Method: We obtained DNA methylation profiles of cord blood using the Illumina HumanMethylation27k BeadChip array for 348 neo-nates, ranging from 25.2857143 to 42.86 weeks in gestational age.

Method: We obtained DNA methylation profiles of cord blood using the Illumina HumanMethylation27k BeadChip array for 341 neo-nates, ranging from 30 to 42.86 weeks in gestational age.

We recalculated CpGs for the 27k array that distinguish cell types in adult blood as described in Houseman et al.
**1 Figure1**

Method: We performed cluster analysis on the neonatal cohort DNA methylation profiles on the top 500 CpGs that distinguish cell types on adult blood. We used hierarchical clustering using Euclidean distance on methylation log ratio (M values).

Result: We observed that methylation profiles on our cohort segregate on these blood cell type specific CpGs with two clearly defined groups (Figure 1).
Result: We observed that the two clusters have distinct general methylation patterns in these 500 CpGs, with one group showing relative hyper-methylation (median probe-level methylation z-scores −0.33 and 0.5 respectively). We also observed that the clustering also reflects differences in gestational age since the cluster showing relative hyper-methylation of celltype marker CpGs contains a larger proportion of samples with earlier gestational age (Figure 2).
3 Figure 3

```r
> print(welch <- t.test(pd$GEAA[g1], pd$GEAA[g2], var.equal=FALSE))

Welch Two Sample t-test

data: pd$GEAA[g1] and pd$GEAA[g2]
t = 6.9042, df = 211.92, p-value = 5.773e-11
alternative hypothesis: true difference in means is not equal to 0
95 percent confidence interval:
 1.243023 2.236451
sample estimates:
mean of x mean of y
 39.06666  37.32692

> print(wilcox <- wilcox.test(pd$GEAA[g1], pd$GEAA[g2]))

Wilcoxon rank sum test with continuity correction

data: pd$GEAA[g1] and pd$GEAA[g2]
W = 19935, p-value = 1.253e-11
alternative hypothesis: true location shift is not equal to 0

> print(sprintf("Group sds: %.3f, %.3f", sd(pd$GEAA[g1]), sd(pd$GEAA[g2])))

[1] "Group sds: 1.720, 2.576"

> v1 = var(pd$GEAA[g1])
> v2 = var(pd$GEAA[g2])
> fstat = v2/v1
> fpval = 1-pf(fstat, sum(g2)-1, sum(g1)-1)
> cat("fstat: ", fstat, " pval: ", fpval, "\n")

fstat: 2.242794 pval: 8.856527e-08
```

Cluster groups

Hypomethylated

Hypermethylated

Cluster groups

Gestational Age (Weeks)

30 32 34 36 38 40 42

Hypomethylated

Hypermethylated
**Method:** To determine if there is a significant difference in gestational age between the two resulting groups from the clustering, we divided the cohort into two groups as defined by the hierarchical clustering on the top 500 blood cell-type specific CpGs and performed a t-test to determine significance.

**Result:** We found that there is a significant difference in gestational age between the two groups obtained from cluster analysis (Figure 3, 39 weeks vs. 37.3 weeks, \(P=5.77\times10^{-11}\)). This suggests that differences in gestational age are concomitant with a difference in blood cell type composition in samples obtained from cord blood. Also noticeable is a difference in the variability of gestational age within each of the cluster groups, where the cluster corresponding to late gestational age has significantly lower variance (std. dev: 1.72 vs. 2.58).

```r
> g1cut <- boxplot.stats(pd$GEAA[g1])$stats[1]
> g2cut <- boxplot.stats(pd$GEAA[g2])$stats[1]
> g1outliers <- which(g1)[pd$GEAA[g1]<g1cut]
> g2outliers <- which(g2)[pd$GEAA[g2]<g2cut]
> outliers <- as.data.frame(pData(mset[, keep]))[c(g1outliers, g2outliers),]
> write.csv(outliers, file="fig1_outliers.csv")
```
**Method:** We next used the cell type deconvolution method described by Houseman to obtain an estimate of the proportion of each of six blood cell types (Gran, Mono, B-cell, NK, CD4-T and CD8-T) in each of the samples in our cohort. Note that we obtain these estimates using the methylation profiles provided by Houseman for the 27k array on adult whole blood.

**Result:** We observed that the blood cell type composition of the two groups obtained by the clustering shown in Figure 1 shows distinct profiles (Figure 4). The composition of the cluster corresponding to late gestational age is dominated by Granulocytes, whereas for early gestational age, the presence of Granulocytes is diminished and an increase in CD4-T (spell out) B-cell and NK (spell out) is observed.

**NOTE: IS THIS EXPECTED IN DEVELOPMENT OF IMMUNE SYSTEM?**
5 Combined Fig. 1 and 2 (in paper)

The new way of doing this, cluster on cell type composition, then do gestational age analysis on resulting cluster

> print(welch <- t.test(pd$GEAA[g1], pd$GEAA[g2], var.equal=FALSE))

Welch Two Sample t-test
data: pd$GEAA[g1] and pd$GEAA[g2]  
t = 5.0056, df = 229.52, p-value = 1.109e-06  
alternative hypothesis: true difference in means is not equal to 0  
95 percent confidence interval:  
0.7761357 1.7837955  
sample estimates:  
mean of x mean of y  
38.89590 37.61593

> print(wilcox <- wilcox.test(pd$GEAA[g1], pd$GEAA[g2]))

Wilcoxon rank sum test with continuity correction

data: pd$GEAA[g1] and pd$GEAA[g2]  
W = 18240, p-value = 2.161e-06  
alternative hypothesis: true location shift is not equal to 0

> print(sprintf("Group sds: %.3f, %.3f", sd(pd$GEAA[g1]), sd(pd$GEAA[g2])))

[1] "Group sds: 1.847, 2.589"

> v1 = var(pd$GEAA[g1])
> v2 = var(pd$GEAA[g2])
> fstat = v2/v1
> fpval = 1-pf(fstat, sum(g2)-1, sum(g1)-1)
> cat("fstat: ", fstat, " pval: ", fpval, "\n")

fstat: 1.965803 pval: 6.06914e-06
6 Regression analysis of gestational age vs. cell type proportion

```r
> load("../Houseman_SBraid/targetBoot.rda")
> # View summary with bootstraps
> (tab <- summary(targetEst, targetBoot))

<table>
<thead>
<tr>
<th></th>
<th>Est</th>
<th>StdErr0</th>
<th>StdErr1</th>
<th>StdErr2</th>
<th>Zscore</th>
<th>Pvalue</th>
</tr>
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<td>GEAA</td>
<td>Intercept</td>
<td>-0.14415</td>
<td>0.05002</td>
<td>0.04514</td>
<td>0.04591</td>
<td>-3.1396</td>
</tr>
<tr>
<td></td>
<td>CD8T</td>
<td>0.44923</td>
<td>0.16599</td>
<td>0.07422</td>
<td>0.09082</td>
<td>4.9465</td>
</tr>
<tr>
<td></td>
<td>CD4T</td>
<td>-1.03859</td>
<td>0.15915</td>
<td>0.14303</td>
<td>0.14332</td>
<td>-7.2469</td>
</tr>
<tr>
<td></td>
<td>NK</td>
<td>-0.49186</td>
<td>0.08196</td>
<td>0.11640</td>
<td>0.11633</td>
<td>-4.2283</td>
</tr>
<tr>
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<td>Bcell</td>
<td>-0.53810</td>
<td>0.05761</td>
<td>0.08855</td>
<td>0.08914</td>
<td>-6.0367</td>
</tr>
<tr>
<td></td>
<td>Mono</td>
<td>0.05631</td>
<td>0.08027</td>
<td>0.06950</td>
<td>0.07106</td>
<td>0.7923</td>
</tr>
<tr>
<td></td>
<td>Gran</td>
<td>1.76884</td>
<td>0.07989</td>
<td>0.23753</td>
<td>0.23276</td>
<td>7.5993</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>Est</th>
<th>StdErr0</th>
<th>StdErr1</th>
<th>StdErr2</th>
<th>Zscore</th>
<th>Pvalue</th>
</tr>
</thead>
<tbody>
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<td>0.1244</td>
<td>0.1955</td>
<td>0.1943</td>
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<tr>
<td></td>
<td>CD8T</td>
<td>-0.62570</td>
<td>0.4129</td>
<td>0.3401</td>
<td>0.3363</td>
<td>-1.8603</td>
</tr>
<tr>
<td></td>
<td>CD4T</td>
<td>1.99117</td>
<td>0.3959</td>
<td>0.6517</td>
<td>0.6446</td>
<td>3.0888</td>
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<tr>
<td></td>
<td>NK</td>
<td>1.25886</td>
<td>0.2039</td>
<td>0.4886</td>
<td>0.4850</td>
<td>2.5955</td>
</tr>
<tr>
<td></td>
<td>Bcell</td>
<td>0.82912</td>
<td>0.1433</td>
<td>0.3826</td>
<td>0.3857</td>
<td>2.1497</td>
</tr>
<tr>
<td></td>
<td>Mono</td>
<td>0.46291</td>
<td>0.1997</td>
<td>0.3222</td>
<td>0.3235</td>
<td>1.4309</td>
</tr>
<tr>
<td></td>
<td>Gran</td>
<td>-3.65631</td>
<td>0.1987</td>
<td>1.0543</td>
<td>1.0419</td>
<td>-3.5092</td>
</tr>
</tbody>
</table>

Inference based on double bootstrap standard errors (StdErr2) from 250 bootstrap iterations

Proportion of total variation explained by WBC: 0.08209
Proportion of stage 1 model explained by WBC: 0.8493

```r
> est <- tab$Coef[, "GEAA"] * 100
> zscore <- tab$Coef[, "GEAA"] / tab$SD.Double[, "GEAA"]
> pvals <- 2*pnorm(-abs(zscore))
> apvals <- p.adjust(pvals, method="bonferroni")
> restab <- data.frame(Est=round(est, 3),
                     Zscore=round(zscore, 3),
                     Pvalue=round(pvals, 5),
                     adj.Pvalue=round(apvals, 5))

> write.csv(restab, file="geaa_tab.csv")

> pctSystematic <- 1-sum(targetEst$sse)/sum(targetEst$ss0)
> cat("Pct. systematic variation", sprintf("%.2f\%", pctSystematic*100))

Pct. systematic variation 9.67%

Est = regression coefficient estimate (x 100%); StdErr0 = naive (x 100%); StdErr1 = single bootstrap (x 100%); StdErr2 = double bootstrap (x 100%); the p-values are based on StdErr2 (x 100%).
Plot weights vs. GEAA

**CD4 T-cells**

- Y-axis: Cell-type Proportion
- X-axis: Gestational age (weeks)
- Open circles: female
- Filled circles: male

**CD8 T-cells**

- Y-axis: Cell-type Proportion
- X-axis: Gestational age (weeks)
- Open circles: female
- Filled circles: male
Natural Killer Cells

B cells
Monocytes

Gestational age (weeks)

Cell-type Proportion

female
male

Granulocyte

Gestational age (weeks)

Cell-type Proportion

female
male
8 Horvath Analysis

We downloaded the list of 353 age prediction CpGs (Additional File 23) from http://www.genomebiology.com/2013/14/10/R115/additional.

```r
> horvath_anno_1 <- read.csv("../horvath_data/gb-2013-14-10-r115-s21.csv")
> horvath_anno_2 <- read.csv("../horvath_data/gb-2013-14-10-r115-s22.csv")
> horvath_coefs <- read.csv("../horvath_data/gb-2013-14-10-r115-s23.csv")
> horvath_mset <- mset[auIndex,!(mset$GEAA < 30)]
> horvath_pd <- pData(horvath_mset)
> horvath_mmat <- getM(horvath_mset)
> inOurs <- rownames(horvath_mmat) %in% DMRselection
> inHorvath <- rownames(horvath_mmat) %in% horvath_coefs$CpGmarker[-1]
> table(inOurs, inHorvath)

inHorvath
inOurs FALSE TRUE
FALSE 25642 344
TRUE 491 9
```

We compared the list of top 500 celltype-specific CpGs used in our study to the 353 CpGs used for age prediction by Horvath et al. We found that only 9 of the 500 celltype-specific CpGs are included in the list of age-predictable CpGs. Furthermore

```r
> design1 <- model.matrix(~1+GEAA, data=horvath_pd)
> fit <- lmFit(horvath_mmat, design1)
> ebfit <- eBayes(fit)
> topTab1 <- topTable(ebfit, coef=2, number=nrow(horvath_mmat), p.value=0.1)
> inOurs <- rownames(horvath_mmat) %in% rownames(topTab1)
> tab <- table(inOurs, inHorvath)
> print(tab)

inHorvath
inOurs FALSE TRUE
FALSE 17948 235
TRUE 8185 118
```

Fisher's Exact Test for Count Data

```r
> fisher.test(tab)

Fisher's Exact Test for Count Data

data: tab
p-value = 0.4188
alternative hypothesis: true odds ratio is not equal to 1
95 percent confidence interval:
0.8736117 1.3816340
sample estimates:
odds ratio
1.101053
```

We also performed a regression analysis to determine probes where differences in methylation were associated with differences in gestational age (without controlling for celltype composition) and found 8303 probes associated with gestational age, 0 of which are in the set of 353 age-predictable CpGs. No evidence for enrichment was detected (Fisher test OR=1.1, P=0.41). This suggests that the rapid changes observed in epigenetic state during gestation are due to mechanisms that are potentially distinct to those previously identified as age-related in adults.
9 Steroid use, gestational age and blood composition

We obtained data on anti-natal steroid use for 60 of the samples in our cohort. Anti-natal steroids were applied in NA of these cases. We observed an association between gestational age and antenatal steroid application, as well as an association between antenatal steroid application and Granulocyte and CD4 T-cell proportion.
```r
> steroid_mset <- mset[auIndex,!(mset$GEAA < 30)]
> keep <- steroid_mset$ID %in% placental_tab$id
> steroid_mset <- steroid_mset[,keep]
> placental_ObsMix <- ObsMix[,keep]
>
> m <- match(steroid_mset$ID, placental_tab$id)
> steroid_mset$steroid <- factor(placental_tab$Ant_st[m])
>
> drop <- is.na(steroid_mset$steroid)
> steroid_mset <- steroid_mset[,!drop]
> placental_ObsMix <- placental_ObsMix[,!drop]
>
> tmpPd <- pData(steroid_mset)
> tmpPd <- tmpPd[,c("steroid", "GEAA", "SEX")]
> tmpPd <- bind(tmpPd, DataFrame(placental_ObsMix[,c("Gran","CD4T")]))
>
> fit <- glm(steroid ~ I(scale(GEAA))+SEX+I(scale(ilogit2(Gran)))+I(scale(ilogit2(CD4T))), data=tmpPd, family="binomial")

Call:
glm(formula = steroid ~ I(scale(GEAA)) + SEX + I(scale(ilogit2(Gran)))+I(scale(ilogit2(CD4T))), data = tmpPd, family = "binomial")
```
We found a strong association between antenatal steroid application and both younger gestational age (z-score -3.270, P=0.001) and lower CD4 T-cell concentration (z-score -2.027, P=0.042) as estimated from DNAm using Houseman’s approach.

```
Deviance Residuals:
     Min       1Q     Median       3Q      Max
-2.3108  -0.6562  -0.3625   0.7534   2.1263

Coefficients:
                           Estimate Std. Error z value Pr(>|z|)
(Intercept)                -0.7747    0.4847  -1.598  0.10997
I(scale(GEAA))             -1.5546    0.4754  -3.270  0.00108 **
SEX                        -0.6462    0.7492  -0.862  0.38843
I(scale(ilogit2(Gran)))    -0.7844    0.4995  -1.570  0.11632
I(scale(ilogit2(CD4T)))    -1.0023    0.4944  -2.027  0.04262 *
---
Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

(Dispersion parameter for binomial family taken to be 1)

    Null deviance: 74.92 on 59 degrees of freedom
    Residual deviance: 52.44 on 55 degrees of freedom
    AIC: 62.44

Number of Fisher Scoring iterations: 5
```

We regressed methylation log-ratio for each CpG on antenatal steroid use, gestational age, and CD4 T-cell concentration. As illustration of the effect of confounding gestational age, cord blood cell composition

```
> mmat <- getM(steroid_mset)
> design1 <- model.matrix(~1+steroid+GEAA*CD4T, data=tmpPd)
> fit <- lmFit(mmat, design1)
> ebfit <- eBayes(fit)
> topTab1 <- topTable(ebfit, coef=2, number=nrow(mmat), p.value=0.1)
> design0 <- model.matrix(~1+steroid, data=tmpPd)
> fit <- lmFit(mmat, design0)
> ebfit <- eBayes(fit)
> topTab0 <- topTable(ebfit, coef=2, number=nrow(mmat), p.value=0.1)
> design1b <- model.matrix(~1+steroid+GEAA+CD4T, data=tmpPd)
> fit <- lmFit(mmat, design1b)
> ebfit <- eBayes(fit)
> topTab1b <- topTable(ebfit, coef=2, number=nrow(mmat), p.value=0.1)
> print(topTab1)

  logFC AveExpr  t  P.Value adj.P.Val
cg09068993 0.4492735 -3.392372 5.718052 3.338059e-07 0.008841184 B
cg09068993 5.182328

> print(topTab1b)

  logFC AveExpr  t  P.Value adj.P.Val
cg09068993 0.4612523 -3.392372 5.97322 1.187321e-07 0.003144738 B
cg09068993 6.314831
```

We regressed methylation log-ratio for each CpG on antenatal steroid use, gestational age, and CD4 T-cell concentration. As illustration of the effect of confounding gestational age, cord blood cell composition
and antenatal steroid treatment we compared three models as follows. When only considering antenatal steroid application in this cohort, we found 127 CpGs that show significant differences. On the other hand, only 1 CpG show significant differences due to antenatal steroid application when controlling for gestational age and CD4 T-cell concentration (logFC=0.45, t-statistic=5.71, adj P-value=0.008).

```r
> anno <- getAnnotation(steroid_mset)

Loading required package: IlluminaHumanMethylation27kanno.ilmn12.hg18

> anno1 <- anno[rownames(topTab1),]
> anno0 <- anno[rownames(topTab0),]
> beta <- getBeta(steroid_mset)
> y0 <- beta[rownames(anno0)[1],]
> y1 <- beta[rownames(anno1)[1],]
> print(anno1)

DataFrame with 1 row and 34 columns

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<th>strand</th>
<th>Name</th>
</tr>
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<td>95452163</td>
<td>* cg09068993</td>
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<td>AddressB</td>
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| CACACCCCTTTCCCTTTCCCTATATACCAAATTACCCCCAATACCA |
| ProbeSeqB |
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| cg09068993 |
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|
Type | NextBase | Color |
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| cg09068993 | TypeI | T Red |
|
Islands_Name | Relation_to_Island | Ploidy |
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| cg09068993 | 10:95451797-95452738 | Island diploid |
|
Species | Source | SourceVersion | SourceStrand |
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| Homo sapiens | NCBi:RefSeq | 36.1 BOT |
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SourceSeq |
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| TopGenomicSeq |
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TSS_Coordinate | Gene_Strand | Gene_ID | Symbol |
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| cg09068993 | 95452319 | GeneID:118924 | C10orf4 |
| Synonym | Accession | GID |
| <character> | <character> | <character> |
| cg09068993 | FRA10AC1; F26C11.1-like; | NM_203440.2 GI:50408561 |
|
Product | Distance_to_TSS |
| <character> | <character> |
| cg09068993 | isoform FRA10AC1-3.1 is encoded by transcript variant FRA10AC1-3.1; rare folic acid-type fragile site FRA(10)(q23.3); candidate gene 1; fragile site 10q23.3; go_component: nucleus; go_function: protein binding |
|
MIR_CPG_ISLAND | MIR_NAMES | X |
| <character> | <character> | <character> |
| cg09068993 | FRA10AC1 protein isoform FRA10AC1-3.1 |
| X.1 |
| <integer> |
| cg09068993 | NA |
```

18
> steroid_cpg <- rownames(annol)[1]

> plot(tmpPd$GEAA, y0, col=tmpPd$steroid, main="not controlling", pch=19, cex=1+tmpPd$CD4T/.3)

> plot(tmpPd$GEAA, y1, col=tmpPd$steroid, main="CpG in FRA10AC1 (chr10:95452163)", pch=19, cex=1+tmpPd$CD4T/.3)

> legend("topleft", pch=19, col=2:1, legend=c("Steroid Applied", "Steroid not applied"), pt.cex=1, cex=.6)
This CpG (cg09068993) showing increased DNAm associated with antenatal steroid application is located (chr10:95452163) in the promoter region of isoform gene FRA10AC1 within the rare FRA10A folate-sensitive fragile site [PMID:15203205,6638066]. Hypermethylation in this region serves to counteract folate-sensitive fragility. We observed that subjects to which antenatal steroids were applied had consistently higher methylation at similar gestational ages, and a general increase in methylation due to increased gestational age (Figure 2).

```r
> discrete_geaa <- cut(tmpPd$GEAA,breaks=5)
> fac <- factor(paste0(discrete_geaa,":",tmpPd$steroid))
> library(stringr)
> matches <- str_match(levels(fac), "\((.*),(.*):(.*))"
> at <- apply(matches[,2:3],1,median)
> col <- ifelse(matches[,4]==0,"black","red")
> pch <- ifelse(tmpPd$SEX==0,1,19)
> print(table(tmpPd$SEX))

0 1
29 31

> plot(tmpPd$GEAA, y1, col=tmpPd$steroid, main="CpG in FRA10AC1 (chr10:95452163)", pch=pch,ylab="Methylation Pct.",xlab="Gestational Age (wks)"
> boxplot(y1~fac, main="CpG in FRA10AC1 (chr10:95452163)",ylab="Methylation Pct.", xlab="Gestational Age (wks)"
> points(tmpPd$GEAA,y1,pch=pch,col=tmpPd$steroid,cex=1.2+tmpPd$CD4T/.6)
> legend("topleft", pch=19, col=2:1, legend=c("Steroid applied", "Steroid not applied"),pt.cex=1,
> legend("bottomright", pch=c(1,19), legend=c("female", "male"), cex=0.9)
```
The distribution of methylation in this data is

```
> range(beta)
[1] 0.005425549 0.990438031
```

```
> sessionInfo()
R version 3.3.1 (2016-06-21)
Platform: x86_64-apple-darwin13.4.0 (64-bit)
Running under: OS X 10.11.4 (El Capitan)
locale:

attached base packages:
[1] stats4 parallel stats graphics grDevices utils
[7] datasets methods base

other attached packages:
[1] stringr_1.0.0
[2] illuminaHumanMethylation27kanno.ilmn12.hg18_0.0.99
[3] quadprog_1.5-5
[4] gplots_3.0.1
[5] limma_3.28.15
[6] minfi_1.18.2
[7] bumphunter_1.12.0
[8] locfit_1.5-9.1
[9] iterators_1.0.8
[10] foreach_1.4.3
[12] XVector_0.12.0
[13] SummarizedExperiment_1.2.3
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GenomeInfoDb_1.8.3
IRanges_2.6.1
S4Vectors_0.10.2
lattice_0.20-33
Biobase_2.32.0
BiocGenerics_0.18.0
knitr_1.13
hcbpack_0.1.0
RCcolorBrewer_1.1-2
BiocInstaller_1.22.3

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mclust_5.2
Rcpp_0.12.6
Rsamtools_1.24.0
R6_2.1.2
chron_2.3-47
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data.table_1.9.6
Matrix_1.2-6
splines_3.3.1
RCurl_1.95-4.8
rtracklayer_1.32.1
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GEOquery_2.38.4
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reshape_0.8.5
MASS_7.3-45
grid_3.3.1
xtable_1.8-2
DBI_0.4-1
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stringi_1.1.1
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illumininao_0.14.0
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plyr_1.8.4
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nlme_3.1-128
registry_0.3
magrittr_1.5
KernSmooth_2.23-15
genefilter_1.54.2
nor1mix_1.2-1
tools_3.3.1
rngtools_1.2.4
AnnotationDbi_1.34.4
beanplot_1.2