

# Differential Expression Analysis Methods Comparison

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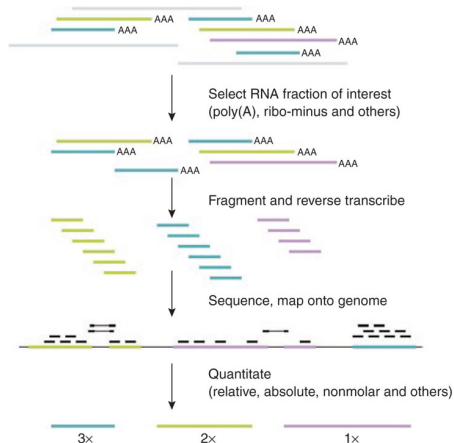
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# Outline

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# RNA fragmentation, sequencing, and alignment



(Pepke, Wold, and Mortazavi (2009) [http://www.nature.com/nmeth/journal/v6/n11s/fig\\_tab/nmeth.1371\\_F5.html](http://www.nature.com/nmeth/journal/v6/n11s/fig_tab/nmeth.1371_F5.html))

# RNAseq data

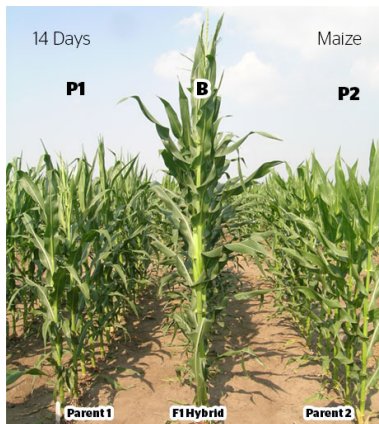
Genes	B73 Rep1	B73 Rep2	B73 Rep3	B73 Rep4	Mo17 Rep1	Mo17 Rep2	Mo17 Rep3	Mo17 Rep4
<b>AC148152.3_FG001</b>	3	4	6	0	8	17	18	20
<b>AC148152.3_FG008</b>	3	3	4	1	31	40	45	49
<b>AC152495.1_FG002</b>	33	46	18	13	4	0	2	6
<b>AC152495.1_FG017</b>	41	44	16	13	2	2	2	0
<b>AC184130.4_FG012</b>	24	47	18	21	110	144	121	96
<b>AC184133.3_FG001</b>	0	1	1	0	14	13	4	9
AC148152.3_FG005	2323	1533	1932	1945	2070	1582	2196	1882
AC148167.6_FG001	672	598	728	713	743	655	821	824
AC149475.2_FG002	459	438	451	483	467	448	634	532
AC149475.2_FG003	1184	976	1131	1206	891	743	1288	1107
AC149475.2_FG005	551	535	360	353	550	524	492	440
AC149475.2_FG007	245	214	169	159	297	262	210	302

- DE genes: expression in Genotype Variety *B73* is different from that in another Genotype Variety *Mo17*

# Differential Expression Genes

## Definition

A gene is regarded as differentially expressed (DE) when the expected count reads of this gene corresponding to one genotype variety differs from that of another genotype variety.



# Differential expression analysis

## Definition

For a given gene, we use statistical testing to decide whether an observed difference in read counts is significant, i.e., whether it is greater than what would be expected just due to natural random variation.

- Normalization  
Estimated normalization factors should ensure that a gene with the same expression level in two samples is not detected as DE.
- Assumed distribution  
Negative binomial
- Parameter estimation  
Mean, Dispersion
- Test for DE  
Exact test, Wald test

# Negative Binomial Model in Generalized Linear Model Framework (Part 1)

Let

- $g$  ( $g = 1, \dots, G$ ) identify the gene,
- $i$  ( $i = 1, 2$ ) identify the genotype variety,
- $j$  ( $j = 1, 2, 3, 4$ )
- $Y_{gij}$  be the RNAseq counts of gene  $g$ , genotype variety  $i$ , replicate  $j$

We assume

$$Y_{gij} \overset{ind}{\sim} \text{NB}(\mu_{gij}, \phi_g) \quad (1)$$

where

- $\mu_{gij}$  are means of read counts of gene  $g$  genotype  $i$  replicate  $j$ ,
- $\phi_g$  allow for gene-specific overdispersion

# Negative Binomial Model in Generalized Linear Model Framework (Part 2)

In the generalized linear model (GLM) setting, the mean response,  $\mu_{gij}$  is linked to a linear predictor with natural log link:

$$\log(\mu_{gij}) = x_i^T \beta_g + \log(N_{ij}) \quad (2)$$

where

- $x_i$  is row of the design matrix containing the covariates indicating this sample belongs to variety  $i$ ,
- $\beta_g = (\beta_{g1}, \beta_{g2})$  is a vector of regression parameters
- $N_{ij}$  is the normalized library size of replicate  $j$  in variety  $i$



# Hierarchical model for RNA-seq counts

We assume

$$Y_{gij} \stackrel{ind}{\sim} \text{NB}(\mu_{gij}, \phi_g)$$

where

- $\mu_{gij} = \exp(x_i^T \beta_g + \log(N_{ij}))$
- $\lambda_{gi} = x_i^T \beta_g$ ,  $\gamma_{ij} = \log(N_{ij})$ , then  $\gamma_{ij}$  are normalization factors
- $\phi_g = \exp(\psi_g)$  allow for gene-specific overdispersion

We reparameterized the mean dispersion structure into the genespecific average  $\beta_{g1}$  and half-variety difference  $\beta_{g2}$

$$\beta_{g1} = \frac{\lambda_{g1} + \lambda_{g2}}{2}, \beta_{g2} = \frac{\lambda_{g1} - \lambda_{g2}}{2} \quad (3)$$

we also assume

$$\beta_{g1} \stackrel{ind}{\sim} \text{N}(\eta_{\beta_1}, \sigma_{\beta_1}^2), \beta_{g2} \stackrel{ind}{\sim} \text{N}(\eta_{\beta_2}, \sigma_{\beta_2}^2), \psi_g \stackrel{ind}{\sim} \text{N}(\eta_{\psi}, \sigma_{\psi}^2) \quad (4)$$

$\beta_{g1}, \beta_{g2}, \psi_g$  are independent to each other.

# Empirical Bayes Method

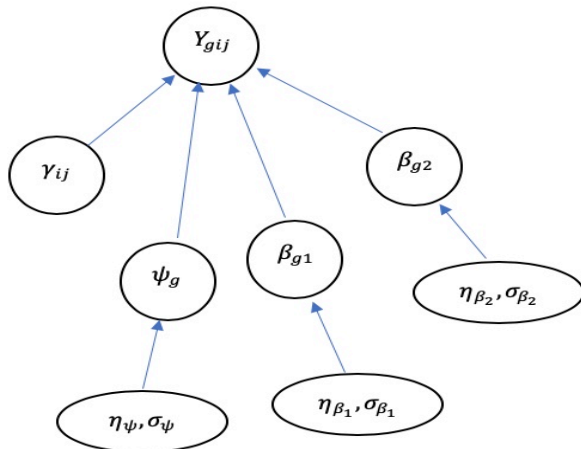
Let

- $\theta = (\theta_1, \dots, \theta_G)$  ( $g = 1, \dots, G$ ) where  $\theta_g = (\beta_{g1}, \beta_{g2}, \psi_g)$ ,
- $\gamma_{ij}$  is the normalized factor for replicate  $j$  in variety  $i$ ,
- $\pi = (\eta, \sigma)$ , where  $\eta = (\eta_{\beta_1}, \eta_{\beta_2}, \eta_{\psi})$ ,  $\sigma = (\sigma_{\beta_1}, \sigma_{\beta_2}, \sigma_{\psi})$

Then,

- $\hat{\gamma}$  was obtained from trimmed mean of  $M$  values (TMM)
- $\hat{\psi}_g$  was got through the adjusted profile likelihood (APL)
- $\hat{\beta}_{g1}, \hat{\beta}_{g2}$  was retrieved by fitting the generalized linear model with log link function
- Using  $\hat{\theta}_g = (\hat{\beta}_{g1}, \hat{\beta}_{g2}, \hat{\psi}_g)$ , we estimated hyperparameters for the location and scale parameters in the hierarchical model using a central method of moments approach, as  $\hat{\pi} = (\hat{\eta}, \hat{\sigma})$ , where  $\hat{\eta} = \sum_{g=1}^G \hat{\beta}_g / G$ ,  $\hat{\sigma}^2 = \sum_{g=1}^G (\hat{\beta}_g - \hat{\eta})^2 / (G - 1)$

# Empirical Bayes Method (cont)



# Empirical Bayes Method Posterior Probability of Parameters

Condition on the estimated normalization factor  $\hat{\gamma}$  and hyperparameters  $\hat{\pi}$ , we perform a Bayesian analysis to re-estimate the  $\theta$  as:

$$p(\theta|y, \hat{\pi}, \hat{\gamma}) \propto \prod_{g=1}^G \prod_{i=1}^2 \prod_{j=1}^{n_i} p(y_{gij} | \hat{\mu}_{gij}, \hat{\phi}_g) p(\hat{\beta}_{g1} | \hat{\eta}_{\beta_1}, \hat{\sigma}_{\beta_1}^2) p(\hat{\beta}_{g2} | \hat{\eta}_{\beta_2}, \hat{\sigma}_{\beta_2}^2) p(\hat{\psi}_g | \hat{\eta}_{\psi}, \hat{\sigma}_{\psi}^2) \quad (5)$$

where  $\hat{\mu}_{gij} = \exp(\lambda_{gi} + \hat{\gamma}_{ij})$ ,  $\hat{\phi}_g = \exp(\hat{\psi}_g)$ , and  $\hat{\beta}_{g1} \stackrel{ind}{\sim} N(\hat{\eta}_{\beta_1}, \hat{\sigma}_{\beta_1}^2)$ ,  $\hat{\beta}_{g2} \stackrel{ind}{\sim} N(\hat{\eta}_{\beta_2}, \hat{\sigma}_{\beta_2}^2)$ ,  $\psi_g \stackrel{ind}{\sim} N(\hat{\eta}_{\psi}, \hat{\sigma}_{\psi}^2)$ .

# Null Hypothesis for DE Analysis

$$H_0 : \beta_{g2} = 0$$

which is equivalent to  $\lambda_{g1} = \lambda_{g2}$

Statistics used to do the DE analysis is based on the posterior probabilities of  $\beta_{g2}$  as

$$P(DE_g|y, \hat{\pi}, \hat{\gamma}) = \min(P(\beta_{g2} < 0|y, \hat{\pi}, \hat{\gamma}), P(\beta_{g2} > 0|y, \hat{\pi}, \hat{\gamma}))$$

where  $P(\beta_{g2} < 0|y, \hat{\pi}, \hat{\gamma}) = \frac{1}{M} \sum_{m=1}^M I(\beta_{g2}^{(m)} < 0)$ , and

$$P(\beta_{g2} > 0|y, \hat{\pi}, \hat{\gamma}) = \frac{1}{M} \sum_{m=1}^M I(\beta_{g2}^{(m)} > 0)$$

# Alternative Methods

## Normalization

edgeR used gene-wise trimmed median of means (TMM), while DESeq, DESeq2, sSeq, EBSeq used sample-wise size factor.

## Dispersion estimation

edgeR used Cox-Reid approximate conditional inference (CRACI) moderate towards the mean while DESeq, DESeq2 used CRACI with focus on maximum individual dispersion estimate; sSeq estimated dispersion by pooling all the samples using the method of moments(MM), and then shrinking the gene-wise estimates through minimizing the mean-square error; EBSeq also estimated the gene-specific variances via MM.

## Test for DE

edgeR, sSeq used exact test for 2 factors; DESeq, DESeq2 used Wald test for 2 factors;

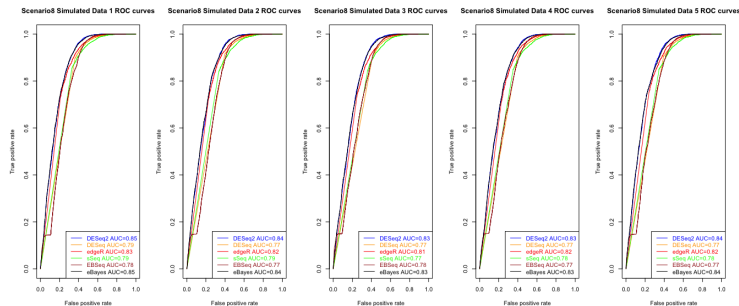
# Simulation studies

Parameter estimation use edgeR:  $\hat{\beta}_{g1}, \hat{\beta}_{g2}, \hat{\phi}_g$ , and the normalized library sizes  $N_{ij}$

Simulation scenario set up: nGenes, nSamples, pDiff

Simulation model:  $Y_{gij} \overset{ind}{\sim} \text{NB}(\mu_{gij}, \phi_g)$  with  $\mu_{gij} = \exp(x_i^T \beta_g + \log(N_{ij}))$  where  $N_{ij}$  is the normalized library size. For non-DE genes, we set  $\mu_{g1} = \mu_{g2}$ .

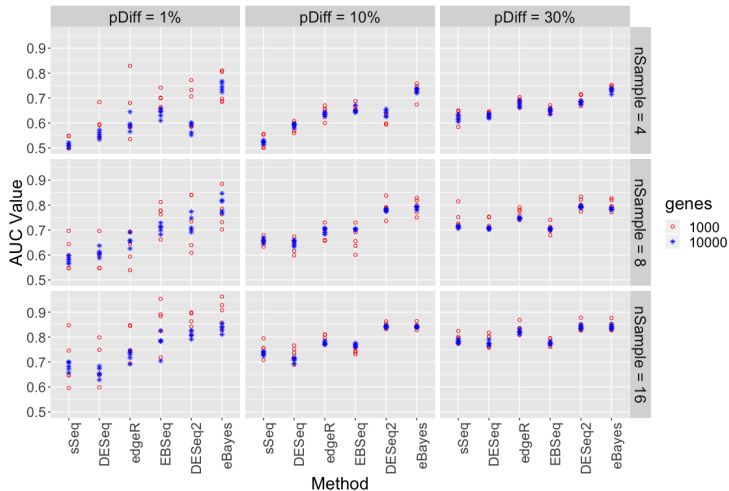
# ROC Curves of One Scenario



nGenes=10000, nSample=16, pDiff=30% Scenario ROC Curves



# AUC Plot



# Summary of the Results

Effect of nGenes: not obvious

Effect of pDiff: smaller pDiff  $\rightarrow$  larger differences between eBayes and other methods

Effect of nSample: smaller nSample  $\rightarrow$  larger differences between eBayes and other methods

# Discussion

For the future research, we could:

- (1) add more methods: baySeq, ShrinkSeq, NOISeq, SAMseq;
- (2) include more varieties;
- (3) consider the flow cell effects;
- (4) improve the eBayes by refining the hierarchical model