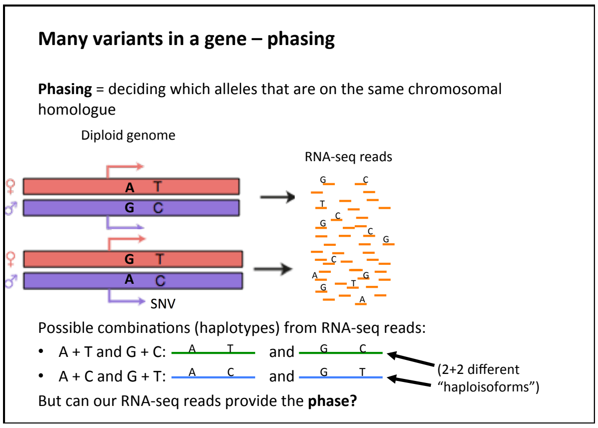


Many variants in a gene

But: RNA-seq does not necessarily capture the relation between the two SNVs.

Possible combinations (haplotypes) from RNA-seq reads:

- A + T and G + C
- A + C and G + T



Many variants in a gene – phasing

Phasing = deciding which alleles that are on the same chromosomal homologue

Possible combinations (haplotypes) from RNA-seq reads:

- A + T and G + C: A T and G C
- A + C and G + T: A C and G T

(2+2 different “haploisoforms”)

But can our RNA-seq reads provide the **phase?**

Phasing is useful but not necessary to detect ASE

Phasing information typically achieved by sequencing the genomes of the parents of the subject. Direct haplotype sequencing also possible.

If you **don't** know the phase (and for most RNA-seq data sets, you don't):

- Try to infer it from
  (a) your RNA-seq data – possible, but typically only partial phasing
  (b) existing population data (LD) – not applicable on new variants
- Disregard from it and calculate ASE anyway

Phasing

- reduces mapping bias
- enables the detection of haploisoform expression (isoforms representing the two homologous chromosomes)
- but is **not necessary** to detect ASE in genes with >1 SNV

[5] ASE tools

ASE tools

A list of tools that can detect ASE, given specified input data:

- cisASE – paired genomic+transcriptomic data, Liu *et al.*, 2016
- MutRSeq – nonsynonomous SNVs from RNA-seq data, Fu *et al.*, 2016
- GeneiASE – unphased RNA-seq data, Edsgärd *et al.*, 2016
- ASE-TIGAR – parental data required, bayesian, Nariai *et al.*, 2016
- ASEQ – paired genomic+transcriptomic data, Romanel *et al.*, 2015
- MBASED – phased or unphased RNA-seq data, Mayba *et al.*, 2015
- Allim – parental data required, Pandey *et al.*, 2013
- MMSEQ – attempts haploisoform identification, Turro *et al.*, 2011
- (Skelly) – requires phased data, Skelly *et al.*, 2011
- AlleleSeq – requires genomic sequence, Rozowsky *et al.*, 2011
- (AlleleDB – database for ASE etc. of 1000genomes, Chen *et al.*, 2016)

ASE tools – where only RNA-seq data from a single individual is required.

- cisASE – paired genomic+transcriptomic data, Liu *et al.*, 2016
- MutRSeq – nonsynonomous SNVs from RNA-seq data, Fu *et al.*, 2016
- **GeneiASE – unphased RNA-seq data, Edsgärd *et al.*, 2016**
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- AlleleSeq – requires genomic sequence, Rozowsky *et al.*, 2011
- (AlleleDB – database for ASE etc. of 1000genomes, Chen *et al.*, 2016)

[6] GeneiASE

GeneiASE

GeneiASE detects genes with significant ASE, in single individuals and based only on RNA-seq data. Haplotype information (phasing) is not needed.

Data required:

- RNA-seq data

Pre-processing required:

- Mapping and quality control of reads
- Variant detection (e.g., GATK)
- Filter variants if desired
- Allele counts for variants extracted into custom input text file

Availability:

- Edsgärd *et al.*, *Scientific Reports* **6**:21134, 2016
- https://github.com/edsgard/geneiase (GNU GPL3 license)

GeneiASE

The situation:

- unphased data
- non-uniform effect within gene
- technical variability

Gene *i*	SNV_1	SNV_2	SNV_3	SNV...
Ref	60	20	70	...
Alt	40	80	30	...

The GeneiASE solution:

1. For each gene, loop over all its SNVs and their 2x1 matrix of read counts
2. Calculate a test statistic (s_{ij}) for each SNV, based on read counts
3. Combine the test statistics for the SNVs within a gene => test statistic for entire gene (g_i) asdf
4. Resample from parametric null SNV model (estimated from DNA data) 10^5 times, calculate the resulting distribution of gene test statistic (g^0_i).
5. Compare g_i *to* g^0_i and calculate a p-value for gene *i*.

GeneiASE – calculate SNP-based gene-wise test statistic

1. Count reads for each SNV in a gene; add pseudo counts if required

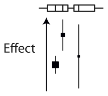
	U	T
a	0	34
A	19	45

	U	T
a	1	35
A	20	46

2. Calculate **SNV** test statistic s_{ij} based on absolute value of effect size, *eff.*

$$s_{ij} = \frac{|eff|}{SE(eff)}, eff = \begin{cases} log(odds(\hat{p})), & \text{if static-ASE} \\ log(OR(\hat{p}|_{t=1}, \hat{p}|_{t=0})), & \text{if icd-ASE} \end{cases}$$

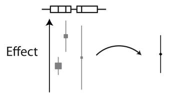
$$\hat{p} = \frac{c_{ij}|_{a=alt}}{c_{ij}|_{a=alt} + c_{ij}|_{a=ref}}$$



Absolute value of effect size => Undirected effect

3. Calculate **gene** test statistic g_i using Stouffer-Liptak method; *k* is number of SNVs in gene *i*

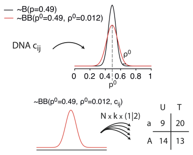
$$g_i = \frac{\sum_{j=1}^{k} s_{ij}}{\sqrt{k}}$$



GeneiASE – null model, and gene-wise *p*-value calculation

0. Estimate SNV null model parameters

DNA based estimate of the technical variability



For each gene (gene *i*):

1. Sample allele counts from null SNV model

(Random effect model)

2. Calculate *k* SNV test statistic

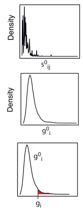
k= number of SNVs in gene *i*

k x

3. Calculate gene test statistic (Stouffer-Liptak)

4. Reiterate 1-3 N times (default: 10^5)

5. Calculate *p*-value for gene *i*



Running GeneiASE – input

Static ASE

- geneiase -cvtl -i cvtl.test.input.tab

```
gene    snp.id  alt.dp  ref.dp
10.9    1       4       6
10.9    2       6       4
10.9    3       5       5
10.9    4       0       10
10.9    5       9       1
10.9    6       5       5
10.9    7       3       7
10.9    8       8       2
```

Condition-dependent ASE

- geneiase -icd -i icd.test.input.tab

```
gene    snp.id  U.alt.dp  U.ref.dp  T.alt.dp  T.ref.dp
1.11    1       8         2         7         3
1.11    2       3         7         4         6
1.11    3       8         2         6         4
1.11    4       5         5         7         3
1.11    5       6         4         1         9
1.11    6       9         1         5         5
1.11    7       4         6         5         5
```

Running GeneiASE – output

One line per gene.

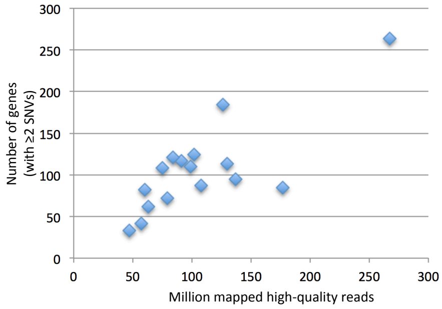
Output columns:

- **feat**: FeatureID as specified in the input file (typically a gene identifier)
- **n.vars**: Number of variants within the gene
- **mean.s**: Mean of *s* across the variants within the gene
- **median.s**: Median of *s* across the variants within the gene
- **sd.s**: Standard deviation of *s* across the variants within the gene
- **cv.s**: Coefficient of variation of *s* across the variants within the gene
- **liptak.s**: Stouffer-Liptak combination of *s* (called *g* on previous slides)
- **p.nom**: Nominal p-value
- **fdr**: Benjamini-Hochberg corrected p-value

(Reminder: *s* is the effect size-based test statistic for each SNV in a gene).

Running GeneiASE – results

The number of genes with significant (fdr<0.05) ASE as detected by GeneiASE from 16 RNA-seq samples (primary white blood cells).



Thank you for your attention

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