Targeted reduction of highly abundant transcripts with *pseudo-random* primers

Ophélie Arnaud¹, Sachi Kato¹, Stéphane Poulain¹, Charles Plessy¹

1. RIKEN Center for Life Science Technologies, Division of Genomic Technologies, Yokohama, Kanagawa, 230-0045 Japan

Corresponding authors: Charles Plessy: RIKEN Center for Life Science Technologies, Division of Genomic Technologies, Yokohama, Kanagawa, 230-0045 Japan;

plessy@riken.jp

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Abstract

Transcriptome studies based on quantitative sequencing estimate gene expression levels by measuring the abundance of target RNAs in libraries of sequence reads. The sequencing cost is proportional to the total number of sequenced reads. Therefore, in order to cover rare RNAs, considerable quantities of abundant and identical reads have to be sequenced. This major limitation can be lifted by strategies used to deplete the library from some of the most abundant sequences. However, these strategies involve either an extra handling of the input RNA sample, or the use of a large number of reverse-transcription primers (termed “not-so-random primers”), which are costly to synthesize and customize. Here, we demonstrate that with a precise selection of only 40 “pseudo-random” reverse-transcription primers, it is possible to decrease the rate of undesirable abundant sequences within a library without affecting the transcriptome diversity. “Pseudo-random” primers are simple to design, and therefore are a flexible tool for enriching transcriptome libraries in rare transcripts sequences.

Methods summary

The precise selection and the use of pseudo-random primers allows for reducing the detection of undesirable sequences within libraries and so increase the effective depth of the sequencing. Our study also concludes that, instead of the 4096 random primers currently used, only 40 pseudo-random primers are enough.

Introduction

In transcriptome studies using quantitative sequencing, highly abundant sequences within a library limit the coverage and increase the difficulty to detect transcripts of
interest. For example, ribosomal RNAs (rRNA) can represent the majority of a sequence library, which means that most of the money spent on sequencing would be for reads that are irrelevant in the downstream analysis. For this reason, transcriptome analysis methods often include a step for removing rRNA.

At present, several methods exist to deplete rRNA, for example, by priming the cDNAs or enriching the mRNAs with poly-T oligonucleotides, by capturing and removing the rRNAs with hybridization probes and magnetic beads (Ribo-Zero kit) (1) or antibodies directed against DNA:RNA hybrids (GeneReadrRNA depletion kit) (2), by capturing first-strand cDNAs synthesized from capped transcripts (CAP Trapper) (3), by selectively degrading the 5′-phosphate RNAs ("Terminator" enzyme) (Epicentre), or by biasing the reverse-transcription primers against the rRNA sequences (4).

In this last method, termed "not-so-random primers" (NSR), the cDNAs are primed with a mixture of the 749 out of 4096 random hexamers that do not have a direct match with the human ribosomal RNAs, leading to a reduction of these sequences from 78% to 13% (4). The major drawback of this method is that the pool of primers is prepared by synthesizing each primer individually, which makes customization costly when adding a linker tail or changing the target for depletion (for instance hemoglobin) (5).

Here, we present a dramatic simplification of the not-so-random primers concept, which we term "pseudo-random primers" (PS). Following the initial observation of Mizuno et al. (1999) that the reverse-transcriptase tolerates even two mismatches at the priming site (6), we reasoned that a large number of not-so-random primer sequences are functionally redundant and that it would be possible to dramatically reduce their number, thus facilitating the development and testing of custom sets.
Materials and methods

Selection of PS primers

The 40 PS primers were selected to bind neither to the human rRNA nor to the linker sequence of the template-switching oligonucleotide used in our experiments (See supplemental information 1).

The 40 primers were individually synthetized (Invitrogen) with standard desalting purification grade, resuspended at 100 µM in ultra-pure water and mixed equimolarly.

Selection of PS_Hb primers

The 33 PS_Hb primers were selected as described in supplemental material 1, by discarding hexamers sequences targeting human α-globin RNA and human β-globin RNA.

Library preparation

NanoCAGE libraries were prepared according to Salimullah et al., 2011 using 50 ng of total RNA extracted from HeLa and THP-1 cells lines (7). Technical triplicates of each nanoCAGE library were prepared from each RNA sample. Four libraries were made, to compare 1) Random hexamers (RanN6) versus PS primers, 2) RanN6, PS and 40 randomly picked RanN6 (40N6) primers, 3) RanN6, PS, 3 subsets of 20 PS and 1 subset of 10 PS primers, and 4) RanN6 versus PS_Hb primers. Thus, differences between RanN6 and PS primers, depleting rRNA and artifacts, were replicated in three independent experiments. Details of each nanoCAGE library are available in supplementary table 1.
Data processing and analysis

The prepared libraries were individually paired-end sequenced on a MiSeq sequencer (Illumina) using the standard nanoCAGE sequencing primers (7). The sequencing data were analyzed using the workflow manager Moirai (8). Briefly, the reads were demultiplexed and trimmed to 15 bases with FASTX-Toolkit (http://hannonlab.cshl.edu/fastx_toolkit/). Then, the reads coming from rRNA or oligo-artifacts were removed with TagDust (version 1.13) (9) and the remaining reads were aligned to the human genome (hg19) with BWA (version 0.7) (10). Then, the non-proper paired reads and the PCR duplicates were filtered out with samtools (version 0.1.19) (11). Finally, the properly paired reads were clustered and analyzed as in Harbers et al., 2013 (12) (the scripts used for the analysis are provided in supplemental materials 2).

Results and discussion

We tested the pseudo-random primers concept using the nanoCAGE method for transcriptome profiling (13). In this method, 5' adapters are introduced by template-switching oligonucleotides during the reverse transcription, where random primers are used to cover the non-polyadenylated transcriptome. Thus, the undesirable sequences in nanoCAGE libraries come mostly from 2 sources: the ribosomal RNA and primers-primers artifacts. The rate of these undesired sequences becomes especially problematic when the quantity of starting material is lower than a nanogram. We therefore designed pseudo-random primers to reduce rRNA and primer-primers artifacts at the same time. Using scripts written in the R language (see Supplemental Information 1), we identified 40 hexamers that do not fully match with the human rRNA reference sequences, and do not show similarities up to 2 mismatches with the
nanoCAGE linker sequence. We prepared a mixture of 40 reverse-transcription primers containing these hexamers (PS), to replace the standard reverse-transcription random primers (RanN6).

We tested the PS primers on three sets of triplicated libraries prepared from HeLa and THP-1 cell line total RNA. Using nanoCAGE libraries prepared with RanN6 primers as a control (Figure S1), we observed a significant decrease in reads matching to ribosomal RNA (Fig 1A). Primer artifacts were also reduced (Figure 1B), but the difference was only statistically significant for the THP-1 libraries: for one HeLa set of triplicates, there was no diminution, but the overall amount of artifacts was uniformly low, making it difficult to see any effect of the PS primers. To exclude the possibility that the observed effect of the PS primers comes only from the reduction of the hexamer diversity regardless of our selection, we included a control using 40 randomly picked hexamers (40N6). These libraries did not significantly deplete rRNA reads, but had an impact on primer artifacts. We explain this effect by the fact that only a few hexamers match to the linker sequences of the nanoCAGE primers, and therefore the 40N6 set was depleted by chance. Indeed, only 32% of them match the linkers with no or 1 mismatch (Figure S2). This confirms the efficiency of our precise selection of the PS primers to decrease the detection of the undesired sequences within nanoCAGE libraries.

We then verified that the two-fold reduction of the number of different hexamers did not impair genes detection. After normalizing the libraries to the same number of aligned reads (supplemental material 2), we detected between 3348 and 4235 genes per replicate (supplemental table 1). Not only the number of genes detected was not reduced with the use of only 40 primers, but also we detected significantly more
genes with the PS primers than with the RanN6 primers, in both cell lines tested (Figure 2A). One simple explanation could be that PS primers that don’t bind to the ribosomal RNA are free to bind transcripts of interest, which would increase the likelihood of less abundant RNAs reverse-transcription. This is corroborated by the observation that libraries using the 40N6 primers, not selected against rRNA, do not allow for higher gene detection rate in comparison with the RanN6 primers. Importantly, because we normalized the number of aligned reads after filtering out the ones aligning on the rRNA, the effect of the PS primers can not be explained by the higher coverage at an equal number of raw reads. Altogether, our results show that the libraries prepared with PS primers cover more genes than the libraries prepared with RanN6 primers.

To investigate the reliability of the expression values measured in PS-primed libraries, we compared our experiments pairwise after averaging the triplicates (supplemental material 2). Samples prepared from the same RNAs correlated better than samples prepared with the same RT primers set, but the correlation coefficients still suggested important differences induced by the change of primers (Fig 2B). Indeed, inspection of the pairwise plots shows that the most highly expressed genes deviate strongly from the diagonal when comparing the PS and RanN6 primers on the same RNA (Fig 2B). Given that the PS primers are strongly selected, this was expectable, and we reasoned that the bias should be systematic. To demonstrate that fact, we compared the fold change of expression levels between HeLa and THP-1 RNA in each set of primers, and showed that they were conserved (Fig 2C). Thus, libraries made with PS primers can be compared with libraries made with other RT primers by looking at fold changes with a common reference, like in transcriptome platform comparisons (14).
According to the good transcriptome coverage obtained with only 40 PS primers, we next wondered how many PS primers are required to conserve the same transcriptome diversity? The original number of 40 was set empirically from the matches on rRNA and nanoCAGE linkers, but the lower limit is unknown. We therefore prepared libraries with subsets of 20 or 10 PS primers (supplemental table 2). A similar number of genes (around 4000 genes per sample, supplemental table 1) could be detected in the libraries. We also observed a systematic bias in these libraries, but because they were made with subsets of the original PS primers, they all had a stronger similarity with each other than with RanN6 libraries (Fig 3). Thus, it appears possible to prepare whole-transcriptome libraries with as few as 10 pseudo-random primers.

Finally, we sought to demonstrate that the PS primers concept could be applied on other targets than the rRNAs. In total RNA extracted from blood, up to 60% of the transcripts come from hemoglobin genes, (15). Hence, we have selected 33 PS primers (PS_Hb) that did not match on hemoglobin sequences (with 2 or more mismatches) (supplemental material 1) and prepared nanoCAGE libraries with either these primers or standard RanN6 primers. The selection drastically reduced the number of tag per hemoglobin genes (Fig 4A), without reducing the number of detected genes (Fig 4B), thus demonstrating the possibility of designing PS primers against other targets.

In conclusion, despite several methods already exists to eliminate the sequences coming from ribosomal RNA in transcriptome studies, lots of them require an extra step in the protocol. Moreover, none of them is also able to eliminate, in a single step, multiple unrelated undesirable sequences. Here, we report that in transcriptome studies a drastic selection of the primers used during the reverse transcription is
effective for eliminating specific sequences without reducing gene coverage. Moreover, our data supports the idea that the number of PS primers required is low, leading to a real cost-saving effect in the experiments. Finally, while tested here with the nanoCAGE protocol, this strategy is not limited to it and should be applicable to any kind of transcriptome studies.
Authors contributions

CP conceived the project; OA, SK and CP designed the experiments; OA, SK and SP performed the experiments; OA and CP analyzed the data, OA and CP wrote the manuscript. All authors read and approved the final manuscript.

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Competing Interests statement

The authors declare no competing financial interests.

References


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Figures

Figure 1: Depletion of ribosomal sequences and artifacts

Rate of ribosomal RNA (A) and artifacts (B) detected with the 40N6, PS or RanN6 primers sets. Each point corresponds to the mean of 3 technical replicates in the same experiment. Statistical test: t.test paired between the mean of PS and RanN6 data sets, non-paired with the raw value of 40N6 data set. * P-value<0.05; ** p-value<0.01; *** p-value<0.001.

Figure 2: Coverage of transcriptome diversity

A. Percentage of genes detected with the 40N6 and PS primers compared to the RanN6 primers, set arbitrarily to 100 % in HeLa and THP-1 respectively. Each point corresponds to the mean of 3 technical replicates in the same experiment. The data were normalized by sub-sampling to 8700 tags per sample. Statistical test: t.test paired between the mean of RanN6 and PS data sets, non-paired with the raw value of 40N6 data set. * P-value<0.05; ** p-value<0.01; *** p-value<0.001

B. Pairwise comparison between the PS and RanN6 libraries from the 2 cell lines. Each plot is the mean of 3 experiments with 3 technical replicates. Upper part:
expression plots where the reads are aligned to the reference gene model. Lower part:

Pearson correlation of each pair.

C. HeLa-THP1 fold change in gene expression.

**Figure 3: Transcriptome coverage with small number of primers**

Hierarchical clustering of the detected genes (after normalization to 8700 tags per sample). The red value is the Approximately Unbiased (AU) p-value and the green value is the Bootstrap Probability (BP) value. The red box represent the cluster significantly established (AU p-value<0.05). All the samples were prepared in the same experiment (library NC_17).

**Figure 4: Targeted depletion of hemoglobin sequences**

A. Measured expression levels (in counts per million) of hemoglobin genes with the PS_Hb and the RanN6 primers. Each bar represents a technical replicates of one experiment.

B. Number of genes detected with the use of PS_Hb versus RanN6 primers. Each point corresponds to a technical replicate of the same experiment. The data were normalized to 3190 tags per sample.

**Supplemental material:**

- Supplemental material 1: scripts and programs used for the primers selection

- Supplemental material 2: Scripts and programs used in the data analysis:
Link 1: general commands creating the files used in downstream analysis
Link 2: analysis of the first experiment, NCms10058
Link 3: analysis of the second experiment, NC12
Link 4: analysis of the third experiment, NC17
Link 5: common analysis of the three experiments
Link 6: Statistical analysis
Link 7: analysis of the fourth experiment regarding the RNA extracted from blood, NC22

• Supplemental material 3: Figure S2: Reads genomic features
  Percentage of reads aligned to each feature of the genome (promoter, exon, intron, intergenique section, rDNA) and the artifacts. Each row is the average of the technical triplicates of the same library.

• Supplemental material 4: Figure S1: Maximal distance of the 40N6 primers with the template-switching primer
  Number of mismatches between the hexamers of the 40N6 primers and the template switching primers.

• Supplemental material 5: Table S1: Summary table
  Extensive summary for each sample tested. It includes the experiment name, the origin of the RNA, the barcode and index added, the primer set used and the sequencing results.

• Supplemental material 6: Table S2: sequences of the 20 PS and 10
PS primers sets.

Pseudo-random primers sequences composing the different sets of pseudo-random tested.
pseudo-random primers

2B

HeLa_RanN6

0.85

HeLa_pseudoRan

0.76  0.70

THP1_RanN6

0.70  0.76  0.86

THP1_pseudoRan
Cluster dendrogram with AU/BP values (%) 

Distance: correlation  
Cluster method: average
Selection of Pseudo-random primers

rRNA

Human:

hsu13369.fasta file produced with the command extractfeat -type rRNA U13369.gb. (from the EMBOSS package)

<table>
<thead>
<tr>
<th>Sequence</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>HSU13369_3657_5527</td>
<td>[rRNA] Human ribosomal DNA complete repeating unit.</td>
</tr>
<tr>
<td>HSU13369_6623_6779</td>
<td>[rRNA] Human ribosomal DNA complete repeating unit.</td>
</tr>
<tr>
<td>HSU13369_7935_12969</td>
<td>[rRNA] Human ribosomal DNA complete repeating unit.</td>
</tr>
</tbody>
</table>

Mitochondrial

<table>
<thead>
<tr>
<th>Sequence</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>NC_012920_648_1601</td>
<td>[rRNA] Homo sapiens mitochondrion, complete genome.</td>
</tr>
<tr>
<td>NC_012920_1671_3229</td>
<td>[rRNA] Homo sapiens mitochondrion, complete genome.</td>
</tr>
</tbody>
</table>

Combination

```
(cat nc_012920.fasta hsu13369.fasta | revseq -filter | grep -v '>' | perl -pe chomp ; echo) > ribo.txt
```

R code
acgt <- c('A', 'C', 'G', 'T')
LINKER <- 'CCCTATAAGATCGGAAGAGCGGTTCGGAGACCTTCAGTTCGACTA'
BARCODES <- scan('barcodes.txt', what='character')
RIBO <- scan('ribo.txt', what='character')  # See below in the wiki about the file 'ribo.txt'.

hexamers <- apply(expand.grid(acgt, acgt, acgt, acgt, acgt, acgt), 1, paste, collapse='')
hexamers <- data.frame(row.names=hexamers)

hexamers[,c('LINKER_0', 'LINKER_1', 'LINKER_2', 'LINKER_3', 'RIBO_0', 'RIBO_1', 'BARCODE')] <- c(rep(FALSE, 7))
hexamers[names(unlist(sapply(rownames(hexamers), function(X) {agrep(X, LINKER, 0, ignore.case=T)}))), "LINKER_0"] <- TRUE
hexamers[names(unlist(sapply(rownames(hexamers), function(X) {agrep(X, LINKER, 1, ignore.case=T)}))), "LINKER_1"] <- TRUE
hexamers[names(unlist(sapply(rownames(hexamers), function(X) {agrep(X, LINKER, 2, ignore.case=T)}))), "LINKER_2"] <- TRUE
hexamers[names(unlist(sapply(rownames(hexamers), function(X) {agrep(X, LINKER, 3, ignore.case=T)}))), "LINKER_3"] <- TRUE
hexamers[names(unlist(sapply(rownames(hexamers), function(X) {agrep(X, RIBO, 0, ignore.case=T)}))), "RIBO_0"] <- TRUE
hexamers[names(unlist(sapply(rownames(hexamers), function(X) {agrep(X, RIBO, 1, ignore.case=T)}))), "RIBO_1"] <- TRUE
hexamers[BARCODES, "BARCODE"] <- TRUE

summary(hexamers)
LINKER_0       LINKER_1       LINKER_2       LINKER_3         RIBO_0
RIBO_1         BARCODE
Mode :logical   Mode :logical   Mode :logical   Mode :logical   Mode :logical
FALSE:4056      FALSE:3082      FALSE:259       TRUE:4096      FALSE:719
TRUE:4096      FALSE:4000      TRUE:3837      NA's:0         TRUE:3377
NA's:0         TRUE :96        TRUE :1014      TRUE :3837      NA's:0         TRUE :3377
NA's:0

with(hexamers, rownames(hexamers)[! (LINKER_2 | RIBO_0 | BARCODE)])

Selection PS_Hb

Haemoglobin sequences

(wh...

The 2 fasta files are combined in 1 file named Hb.txt

R Code

```r
acgt <- c('A', 'C', 'G', 'T')
Hb <- scan('Hb.txt', what='character')
hexamers <- apply(expand.grid(acgt, acgt, acgt, acgt, acgt, acgt), 1, paste,
collapse='')
hexamers <- data.frame(row.names=hexamers)
hexamers[,c('Hb_0', 'Hb_1', 'Hb_2')] <- c(rep(FALSE,3 ))
hexamers[names(unlist(sapply(rownames(hexamers), function(X) {agrep(X, Hb, 0, ignore.case=T)})))], "Hb_0"] <- TRUE
hexamers[names(unlist(sapply(rownames(hexamers), function(X) {agrep(X, Hb, 1, ignore.case=T)})))], "Hb_1"] <- TRUE
hexamers[names(unlist(sapply(rownames(hexamers), function(X) {agrep(X, Hb, 2, ignore.case=T)})))], "Hb_2"] <- TRUE

summary(hexamers)

     Hb_0     Hb_1     Hb_2
Mode:logical Mode:logical Mode:logical
FALSE:3154 FALSE:33   TRUE:4096
TRUE :942   TRUE :4063 NA's:0
NA's:0

with(hexamers, rownames(hexamers)[! (Hb_1)])

[1] "GTAAA" "CGCACA" "GGATAA" "GTATAA" "CTACGA" "TATCGA" "CGAATA" "GATATA"
[9] "CTATTA" "GTACTA" "TACCTA" "ATCGTA" "CTCGTA" "TCGTTA" "TAAAAC" "TACAAC"
[17] "ATTATC" "AAACC" "TAATGC" "AAGTGC" "CTACAT" "ATTAGG" "GATCTG" "GATTCG"
[25] "TACGAT" "ATCGAT" "ATCTAT" "TCGTAT" "CTAATT" "TCCATT" "CCGATT" "TGCATT"
[33] "CGATTT"
```

Selection of 40N6 primers

R code

```r
acgt <- c('A', 'C', 'G', 'T')
hexamers <- apply(expand.grid(acgt, acgt, acgt, acgt, acgt, acgt), 1, paste,
collapse='')
sample(hexamers,40)

[1] "CCGTC" "CCCTTC" "TTTTTT" "CTTGTA" "TGACCG" "TGTGAT" "AACCTT" "AGGCCG"
[9] "TGCTT" "CTAACA" "GTACCC" "CAGAGG" "GTGTCT" "GTGTTG" "AAGACT" "CGGCGG"
[17] "AAGAGA" "GAGGCT" "GCTCTT" "GGTCTG" "GCAGCT" "TGAGCT" "GGGGCG" "GAGAGG"
[25] "CTCTCG" "TAAGTT" "ATCTGC" "ACTTAA" "CACAGC" "AGATGA" "GGTAGC" "AAGGCC"
[33] "CGCAGG" "AAACCT" "CAGTTG" "ATTCCC" "AGATGG" "GGCGGC" "CTGCGG" "CTTCAC"
```
Common analysis for all the experiments

configuration

Use appropriate names instead of xxx (see detailed commands for each experiment)

```r
library(plyr)

exportInEnv <- function(X) {
  Name <- X
  Value <- get(X)
  .Internal(Sys.setenv(Name, Value))
  cat( paste0("export ", paste(Name, Value, sep='='), "\n"))
}

LIBRARY <- 'xxx'
MOIRAI_USER <- 'xxx'
MOIRAI_PROJECT <- 'xxx'
GROUP_SHARED <- 'xxx'
WORKDIR <- '.'
GENE_SYMBOLS <- paste(GROUP_SHARED, 'annotation/homo_sapiens/gencode-14/gencode.v14.annotation.genes.bed', sep='/')
ANNOTATION <- paste(GROUP_SHARED, 'annotation/homo_sapiens/100712hg19/100712hg19', sep='/')
PROCESSED_DATA <- dirname( system( paste( 'ls -d /osc-fs_home/scratch/moirai/'
                                      , MOIRAI_USER
                                      , '/project/
                                      , MOIRAI_PROJECT
                                      , '/
                                      , LIBRARY
                                      , '*/Moirai.config'
                                      , sep=''
                                      , intern=TRUE)[1] ))

lply( c("LIBRARY", "MOIRAI_USER", "MOIRAI_PROJECT", "GROUP_SHARED",
        "WORKDIR", "GENE_SYMBOLS", "ANNOTATION",
        "PROCESSED_DATA")
     , exportInEnv )
```

Cluster with the PromoterPipeline

Level 1

Transform the paired-end alignments into level 1 clusters, sort the file and index it. Select
only BAM files that contain aligned reads.

```bash
ALIGNED_DATA=$(for BAM in $PROCESSED_DATA/properly_paired_rmdup/*bam; do samtools flagstat $BAM | grep -Lq '^0 + 0 mapped' || echo $BAM ; done)

level1.py --help | head -n1

level1.py -o /dev/stdout -f 66 -F 516 $ALIGNED_DATA |
  bgzip > $LIBRARY.l1.gz

cat <(zgrep \# -A1 $LIBRARY.l1.gz) <(zgrep -v \#
  $LIBRARY.l1.gz | sed '1d' |
  sort --field-separator '$t' -k2.4,2n -k 2.4,2.4 -k3,3n
  -k4,4n -k5,5) |
  bgzip |
  sponge $LIBRARY.l1.gz
#tabix -s2 -b3 -e4 $LIBRARY.l1.gz
```

**Level 2**

Same for level 2 clusters.

Needs a version of level2.py that is more recent than 20120628, where the “Output” message is sent to stderr.

```bash
level2.py --help | head -n1

level2.py -o /dev/stdout -t 0 $LIBRARY.l1.gz |
  bgzip > $LIBRARY.l2.gz

cat <(zgrep \# -A1 $LIBRARY.l2.gz) <(zgrep -v \#
  $LIBRARY.l2.gz | sed '1d' |
  sort --field-separator '$t' -k2.4,2n -k 2.4,2.4 -k3,3n
  -k4,4n -k5,5) |
  bgzip |
  sponge $LIBRARY.l2.gz
#tabix -s2 -b3 -e4 $LIBRARY.l2.gz
```

**Intersections**

Convert level 1 and 2 files to BED format, and intersect them with pre-defined annotation files.
function osc2bed {
    zcat $1 |
    grep -v \# |
    sed 1d |
    awk '{OFS="\t"}{print $2, $3, $4, "l1", "1000", $5}'}
}

function bed2annot {
    bedtools intersect -a $1 -b $ANNOTATION.annot -s -loj |
    awk '{OFS="\t"}{print $1":"$2"-"$3$6,$10}'} |
    bedtools groupby -g 1 -c 2 -o collapse
}

for LEVEL in l1 l2
do
    osc2bed $LIBRARY.$LEVEL.gz | tee $LIBRARY.$LEVEL.bed |
    bed2annot - > $LIBRARY.$LEVEL.annot
done

Gene symbols

function bed2symbols {
    bedtools intersect -a $1 -b $GENE_SYMBOLS -s -loj |
    awk '{OFS="\t"}{print $1":"$2"-"$3$6,$10}'} |
    bedtools groupby -g 1 -c 2 -o distinct >
    $LIBRARY.l2.genes
}

if [ $GENE_SYMBOLS ]
then
    bed2symbols $LIBRARY.l2.bed > $LIBRARY.l2.genes
fi
Analyze of the first experiment: NCms10058

Configuration

```r
library(plyr)

exportInEnv <- function(X) {
  Name <- X
  Value <- get(X)
  .Internal(Sys.setenv(Name, Value))
  cat(paste0("export ", paste(Name, Value, sep='='), "\n"))
}

LIBRARY <- 'NCms10058_1'
MOIRAI_USER <- 'nanoCAGE2'
MOIRAI_PROJECT <- 'Arnaud'
GROUP_SHARED <- '/osc-fs_home/scratch/gmtu'
WORKDIR <- '.
GENE_SYMBOLS <- paste(GROUP_SHARED, 'annotation/homo_sapiens/gencode-14/gencode.v14.annotation.genes.bed', sep='/')
ANNOTATION <- paste(GROUP_SHARED, 'annotation/homo_sapiens/100712hg19/100712hg19', sep='/')
PROCESSED_DATA <- dirname(system(paste('ls -d /osc-fs_home/scratch/moirai/
',
  , MOIRAI_USER
  , '/project/
  , MOIRAI_PROJECT
  , '/
  , LIBRARY
  , '*/Moirai.config'
  , sep='')
  , intern=TRUE)[1])

l_ply( c("LIBRARY", "MOIRAI_USER", "MOIRAI_PROJECT", "GROUP_SHARED"
  , "WORKDIR", "GENE_SYMBOLS", "ANNOTATION", "PROCESSED_DATA")
  , exportInEnv )
```

Count the reads

```bash
awk '/raw/ {print $3}' $PROCESSED_DATA/text/summary.txt |
/usr/lib/filo/stats |
grep 'Sum' |
cut -f2 -d':' |
tr -d '[:space:]' |
xargs -0 /usr/bin/printf " # %'d
"
```

```text
##  # 3608777
```

```bash
grep raw $PROCESSED_DATA/text/summary.txt
```

```text
## NCms10058_1.ACAGTG.R1  raw 95519
## NCms10058_1.ACTTGA.R1  raw 76278
## NCms10058_1.ATCACG.R1  raw 53374
## NCms10058_1.CAGATC.R1  raw 103408
## NCms10058_1.CGATGT.R1  raw 73164
## NCms10058_1.CTTGTA.R1  raw 134779
## NCms10058_1.GATCAG.R1  raw 95648
## NCms10058_1.GCCAAT.R1  raw 76012
## NCms10058_1.GGCTAC.R1  raw 56348
## NCms10058_1.TAGCTT.R1  raw 54492
## NCms10058_1.TGACCA.R1  raw 63262
## NCms10058_1.TTAGGC.R1  raw 95230
## NCms10058_1.Undetermined.R1  raw 2631263
```

Analysis with R

Configuration
library(oscR) # See https://github.com/charles-plessy/oscR for oscR.
if (compareVersion(sessionInfo()$otherPkgs$oscr$Version,'0.1.1') < 0) stop('Outdated version of oscR.')

library(smallCAGEqc) # See https://github.com/charles-plessy/smallCAGEqc for smallCAGEqc.
if (compareVersion(sessionInfo()$otherPkgs$smallCAGEqc$Version,'0.6.0') < 0)
  stop('Outdated version of smallCAGEqc')

library(vegan)

## Loading required package: permute
## Loading required package: lattice
## This is vegan 2.0-10

library(ggplot2)

Load data

l2_NCki <- read.osc(paste(LIBRARY,'l2','gz',sep='.'), drop.coord=T, drop.norm =T)
colnames(l2_NCki) <- sub('raw.NCms10058_1.','NCki_',colnames(l2_NCki))
colSums(l2_NCki)

##    NCki_HeLa_PS_A    NCki_HeLa_PS_B    NCki_HeLa_PS_C    NCki_HeLa_RanN6_A    NCki_HeLa_RanN6_B    NCki_HeLa_RanN6_C
##             11800             13969             22764             14137             13556             10430
##    NCki_THP1_PS_A    NCki_THP1_PS_B    NCki_THP1_PS_C    NCki_THP1_RanN6_A    NCki_THP1_RanN6_B    NCki_THP1_RanN6_C
##             15157             15453             13092              8708             14536             17122

Normalization number of read per sample : l2.sub ; libs$genes.sub

In all the 3 libraries used, one contain only few reads tags. The smallest one has 8,708 counts. In order to make meaningful comparisons, all of them are subsapled to 8700 counts.

l2.sub1 <- t(rrarefy(t(l2_NCki),min(8700)))
colSums(l2.sub1)
Moirai statistics

Load the QC data produced by the Moirai workflow with which the libraries were processed. Sort in the same way as the l1 and l2 tables, to allow for easy addition of columns.

```
libs <- loadLogs('moirai')
```

Number of clusters

Count the number of unique L2 clusters per libraries after subsampling, and add this to the QC table. Each subsampling will give a different result, but the mean result can be calculated by using the `rarefy` function at the same scale as the subsampling.

```
libs['l2.sub1'] <- colSums(l2.sub1 > 0)
libs['l2.sub1.exp'] <- rarefy(t(l2_NCki), min(colSums(l2_NCki)))
```

Richness

Richness should also be calculated on the whole data.

```
libs['r100.l2'] <- rarefy(t(l2_NCki),100)
boxplot(data=libs, r100.l2 ~ group, ylim=c(92,100), las=1)
```
Hierarchical annotation

Differences of sampling will not bias distort the distribution of reads between annotations, so the non-subsampled library is used here.

```r
annot.l2 <- read.table(paste(LIBRARY,'l2','annot',sep='.'), head=F, col.names =c('id', 'feature'), row.names=1)
annot.l2 <- hierarchAnnot(annot.l2)

rownames(libs) <- sub("HeLa", "NCKi_HeLa", rownames(libs))
rownames(libs) <- sub("THP1", "NCKi_THP1", rownames(libs))

libs <- cbind(libs, t(rowsum(l2_NCKi, annot.l2[, 'class'])))
libs$samplename <- sub("HeLa", "NCKi_HeLa", libs$samplename)
libs$samplename <- sub("THP1", "NCKi_THP1", libs$samplename)
```

Gene symbols used normalisation data
genesymbols <- read.table(paste(LIBRARY,'l2','genes',sep='.'), col.names=c("cluster","symbol"), stringsAsFactors=FALSE)
rownames(genesymbols) <- genesymbols$cluster

g2 <- rowsum(l2_NCki, genesymbols$symbol)

countSymbols <- countSymbols(g2)

libs[rownames(l2_NCki),"genes"] <- (countSymbols)

Number of genes detected in sub-sample

12.sub1 <- data.frame(12.sub1)
g2.sub1 <- rowsum(l2.sub1, genesymbols$symbol)
countSymbols.sub1 <- countSymbols(g2.sub1)
libs[rownames(l2.sub1),"genes.sub1"] <- (countSymbols.sub1)

Table record

save the different tables produced for later analysis

write.table(l2_NCki, "l2_NCki_1.txt", sep = "\t", quote=FALSE)
write.table(l2.sub1, "l2.sub1_NCki_1.txt", sep = "\t", quote=FALSE)
write.table(g2.sub1, 'g2.sub1_NCki_1.txt', sep="\t", quote=F)
write.table(libs, 'libs_NCki_1.txt', sep="\t", quote=F)
Analyze of the second experiment: NC12 configuration

```r
library(plyr)

exportInEnv <- function(X) {
  Name <- X
  Value <- get(X)
  .Internal(Sys.setenv(Name, Value))
  cat(paste0("export ", paste(Name, Value, sep='='), "\n"))
}

LIBRARY <- 'NC12_1'
MOIRAI_USER <- 'nanoCAGE2'
MOIRAI_PROJECT <- 'Arnaud'
GROUP_SHARED <- '/osc-fs_home/scratch/gmtu'
WORKDIR <- '.'
GENE_SYMBOLS <- paste(GROUP_SHARED, 'annotation/homo_sapiens/gencode-14/gencode.v14.annotation.genes.bed', sep='/')
ANNOTATION <- paste(GROUP_SHARED, 'annotation/homo_sapiens/100712hg19/100712hg19', sep='/')
PROCESSED_DATA <- dirname(system(paste('ls -d /osc-fs_home/scratch/moirai/','MOIRAI_USER','/project/','MOIRAI_PROJECT','/','LIBRARY','/*Moirai.config', sep='')))

l_ply( c("LIBRARY", "MOIRAI_USER", "MOIRAI_PROJECT", "GROUP_SHARED",
"WORKDIR", "GENE_SYMBOLS", "ANNOTATION", "PROCESSED_DATA")
, exportInEnv )
```
Count the reads

```bash
awk '/raw/ {print $3}' $PROCESSED_DATA/text/summary.txt | /usr/lib/filo/stats | grep 'Sum' | cut -f2 -d':' | tr -d '[:space:]' | xargs -0 /usr/bin/printf " # %'d\n"
```

```
##  # 3450701
```

```bash
grep raw $PROCESSED_DATA/text/summary.txt
```

```
## NC12_1.ACAGTG.R1 raw 340256
## NC12_1.ATCACG.R1 raw 437139
## NC12_1.CGATGT.R1 raw 274252
## NC12_1.GCCAAT.R1 raw 390496
## NC12_1.TGACCA.R1 raw 287340
## NC12_1.TTAGGC.R1 raw 316502
## NC12_1.Undetermined.R1 raw 1404716
```

Analysis with R

Configuration
library(oscR) # See https://github.com/charles-plessy/oscR for oscR.
if (compareVersion(sessionInfo()$otherPkgs$oscR$Version,'0.1.1') < 0) stop('Outdated version of oscR.')</p>

library(smallCAGEqc) # See https://github.com/charles-plessy/smallCAGEqc for smallCAGEqc.
if (compareVersion(sessionInfo()$otherPkgs$smallCAGEqc$Version,'0.6.0') < 0) stop('Outdated version of smallCAGEqc')

library(vegan)

## Loading required package: permute
## Loading required package: lattice
## This is vegan 2.0-10

library(ggplot2)

### Load data

l2_NC12 <- read.osc(paste(LIBRARY,'l2','gz',sep='.'), drop.coord=T, drop.norm =T)

colnames(l2_NC12) <- sub('raw.NC12_1', 'NC12', colnames(l2_NC12))

colSums(l2_NC12)

##             12154             17411             20790             24065 27215             54835
##             10944             35582             23215              9271 15299             15775
##    NC12.THP1_PS_A    NC12.THP1_PS_B    NC12.THP1_PS_C NC12.THP1_RanN6_A NC12.THP1_RanN6_B NC12.THP1_RanN6_C
##             21303             23454             37395             13356 58890             34922

### Normalization number of read per sample: l2.sub ; libs$genes.sub

In all the 3 libraries used, one contain only few reads tags. The smallest one has 8,708 counts. In order to make meaningful comparisons, all of them are subsapled to 8700 counts.
```r
12.sub1 <- t(rarefy(t(l2_NC12), min(8700)))

colSums(12.sub1)
```

```r
##          NC12.HeLa_40N6_A  NC12.HeLa_40N6_B  NC12.HeLa_40N6_C  NC12.HeLa_PS_A  
## 8700          8700          8700          8700
## 8700          8700
##          NC12.HeLa_RanN6_A  NC12.HeLa_RanN6_B  NC12.HeLa_RanN6_C  NC12.THP1_40N6_A  
## 8700          8700          8700          8700
## 8700          8700
##          NC12.THP1_40N6_B  NC12.THP1_40N6_C  NC12.THP1_RanN6_A  NC12.THP1_RanN6_B  
## 8700          8700          8700          8700
## 8700          8700
```

### Moirai statistics

Load the QC data produced by the Moirai workflow with which the libraries were processed. Sort in the same way as the l1 and l2 tables, to allow for easy addition of columns.

```r
libs <- loadLogs('moirai')
rownames(libs) <- sub('HeLa', 'NC12.HeLa', rownames(libs))
rownames(libs) <- sub('THP1', 'NC12.THP1', rownames(libs))
```

### Number of clusters

Count the number of unique L2 clusters per libraries after subsampling, and add this to the QC table. Each subsampling will give a different result, but the mean result can be calculated by using the rarefy function at the same scale as the subsampling.

```r
libs["l2.sub1"] <- colSums(l2.sub1 > 0)
libs["l2.sub1.exp"] <- rarefy(t(l2_NC12), min(colSums(l2_NC12)))
```

### Richness

Richness should also be calculated on the whole data.

```r
libs["r100.l2"] <- rarefy(t(l2_NC12), 100)

boxplot(data=libs, r100.l2 ~ group, ylim=c(92,100), las=1)
```
Hierarchical annotation

Differences of sampling will not bias distort the distribution of reads between annotations, so the non-subsampled library is used here.

```r
annot.l2 <- read.table(paste(LIBRARY,'l2','annot',sep='.'), head=F, col.names =c('id', 'feature'), row.names=1)
annot.l2 <- hierarchAnnot(annot.l2)

libs <- cbind(libs, t(rowsum(l2_NC12, annot.l2[, 'class'])))
libs$samplename <- sub('HeLa', 'NC12_HeLa', libs$samplename)
libs$samplename <- sub('THP1', 'NC12_THP1', libs$samplename)
```

Gene symbols used normalisation data

```r
genesymbols <- read.table(paste(LIBRARY,'l2','genes',sep='.'), col.names=c("cluster","symbol"), stringsAsFactors=FALSE)
rownames(genesymbols) <- genesymbols$cluster

g2 <- rowsum(l2_NC12, genesymbols$symbol)
countSymbols <- countSymbols(g2)

libs[colnames(l2_NC12),"genes"] <- (countSymbols)
```

Number of genes detected in sub-sample
l2.sub1 <- data.frame(l2.sub1)
g2.sub1 <- rowsum(l2.sub1, genesymbols$symbol)
countSymbols.sub1 <- countSymbols(g2.sub1)
libs[ colnames(l2.sub1), "genes.sub1"] <- (countSymbols.sub1)

**Table record**

save the different tables produced for later analysis

```r
write.table(l2_NC12, "l2_NC12_1.txt", sep = "\t", quote=FALSE)
write.table(l2.sub1, "l2.sub1_NC12_1.txt", sep = "\t", quote=FALSE)
write.table(g2.sub1, 'g2.sub1_NC12_1.txt', sep="\t", quote=F)
write.table(libs, 'libs_NC12_1.txt', sep="\t", quote=F)
```
Analyze of the third experiment: NC17

Configuration

```r
library(plyr)

exportInEnv <- function(X) {
  Name <- X
  Value <- get(X)
  .Internal(Sys.setenv(Name, Value))
  cat( paste0("export ", paste(Name, Value, sep='='), "\n"))
}

LIBRARY <- 'NC16-17_1'
MOIRAI_USER <- 'nanoCAGE2'
MOIRAI_PROJECT <- 'Arnaud'
GROUP_SHARED <- '/osc-fs_home/scratch/gmtu'
WORKDIR <- '.'
GENE_SYMBOLS <- paste(GROUP_SHARED, 'annotation/homo_sapiens/gencode-14/gencode.v14.annotation.genes.bed', sep='/')
ANNOTATION <- paste(GROUP_SHARED, 'annotation/homo_sapiens/100712hg19/100712hg19', sep='/')
PROCESSED_DATA <- dirname( system( paste( 'ls -d /osc-fs_home/scratch/moirai/ ',
                                             ', MOIRAI_USER
                                             , '/project/
                                             , MOIRAI_PROJECT
                                             , '/'
                                             , LIBRARY
                                             , '*Moirai.config'
                                             , sep=''
                                             , intern=TRUE)[1])

l_ply( c("LIBRARY", "MOIRAI_USER", "MOIRAI_PROJECT", "GROUP_SHARED"
          , "WORKDIR", "GENE_SYMBOLS", "ANNOTATION", "PROCESSED_DATA"
          , "exportInEnv")
```
export LIBRARY=NC16-17_1
export MOIRAI_USER=nanoCAGE2
export MOIRAI_PROJECT=Arnaud
export GROUP_SHARED=/osc-fs_home/scratch/gmtu
export WORKDIR=.
export GENE_SYMBOLS=/osc-fs_home/scratch/gmtu/annotation/homo_sapiens/gencode-14/gencode.v14.annotation.genes.bed
export ANNOTATION=/osc-fs_home/scratch/gmtu/annotation/homo_sapiens/100712hg19/100712hg19
export PROCESSED_DATA=/osc-fs_home/scratch/moirai/nanoCAGE2/project/Arnaud/NC16-17_1.CAGEscan_short-reads.20150625154740

Count the reads

awk '/raw/ {print $3}' $PROCESSED_DATA/text/summary.txt | 
/usr/lib/filo/stats | 
grep 'Sum' | 
cut -f2 -d': ' | 
tr -d '[[:space:]]' | 
xargs -0 /usr/bin/printf " # %'d\n"

##  # 4821156

grep raw $PROCESSED_DATA/text/summary.txt

## NC16-17_1.ACAGTG.R1 raw 211404
## NC16-17_1.ACCTTG.A1 raw 189074
## NC16-17_1.ATCACG.R1 raw 544817
## NC16-17_1.CAGATC.R1 raw 214188
## NC16-17_1.CGATGT.R1 raw 490410
## NC16-17_1.CTTGTA.R1 raw 308921
## NC16-17_1.GATCAG.R1 raw 167839
## NC16-17_1.GCCAAT.R1 raw 620406
## NC16-17_1.GGCTAC.R1 raw 150422
## NC16-17_1.TAGCTT.R1 raw 200755
## NC16-17_1.TGACCA.R1 raw 386420
## NC16-17_1.TTAGGC.R1 raw 368814
## NC16-17_1.Undetermined.R1 raw 967686

Analysis with R
library(oscR) # See https://github.com/charles-plessy/oscR for oscR.
if (compareVersion(sessionInfo()$otherPkgs$oscR$Version,'0.1.1') < 0) stop('Outdated version of oscR."

library(smallCAGEqc) # See https://github.com/charles-plessy/smallCAGEqc for smallCAGEqc.
if (compareVersion(sessionInfo()$otherPkgs$smallCAGEqc$Version,'0.6.0') < 0) stop('Outdated version of smallCAGEqc')

library(vegan)

## Loading required package: permute
## Loading required package: lattice
## This is vegan 2.0-10

library(ggplot2)
library(pvclust)

Load data

l2_NC17 <- read.osc(paste(LIBRARY,'l2','gz',sep='.'), drop.coord=T, drop.norm =T)

colnames(l2_NC17) <- sub('raw.NC16.17_1.17', 'NC17', colnames(l2_NC17))

colSums(l2_NC17)
<table>
<thead>
<tr>
<th>Sample</th>
<th>Counts</th>
</tr>
</thead>
<tbody>
<tr>
<td>NC17_HeLa_10PS_A</td>
<td>31006</td>
</tr>
<tr>
<td>NC17_HeLa_10PS_B</td>
<td>29327</td>
</tr>
<tr>
<td>NC17_HeLa_10PS_C</td>
<td>34781</td>
</tr>
<tr>
<td>NC17_HeLa_20PS1_A</td>
<td>18858</td>
</tr>
<tr>
<td>NC17_HeLa_20PS1_B</td>
<td>18469</td>
</tr>
<tr>
<td>NC17_HeLa_20PS1_C</td>
<td>23579</td>
</tr>
<tr>
<td>NC17_HeLa_20PS2_A</td>
<td>24582</td>
</tr>
<tr>
<td>NC17_HeLa_20PS2_B</td>
<td>15882</td>
</tr>
<tr>
<td>NC17_HeLa_20PS2_C</td>
<td>15389</td>
</tr>
<tr>
<td>NC17_HeLa_20PS3_A</td>
<td>29038</td>
</tr>
<tr>
<td>NC17_HeLa_20PS3_B</td>
<td>21308</td>
</tr>
<tr>
<td>NC17_HeLa_20PS3_C</td>
<td>17650</td>
</tr>
<tr>
<td>NC17_HeLa_PS_A</td>
<td>26158</td>
</tr>
<tr>
<td>NC17_HeLa_PS_B</td>
<td>19394</td>
</tr>
<tr>
<td>NC17_HeLa_PS_C</td>
<td>28814</td>
</tr>
<tr>
<td>NC17_HeLa_RanN6_A</td>
<td>14452</td>
</tr>
<tr>
<td>NC17_HeLa_RanN6_B</td>
<td>19870</td>
</tr>
<tr>
<td>NC17_HeLa_RanN6_C</td>
<td>19562</td>
</tr>
<tr>
<td>NC17_THP1_10PS_A</td>
<td>24370</td>
</tr>
<tr>
<td>NC17_THP1_10PS_B</td>
<td>14661</td>
</tr>
<tr>
<td>NC17_THP1_10PS_C</td>
<td>20788</td>
</tr>
<tr>
<td>NC17_THP1_20PS1_A</td>
<td>18173</td>
</tr>
<tr>
<td>NC17_THP1_20PS1_B</td>
<td>20236</td>
</tr>
<tr>
<td>NC17_THP1_20PS1_C</td>
<td>14809</td>
</tr>
<tr>
<td>NC17_THP1_20PS2_A</td>
<td>17447</td>
</tr>
<tr>
<td>NC17_THP1_20PS2_B</td>
<td>17429</td>
</tr>
<tr>
<td>NC17_THP1_20PS2_C</td>
<td>21447</td>
</tr>
<tr>
<td>NC17_THP1_20PS3_A</td>
<td>18173</td>
</tr>
<tr>
<td>NC17_THP1_20PS3_B</td>
<td>20788</td>
</tr>
<tr>
<td>NC17_THP1_20PS3_C</td>
<td>14661</td>
</tr>
<tr>
<td>NC17_THP1_PS_A</td>
<td>22048</td>
</tr>
<tr>
<td>NC17_THP1_PS_B</td>
<td>20236</td>
</tr>
<tr>
<td>NC17_THP1_PS_C</td>
<td>20788</td>
</tr>
<tr>
<td>NC17_THP1_RanN6_A</td>
<td>14661</td>
</tr>
<tr>
<td>NC17_THP1_RanN6_B</td>
<td>20236</td>
</tr>
<tr>
<td>NC17_THP1_RanN6_C</td>
<td>18173</td>
</tr>
</tbody>
</table>

Normalization number of read per sample: l2.sub ;
libs$genes.sub

In all the 3 libraries used, one contain only few reads tags. The smallest one has 8,708 counts. In order to make meaningful comparisons, all of them are subsapled to 8700 counts.

```r
l2.sub1 <- t(rarerefy(t(l2_NC17),min(8700)))
colSums(l2.sub1)
```
Moirai statistics

Load the QC data produced by the Moirai workflow with which the libraries were processed. Sort in the same way as the l1 and l2 tables, to allow for easy addition of columns.

```r
libs <- loadLogs('moirai')
```

Number of clusters

Count the number of unique L2 clusters per libraries after subsampling, and add this to the QC table. Each subsampling will give a different result, but the mean result can be calculated by using the `rarefy` function at the same scale as the subsampling.

```r
libs['l2.sub1'] <- colSums(l2.sub1 > 0)
libs['l2.sub1.exp'] <- rarefy(t(l2_NC17), min(colSums(l2_NC17)))
```

Richness

Richness should also be calculated on the whole data.

```r
libs['r100.l2'] <- rarefy(t(l2_NC17),100)

boxplot(data=libs, r100.l2 ~ group, ylim=c(92,100), las=1)
```
Hierarchical annotation

Differences of sampling will not bias distort the distribution of reads between annotations, so the non-subsampled library is used here.

```r
annot.l2 <- read.table(paste(LIBRARY,'l2','annot',sep='.'), head=F, col.names =c('id', 'feature'), row.names=1)
annot.l2 <- hierarchAnnot(annot.l2)

rownames(libs) <- sub("17_", "NC17_", rownames(libs))

libs <- cbind(libs, t(rowsum(l2_NC17, annot.l2[, 'class'])))
libs$samplename <- sub('17_','NC17_', libs$samplename)
```

Gene symbols used normalisation data

```r
genesymbols <- read.table(paste(LIBRARY,'l2','genes',sep='.'), col.names=c("cluster","symbol"), stringsAsFactors=FALSE)
rownames(genesymbols) <- genesymbols$cluster

g2 <- rowsum(l2_NC17, genesymbols$symbol)
countSymbols <- countSymbols(g2)

libs[rownames(l2_NC17),"genes"] <- (countSymbols)
```

Number of genes detected in sub-sample
l2.sub1 <- data.frame(l2.sub1)
g2.sub1 <- rowsum(l2.sub1, genesymbols$symbol)
countSymbols.sub1 <- countSymbols(g2.sub1)
libs[colnames(l2.sub1), "genes.sub1"] <- (countSymbols.sub1)

Comparison transcriptome

m2 <- data.frame(
  HeLa_RanN6 = rowMeans(g2[, c('NC17_HeLa_RanN6_A', 'NC17_HeLa_RanN6_B', 'NC17_HeLa_RanN6_C')]),
  HeLa_PS = rowMeans(g2[, c('NC17_HeLa_PS_A', 'NC17_HeLa_PS_B', 'NC17_HeLa_PS_C')]),
  HeLa_20PS3 = rowMeans(g2[, c('NC17_HeLa_20PS3_A', 'NC17_HeLa_20PS3_B', 'NC17_HeLa_20PS3_C')]),
  HeLa_20PS1 = rowMeans(g2[, c('NC17_HeLa_20PS1_A', 'NC17_HeLa_20PS1_B', 'NC17_HeLa_20PS1_C')]),
  HeLa_20PS2 = rowMeans(g2[, c('NC17_HeLa_20PS2_A', 'NC17_HeLa_20PS2_B', 'NC17_HeLa_20PS2_C')]),
  HeLa_10PS = rowMeans(g2[, c('NC17_HeLa_10PS_A', 'NC17_HeLa_10PS_B', 'NC17_HeLa_10PS_C')]),
  THP1_RanN6 = rowMeans(g2[, c('NC17_THP1_RanN6_A', 'NC17_THP1_RanN6_B', 'NC17_THP1_RanN6_C')]),
  THP1_PS = rowMeans(g2[, c('NC17_THP1_PS_A', 'NC17_THP1_PS_B', 'NC17_THP1_PS_C')]),
  THP1_20PS3 = rowMeans(g2[, c('NC17_THP1_20PS3_A', 'NC17_THP1_20PS3_B', 'NC17_THP1_20PS3_C')]),
  THP1_20PS1 = rowMeans(g2[, c('NC17_THP1_20PS1_A', 'NC17_THP1_20PS1_B', 'NC17_THP1_20PS1_C')]),
  THP1_20PS2 = rowMeans(g2[, c('NC17_THP1_20PS2_A', 'NC17_THP1_20PS2_B', 'NC17_THP1_20PS2_C')]),
  THP1_10PS = rowMeans(g2[, c('NC17_THP1_10PS_A', 'NC17_THP1_10PS_B', 'NC17_THP1_10PS_C')])
)

results <- pvclust(m2)

## Bootstrap (r = 0.5)... Done.
## Bootstrap (r = 0.6)... Done.
## Bootstrap (r = 0.7)... Done.
## Bootstrap (r = 0.8)... Done.
## Bootstrap (r = 0.9)... Done.
## Bootstrap (r = 1.0)... Done.
## Bootstrap (r = 1.1)... Done.
## Bootstrap (r = 1.2)... Done.
## Bootstrap (r = 1.3)... Done.
## Bootstrap (r = 1.4)... Done.
plot(results)
pvrect(results, alpha=0.95)

Cluster dendrogram with AU/BP values (%)

Distance: correlation
Cluster method: average

Table record
save the different tables produced for later analysis

write.table(l2_NC17, "l2_NC17_1.txt", sep = "\t", quote=FALSE)
write.table(l2.sub1, "l2.sub1_NC17_1.txt", sep = "\t", quote=FALSE)
write.table(g2.sub1, 'g2.sub1_NC17_1.txt', sep="\t", quote=F)
write.table(libs, 'libs_NC17_1.txt', sep="\t", quote=F)
write.table(m2, "m2_NC17_1.txt", sep = "\t", quote = FALSE)
Analyze of the 3 experiments

Analysis with R

Configuration

```r
library(oscR) # See https://github.com/charles-plessy/oscR
if (compareVersion(sessionInfo()$otherPkgs$oscR$Version, '0.1.1') < 0)
  stop('Out of date oscR library')

library(smallCAGEqc) # See https://github.com/charles-plessy/smallCAGEqc
if (compareVersion(sessionInfo()$otherPkgs$smallCAGEqc$Version, '0.6.0') < 0)
  stop('Out of date smallCAGEqc library')

library(gdata)
```

```r
## gdata: read.xls support for 'XLS' (Excel 97-2004) files ENABLED.
## gdata: read.xls support for 'XLSX' (Excel 2007+) files ENABLED.
## Attaching package: 'gdata'
## The following object is masked from 'package:stats':
##   nosb
## The following object is masked from 'package:utils':
##   object.size

library(vegan)
```

```r
## Loading required package: permute
## Loading required package: lattice
## This is vegan 2.0-10

library(ggplot2)
```

Load the data

```r
libs_NC12 <- read.table("libs_NC12_1.txt", sep="\t", head=T)
libs_NCki <- read.table("libs_NCki_1.txt", sep="\t", head=T)
libs_NC17 <- read.table("libs_NC17_1.txt", sep="\t", head=T)
```
Merge 3 tables

The data coming from the 3 experiments are merged in one table to analyzed them together

```r
rownames(libs_NC12) <- sub('NC12\.', 'NC12_', rownames(libs_NC12))

libs <- rbind(libs_NC12, libs_NC17, libs_NCKi)
```

Add the celltype

```r
libs$celltype <- libs$samplename
libs$celltype <- sub('NC\..', '', libs$celltype)
libs$celltype <- sub('\..', '', libs$celltype)
libs$celltype <- factor(libs$celltype)
```

Figure S2

Modification of the table libs (group by triplicates)

```r
libs2 <- libs
libs2$group <- sub('\.$', '', libs2$group)
libs2$group <- factor(libs2$group)

plotAnnot(libs2, 'all', 'pseudo-random primers') + theme_bw()
```

## Using group as id variables

## Warning: Stacking not well defined when ymin != 0
Impact rDNA and artefacts

Calculate means by triplicate

```r
library <- with(libs, 
  data.frame(samplename, group, celltype, 
    promoter = promoter / extracted, 
    exon = exon / extracted, 
    intron = intron / extracted, 
    unknown = unknown / extracted, 
    rDNA = rdna / extracted, 
    artefacts = tagdust / extracted 
  ))
library$triplicates <- sub('_.$', '', library$samplename)
library <- aggregate(library[,c('rDNA', 'artefacts')], list(library$triplicates), mean)
library$rDNA1000 <- (library$rDNA)*1000
library$artefact1000 <- (library$artefacts)*1000
library$group <- library$Group.1
library$group <- sub('NC..', '', library$group)
```

Draw graph

---

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http://dx.doi.org/10.1101/027805

The preprint was first posted online Sep. 29, 2015.
```r
dotsize <- mean(libm$artefact1000) / 5
p <- ggplot(libm, aes(y=artefact1000, x=group)) +
  stat_summary(fun.y=mean, fun.ymin=mean, fun.ymax=mean,
               geom="crossbar", color="gray") +
  geom_dotplot(aes(fill=group), binaxis='y', binwidth=1,
               dotsize=dotsize, stackdir='center') +
  theme_bw() +
  theme(axis.text.x = element_text(size=13, angle=90)) +
  theme(axis.text.y = element_text(size=13)) +
  theme(axis.title.x = element_blank())+
  theme(axis.title.y = element_text(size=14)) +
  scale_y_continuous(breaks =c(0, 50, 100, 150, 200), limits= c(0,200),
                     labels=c("0", "0.05", "0.1", "0.15", "0.2")) +
  ylab("arefacts / extracted")

p + theme(legend.position="none")
```
```
dotsize <- mean(libm$rDNA1000)/10
p <- ggplot(libm, aes(y=rDNA1000, x=group)) +
    stat_summary(fun.y=mean, fun.ymin=mean, fun.ymax=mean,
    geom="crossbar", color="gray") +
    geom_dotplot(aes(fill=group), binaxis='y', binwidth=1,
    dotsize=dotsize, stackdir='center') +
    theme_bw() +
    theme(axis.text.x = element_text(size=13, angle=90)) +
    theme(axis.text.y = element_text(size=13)) +
    theme(axis.title.x = element_blank())+
    theme(axis.title.y = element_text(size=14))+
    scale_y_continuous(limits=c(0,900), breaks =c(0, 200, 400, 600, 800),
    labels=c("0", "0.2", "0.4", "0.6", "0.8")) +
    ylab("rDNA / extracted")
p + theme(legend.position="none")
```
genes_percentage <- libs[,c('sampleName', 'group', 'genes.sub1')]
genes_percentage$group1 <- genes_percentage$samplename
genes_percentage$group1 <- sub('_\.$', '', genes_percentage$group1)
genes_percentage$group1 <- factor(genes_percentage$group1)
genes_percentage <- tapply(genes_percentage$genes.sub1, genes_percentage$group1, mean)

genes_percentage <- sapply(
  c("NC12_Hela", "NC12_THP1", "NC17_Hela", "NC17_THP1", "NCki_Hela", "NCki_THP1"),
  function(experiment) genes_percentage[grep(experiment, names(genes_percentage))] / genes_percentage[paste0(experiment, '_RanN6')] * 100
)
genes_percentage <- unlist(genes_percentage)
names(genes_percentage) <- sub("\.*\."", ", names(genes_percentage))

genes_percentage <- data.frame(genes_percentage)
genes_percentage$group <- rownames(genes_percentage)
genes_percentage$group <- sub('NC.._', '', genes_percentage$group)

dotsize <- mean(genes_percentage$genes_percentage) / 110
p <- ggplot(genes_percentage, aes(x=group, y=genes_percentage)) +
  stat_summary(fun.y=mean, fun.ymin=mean, fun.ymax=mean,
    geom="crossbar", color="gray") +
  geom_dotplot(aes(fill=group), binaxis='y', binwidth=1,
    dotsize=dotsize, stackdir='center') +
    theme_bw() +
    theme(axis.text.x = element_text(size=14, angle=90)) +
    theme(axis.text.y = element_text(size=14)) +
    theme(axis.title.y = element_blank()) +
    theme(axis.title.x = element_text(size=14)) +
    ylim(90,120) +
    ylab("percentage of genes detected")
p + guides(col = guide_legend(nrow = 8))
Transcriptome analysis

Load the data

```r
g2_NC12 <- read.table('g2.sub1_NC12_1.txt', sep="\t", head=T)
g2_NC17 <- read.table('g2.sub1_NC17_1.txt', sep="\t", head=T)
g2_NCki <- read.table('g2.sub1_NCki_1.txt', sep="\t", head=T)
```

Create a new table

```r
g2 <- merge(g2_NC12, g2_NC17, by='row.names', all=T)
rownames(g2) <- g2$Row.names
g2 <- g2[, -1]
g2 <- merge(g2, g2_NCki, by='row.names', all=T)
rownames(g2) <- g2$Row.names
g2 <- g2[, -1]
g2[is.na(g2)] <- 0
g2b <- g2[-1,]
```
\[ \text{RanN6\_HeLa} = c( 'NC12\_HeLa\_RanN6\_A', 'NC12\_HeLa\_RanN6\_B', 'NC12\_HeLa\_RanN6\_C', 'NC17\_HeLa\_RanN6\_A', 'NC17\_HeLa\_RanN6\_B', 'NC17\_HeLa\_RanN6\_C', 'NCki\_HeLa\_RanN6\_A', 'NCki\_HeLa\_RanN6\_B', 'NCki\_HeLa\_RanN6\_C') \]

\[ \text{PS\_HeLa} = c( 'NC12\_HeLa\_PS\_A', 'NC12\_HeLa\_PS\_B', 'NC12\_HeLa\_PS\_C', 'NC17\_HeLa\_PS\_A', 'NC17\_HeLa\_PS\_B', 'NC17\_HeLa\_PS\_C', 'NCki\_HeLa\_PS\_A', 'NCki\_HeLa\_PS\_B', 'NCki\_HeLa\_PS\_C') \]

\[ \text{RanN6\_THP1} = c( 'NC12\_THP1\_RanN6\_A', 'NC12\_THP1\_RanN6\_B', 'NC12\_THP1\_RanN6\_C', 'NC17\_THP1\_RanN6\_A', 'NC17\_THP1\_RanN6\_B', 'NC17\_THP1\_RanN6\_C', 'NCki\_THP1\_RanN6\_A', 'NCki\_THP1\_RanN6\_B', 'NCki\_THP1\_RanN6\_C') \]

\[ \text{PS\_THP1} = c( 'NC12\_THP1\_PS\_A', 'NC12\_THP1\_PS\_B', 'NC12\_THP1\_PS\_C', 'NC17\_THP1\_PS\_A', 'NC17\_THP1\_PS\_B', 'NC17\_THP1\_PS\_C', 'NCki\_THP1\_PS\_A', 'NCki\_THP1\_PS\_B', 'NCki\_THP1\_PS\_C') \]

\[
\text{mx} \leftarrow \text{function}\left( \text{DATA} \right) \\
\{ \\
\text{data.frame} \left( \text{HeLa\_RanN6} = \text{rowMeans(\text{DATA[,RanN6\_HeLa]})} \right) \\
\text{, HeLa\_pseudoRan} = \text{rowMeans(\text{DATA[,PS\_HeLa]})} \right) \\
\text{, THP1\_RanN6} = \text{rowMeans(\text{DATA[,RanN6\_THP1]})} \right) \\
\text{, THP1\_pseudoRan} = \text{rowMeans(\text{DATA[,PS\_THP1]})} \right) \\
\} \\
\text{m2} \leftarrow \text{mx}(\text{g2}) \\
\text{write.table(m2, "m2.txt", sep = "\t", quote = FALSE)}
\]

\[
\text{panel.cor} \leftarrow \text{function}(x, y, digits=2, prefix="", cex.cor, ...) \\
\{ \\
\text{usr} \leftarrow \text{par}\left( "usr" \right); \text{on.exit}(\text{par(usr)}) \\
\text{par(usr = c(0, 1, 0, 1))} \\
\text{r} \leftarrow \text{abs(\text{cor}(x, y))} \\
\text{txt} \leftarrow \text{format(c(r, 0.123456789), digits=digits)[1]} \\
\text{txt} \leftarrow \text{paste(prefix, txt, sep="\"")} \\
\text{if(missing(cex.cor)) cex.cor <- 0.8/\text{strwidth(txt)}} \\
\text{text(0.5, 0.5, txt, cex = cex.cor * r)} \\
\} \\
\]

\[
\text{pointsUnique} \leftarrow \text{function}(x,y,...) \\
\text{points(\text{unique(\text{data.frame}(x,y))),...)} \\
\]

\[
\text{pairPanel} \leftarrow \text{function}(\text{dataframe, title}) \\
\text{\text{pairs( \text{dataframe} , lower.panel=panel.cor}} \\
\text{, upper.panel=\text{pointsUnique}} \\
\text{, main=\text{title}} \\
\text{, pch='.' , cex=4)} \\
\]

\[
\text{pairPanel(\text{log(m2+1)}, 'pseudo-random primers')} \\
\]
```r
plotFoldChange <- function (DATA, COL, MAX, FUN=points, ...) {
  with( DATA[rowSums(DATA) > MAX,] +1
    , FUN( HeLa_RanN6 / THP1_RanN6
           , HeLa_pseudoRan / THP1_pseudoRan
           , col=COL
           , pch='.'
           , cex=5
           , ... ))
}

plotFoldChangeGrays <- function (DATA, TITLE, xlab="Standard N6 random primers", ylab="Pseudo-random primers") {
  plotFoldChange( DATA,'gray90', 0
                 , plot, log='xy', main=TITLE
                 , xlab=xlab, ylab=ylab)
  plotFoldChange(DATA, 'gray70', 10)
  plotFoldChange(DATA, 'gray50', 20)
  plotFoldChange(DATA, 'gray30', 30)
  plotFoldChange(DATA, 'gray10', 40)
  legend( 'bottomright'
    , legend=c(0, 10, 20, 30, 40)
    , col=c('gray90', 'gray70', 'gray50', 'gray30', 'gray10')
    , pch=16, title='min expr.'
  )
}```
u2 <- unique(m2)

Draw graphs

plotFoldChangeGrays(u2, "HeLa - THP-1 fold changes")

plotFoldChange( u2, 'gray10', 0
, plot, log='xy', main="HeLa - THP-1 fold changes"
, xlab="Standard N6 random primers", ylab="Pseudo-random primers")
Statistic tests

statistic tests about sequences coming from ribosomal RNA

Regarding the PS and RanN6 set, we use a paired t.test as the results come from 3 independents experiments.

```r
rDNA <- read.table('rDNA.csv', sep = ',', head = T)
## Warning in read.table("rDNA.csv", sep = ",", head = T): incomplete final line found by readTableHeader on 'rDNA.csv'

rDNA

##   experiments HeLa_40N6   HeLa_PS HeLa_RanN6 THP1_40N6   THP1_PS THP1_RanN6
## 1        NC12 0.7149618 0.2279513  0.4715841 0.7314974 0.2765081  0.480080
## 2        NC17        NA 0.2413637  0.4651863        NA 0.2921605  0.473343
## 3        Ncki        NA 0.2116392  0.4669319        NA 0.1976666  0.455164

t.test(rDNA$HeLa_PS, rDNA$HeLa_RanN6, paired = T)

## Paired t-test
## data:  rDNA$HeLa_PS and rDNA$HeLa_RanN6
## t = -26.2275, df = 2, p-value = 0.001451
## alternative hypothesis: true difference in means is not equal to 0
## 95 percent confidence interval:
##  -0.2804387 -0.2013934
## sample estimates:
## mean of the differences
##         -0.2409161

t.test(rDNA$THP1_PS, rDNA$THP1_RanN6, paired = T)
```
## Paired t-test

- **data:** rDNA$THP1_PS and rDNA$THP1_RanN6  
  - **t:** -9.4525, **df:** 2, **p-value:** 0.01101  
  - **alternative hypothesis:** true difference in means is not equal to 0  
  - **95 percent confidence interval:**  
    - -0.3115324 to -0.1166364  
  - **sample estimates:**  
    - mean of the differences  
      - -0.2140844

```r
rDNA_40N6 <- read.table('rDNA_40N6.csv', sep="","", head=T)
```

```r
# Warning in read.table("rDNA_40N6.csv", sep = ",", head = T): incomplete final line found by readTableHeader on  
# 'rDNA_40N6.csv'
```

Regarding the 40N6 set, we cannot use the paired test as only one experiment has been performed. Thus, we use the 3 replicates of 1 experiment.

```r
t.test(rDNA_40N6$HeLa_RanN6, rDNA_40N6$HeLa_40N6)
```

```r
# Welch Two Sample t-test  
# data: rDNA_40N6$HeLa_RanN6 and rDNA_40N6$HeLa_40N6  
# t = -14.363, df = 3.375, p-value = 0.0003821  
# alternative hypothesis: true difference in means is not equal to 0  
# 95 percent confidence interval:  
# -0.2940671 to -0.1926882  
# sample estimates:  
# mean of x mean of y  
# 0.4715841 0.7149618
```

```r
t.test(rDNA_40N6$HeLa_PS, rDNA_40N6$HeLa_40N6)
```
statistic tests about sequences coming from artefacts

Regarding the PS and RanN6 set, we use a paired t.test as the results come from 3 independents experiments.

```r
artefact <- read.table('artefacts.csv', sep=',', head=T)
```
### Warning in read.table("artefacts.csv", sep = ",", head = T): incomplete final line found by readTableHeader on

### 'artefacts.csv'

artefact

---

### experiments  HeLa_40N6  HeLa_PS  HeLa_RanN6  THP1_40N6  THP1_PS  THP1_RanN6
---

<p>| | | | | | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>NC12</td>
<td>0.01044525</td>
<td>0.01139030</td>
<td>0.08445126</td>
<td>0.01031001</td>
<td>0.01440960</td>
</tr>
<tr>
<td>2</td>
<td>NC17</td>
<td>NA</td>
<td>0.02860542</td>
<td>0.02765798</td>
<td>NA</td>
<td>0.02274970</td>
</tr>
<tr>
<td>3</td>
<td>Ncki</td>
<td>NA</td>
<td>0.02998795</td>
<td>0.07369473</td>
<td>NA</td>
<td>0.02369741</td>
</tr>
</tbody>
</table>

---

t.test(artefact$HeLa_PS, artefact$HeLa_RanN6, paired = T)

---

## Paired t-test

## data:  artefact$HeLa_PS and artefact$HeLa_RanN6
## t = -1.7943, df = 2, p-value = 0.2146
## alternative hypothesis: true difference in means is not equal to 0
## 95 percent confidence interval:
##  -0.13118278  0.05396924
## sample estimates:
## mean of the differences
##             -0.03860677

t.test(artefact$THP1_PS, artefact$THP1_RanN6, paired = T)

---

## Paired t-test

## data:  artefact$THP1_PS and artefact$THP1_RanN6
## t = -5.1377, df = 2, p-value = 0.03586
## alternative hypothesis: true difference in means is not equal to 0
## 95 percent confidence interval:
##  -0.100771328 -0.008913305
## sample estimates:
## mean of the differences
##             -0.05484232

Regarding the 40N6 set, we can not use the paired test as only one experiment has been performed. Thus, we use the 3 replicats of 1 experiment.

artefact_40N6 <- read.table('artefact_40N6.csv', sep="", head=T)
## Warning in read.table("artefact_40N6.csv", sep = ",", head = T): incomplete final line found by readTableHeader on 'artefact_40N6.csv'

<table>
<thead>
<tr>
<th>experiments</th>
<th>HeLa_40N6</th>
<th>HeLa_PS</th>
<th>HeLa_RanN6</th>
<th>THP1_40N6</th>
<th>THP1_PS</th>
<th>THP1_RanN6</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>NC12_A</td>
<td>0.014282432</td>
<td>0.014614436</td>
<td>0.08701817</td>
<td>0.01415881</td>
<td>0.019349645</td>
</tr>
<tr>
<td>2</td>
<td>NC12_B</td>
<td>0.005714088</td>
<td>0.007657406</td>
<td>0.05647798</td>
<td>0.00548908</td>
<td>0.008236931</td>
</tr>
<tr>
<td>3</td>
<td>NC12_C</td>
<td>0.011339241</td>
<td>0.011899050</td>
<td>0.10985765</td>
<td>0.01128215</td>
<td>0.015642227</td>
</tr>
</tbody>
</table>

`t.test(artefact_40N6$HeLa_RanN6, artefact_40N6$HeLa_40N6)`

```
# Welch Two Sample t-test
#
# data: artefact_40N6$HeLa_RanN6 and artefact_40N6$HeLa_40N6
# t = 4.7241, df = 2.106, p-value = 0.03798
# alternative hypothesis: true difference in means is not equal to 0
# 95 percent confidence interval:
#  0.009740573 0.138271448
# sample estimates:
#  mean of x  mean of y
#  0.08445126 0.01044525
```

`t.test(artefact_40N6$HeLa_PS, artefact_40N6$HeLa_40N6)`

```
# Welch Two Sample t-test
#
# data: artefact_40N6$HeLa_PS and artefact_40N6$HeLa_40N6
# t = 0.2928, df = 3.826, p-value = 0.7848
# alternative hypothesis: true difference in means is not equal to 0
# 95 percent confidence interval:
#  -0.008178375 0.010068462
# sample estimates:
#  mean of x  mean of y
#  0.01139030 0.01044525
```

`t.test(artefact_40N6$THP1_RanN6, artefact_40N6$THP1_40N6)`
statistic tests about the numbers of genes detected

Regarding the PS and RanN6 set, we use a paired t.test as the results come from 3 independents experiments.

```r
genes <- read.table('genes.csv', sep = ',', head = T)
```

```r
## Warning in read.table("genes.csv", sep = ",", head = T): incomplete final line found by readTableHeader on 'genes.csv'
genes
```

```r
## experiments HeLa_40N6 HeLa_PS HeLa_RanN6 THP1_40N6 THP1_PS THP1_RanN6
## 1  1 NC12 104.1283 110.6335 100 100.1942 108.2811 100
## 2  2 NC17 NA 106.4641 100 NA 107.6821 100
## 3  3 Ncki NA 109.3965 100 NA 108.8293 100
t.test(genes$HeLa_PS, genes$HeLa_RanN6, paired = T)
```
Paired t-test

data:  genes$HeLa_PS and genes$HeLa_RanN6
t = 7.1433, df = 2, p-value = 0.01904
alternative hypothesis: true difference in means is not equal to 0
95 percent confidence interval:
  3.511913 14.150874
sample estimates:
mean of the differences
  8.831394

t.test(genes$THP1_PS, genes$THP1_RanN6, paired = T)

Paired t-test
data:  genes$THP1_PS and genes$THP1_RanN6
t = 24.9454, df = 2, p-value = 0.001603
alternative hypothesis: true difference in means is not equal to 0
95 percent confidence interval:
  6.83873 9.68958
sample estimates:
mean of the differences
  8.264155

Regarding the 40N6 set, we can not use the paired test as only one experiment has been performed. Thus, we use the 3 replicates of 1 experiment.

genes_40N6 <- read.table('genes_40N6.csv', sep="", head=T)

Warning in read.table("genes_40N6.csv", sep = ",", head = T): incomplete final line found by readTableHeader on 'genes_40N6.csv'

genes_40N6

  experiments  HeLa_40N6  HeLa_PS  HeLa_RanN6  THP1_40N6  THP1_PS  THP1_RanN6
  1     NC12_A  102.3233  108.8375   96.80100  94.68527 106.6037   96.35384
  2     NC12_B  103.3688  110.0974  103.87812 101.91578 106.1005  102.55143
  3     NC12_C  106.6929  112.9658   99.32088 103.98164 112.1391  101.09473

t.test(genes_40N6$HeLa_RanN6, genes_40N6$HeLa_40N6)
```r
### Welch Two Sample t-test
### data: genes_40N6$HeLa_RanN6 and genes_40N6$HeLa_40N6
### t = -1.682, df = 3.391, p-value = 0.1806
### alternative hypothesis: true difference in means is not equal to 0
### 95 percent confidence interval:
###   -11.454223   3.197589
### sample estimates:
###   mean of x   mean of y
###       100.0000       104.1283
```

```r
t.test(genes_40N6$HeLa_PS, genes_40N6$HeLa_40N6)
### Welch Two Sample t-test
### data: genes_40N6$HeLa_PS and genes_40N6$HeLa_40N6
### t = 3.621, df = 3.977, p-value = 0.02255
### alternative hypothesis: true difference in means is not equal to 0
### 95 percent confidence interval:
###   1.506129 11.504326
### sample estimates:
###   mean of x   mean of y
###       110.6335       104.1283
```

```r
t.test(genes_40N6$THP1_RanN6, genes_40N6$THP1_40N6)
### Welch Two Sample t-test
### data: genes_40N6$THP1_RanN6 and genes_40N6$THP1_40N6
### t = -0.0574, df = 3.476, p-value = 0.9574
### alternative hypothesis: true difference in means is not equal to 0
### 95 percent confidence interval:
###   -10.171798   9.783346
### sample estimates:
###   mean of x   mean of y
###       100.0000       100.1942
```

```r
t.test(genes_40N6$THP1_PS, genes_40N6$THP1_40N6)
```
## Welch Two Sample t-test

data:  genes_40N6$THP1_PS and genes_40N6$THP1_40N6

t = 2.3657, df = 3.542, p-value = 0.08558
alternative hypothesis: true difference in means is not equal to 0
95 percent confidence interval:
-1.90779 18.08153
sample estimates:
mean of x mean of y
108.2811 100.1942
Targeted reduction of Hemoglobin cDNAs

Configuration

```r
library(plyr)

exportInEnv <- function(X) {
  Name <- X
  Value <- get(X)
  .Internal(Sys.setenv(Name, Value))
  cat(paste0("export ", paste(Name, Value, sep='='), "\n"))
}

LIBRARY <- 'NC22b'
MOIRAI_USER <- 'nanoCAGE2'
MOIRAI_PROJECT <- 'Arnaud'
GROUP_SHARED <- '/osc-fs_home/scratch/gmtu'
WORKDIR <- '.'
GENE_SYMBOLS <- paste(GROUP_SHARED, 'annotation/homo_sapiens/gencode-14/gencode.v14.annotation.genes.bed', sep='/')
ANNOTATION <- paste(GROUP_SHARED, 'annotation/homo_sapiens/100712hg19/100712hg19', sep='/')
PROCESSED_DATA <- dirname(system(paste('ls -d /osc-fs_home/scratch/moirai/','
  , MOIRAI_USER
  , '/project/'
  , MOIRAI_PROJECT
  , '/'
  , LIBRARY
  , '/Moirai.config'
  , sep='')
  , intern=TRUE)[1])

l_ply( c("LIBRARY", "MOIRAI_USER", "MOIRAI_PROJECT", "GROUP_SHARED"
      , "WORKDIR", "GENE_SYMBOLS", "ANNOTATION", "PROCESSED_DATA")
      , exportInEnv )
```
export LIBRARY=NC22b
export MOIRAI_USER=nanoCAGE2
export MOIRAI_PROJECT=Arnaud
export GROUP_SHARED=/osc-fs_home/scratch/gmtu
export WORKDIR=.
export GENE_SYMBOLS=/osc-fs_home/scratch/gmtu/annotation/homo_sapiens/gencode-14/gencode.v14.annotation.genes.bed
export ANNOTATION=/osc-fs_home/scratch/gmtu/annotation/homo_sapiens/100712hg19/100712hg19
export PROCESSED_DATA=/osc-fs_home/scratch/moirai/nanoCAGE2/project/Arnaud/NC22b.CAGEscan_short-reads.20150625152335

Moirai URL: http://moirai.gsc.riken.jp/osc-fs_home/scratch/moirai/nanoCAGE2/project/Arnaud/NC22b.CAGEscan_short-reads.20150625152335/NC22b.CAGEscan_short-reads.20150625152335.html

Count the reads

```
awk '/raw/ {print $3}' $PROCESSED_DATA/text/summary.txt |
/usr/lib/filo/stats |
grep 'Sum' |
cut -f2 -d':' |
tr -d '[:space:]' |
xargs -0 /usr/bin/printf " # %'d\n"

grep raw $PROCESSED_DATA/text/summary.txt
```

```
##  # 2999748
## NC22b.ACAGTG.R1  raw 181519
## NC22b.ATCACG.R1  raw 211629
## NC22b.CGATGT.R1  raw 82773
## NC22b.GCCAAT.R1  raw 170418
## NC22b.TGACCA.R1  raw 58532
## NC22b.TTAGGC.R1  raw 188190
## NC22b.Undetermined.R1    raw 2106687
```

Analysis with R

Configuration
library(oscR) # See https://github.com/charles-plessy/oscR for oscR.
if (compareVersion(sessionInfo()$otherPkgs$oscR$Version,'0.1.1') < 0) stop('Outdated version of oscR.')

library(smallCAGEqc) # See https://github.com/charles-plessy/smallCAGEqc for smallCAGEqc.
if (compareVersion(sessionInfo()$otherPkgs$smallCAGEqc$Version,'0.6.0') < 0)
  stop('Outdated version of smallCAGEqc')

library(vegan)

## Loading required package: permute
## Loading required package: lattice
## This is vegan 2.0-10

library(ggplot2)

Load data

l1 <- read.osc(paste(LIBRARY,'l1','gz',sep='.'), drop.coord=T, drop.norm=T)
l2 <- read.osc(paste(LIBRARY,'l2','gz',sep='.'), drop.coord=T, drop.norm=T)

colnames(l1) <- sub('raw.NC22b.', '', colnames(l1))
colnames(l2) <- sub('raw.NC22b.', '', colnames(l2))
colSums(l2)

##  22_PSHb_A  22_PSHb_B  22_PSHb_C 22_RanN6_A 22_RanN6_B 22_RanN6_C
##       3786       3196       6805      17433      18864      17218

PSHb <- c('22_PSHb_A', '22_PSHb_B', '22_PSHb_C')
RanN6 <- c('22_RanN6_A', '22_RanN6_B', '22_RanN6_C')

Normalization number of read per sample : libs2.sub

Libraries contain only very few reads tags. The smallest one has 3,191 counts. In order to make meaningful comparisons, all of them are subsapled to 3190 counts.

l2.sub <- t(rrarefy(t(l2),3190))
colSums(l2.sub)

##  22_PSHb_A  22_PSHb_B  22_PSHb_C 22_RanN6_A 22_RanN6_B 22_RanN6_C
##       3190       3190       3190       3190       3190       3190

Moirai statistics
Load the QC data produced by the Moirai workflow with which the libraries were processed. Sort in the same way as the l1 and l2 tables, to allow for easy addition of columns.

```r
libs <- loadLogs('moirai')
libs <- libs[,colnames(l1),]
```

### Number of clusters

Count the number of unique L2 clusters per libraries after subsampling, and add this to the QC table. Each subsampling will give a different result, but the mean result can be calculated by using the `rarefy` function at the same scale as the subsampling.

```r
libs[,"l2.sub"] <- colSums(l2.sub > 0)
libs[,"l2.sub.exp"] <- rarefy(t(l2), min(colSums(l2)))
```

### Richness

Richness should also be calculated on the whole data.

```r
libs[,"r100.l2"] <- rarefy(t(l2),100)
t.test(data=libs, r100.l2 ~ group)
```

```r
##
##  Welch Two Sample t-test
##  ##
##  ## data:  r100.l2 by group
##  ## t = 13.0614, df = 3.836, p-value = 0.0002544
##  ## alternative hypothesis: true difference in means is not equal to 0
##  ## 95 percent confidence interval:
##  ##   7.645323 11.863046
##  ## sample estimates:
##  ## mean in group PS_Hb mean in group RanN6
##  ##            93.44089            83.68671
```

```r
boxplot(data=libs, r100.l2 ~ group, ylim=c(80,100), las=1)
```
Hierarchical annotation

Differences of sampling will not bias distort the distribution of reads between annotations, so the non-subsampled library is used here.

```r
annot.l2 <- read.table(paste(LIBRARY,'l2','annot',sep='.'), head=F, col.names =c('id', 'feature'), row.names=1)
annot.l2 <- hierarchAnnot(annot.l2)

libs <- cbind(libs, t(rowsum(l2, annot.l2[, 'class'])))
```

Gene symbols used normalisation data

```r
genosphiles <- read.table(paste(LIBRARY,'l2','genes',sep='.'), col.names=c("cluster","symbol"), stringsAsFactors=FALSE)
rownames(genosphiles) <- genosphiles$cluster

countSymbols <- function(X) length(unique(genosphiles[X > 0,'symbol']))

libs[rownames(l2.sub),"genes.sub"] <- apply(l2.sub, 2, countSymbols)
libs[rownames(l2), "genes"] <- apply(l2, 2, countSymbols)
```
```r
dotsize <- mean(libs$genes.sub) / 150
par(mar=c(7,10,2,30))
p <- ggplot(libs, aes(x=group, y=genes.sub)) +
stat_summary(fun.y=mean, fun.ymin=mean, fun.ymax=mean,
geom="crossbar", color="gray") +
  geom_dotplot(aes(fill=group), binaxis='y', binwidth=1,
dotsize=dotsize, stackdir='center') +
  theme_bw() +
  theme(axis.text.x = element_text(size=14)) +
  theme(axis.text.y = element_text(size=14)) +
  theme(axis.title.x = element_blank()) +
  theme(axis.title.y = element_text(size=14)) +
  ylim(1300,1600) +
  ylab("Number of genes detected")
p + theme(legend.position="none")
```

**statistical analysis of gene count (with normalized data)**

```r
t.test(data=libs, genes.sub ~ group)
```
### Welch Two Sample t-test

### data: genes.sub by group
### t = 3.9567, df = 3.923, p-value = 0.01736
### alternative hypothesis: true difference in means is not equal to 0
### 95 percent confidence interval:
### 19.52393 113.80940
### sample estimates:
### mean in group PS_Hb mean in group RanN6
### 1472.333 1405.667

---

Analysis of the gene expressed in different sample with different primers - normalized data (l2.sub)

```r
l2_to_g2 <- function(l2) {
  g2 <- rowsum(l2, genesymbols$symbol)
  subset(g2, rowSums(g2) > 0)
}

g2.sub <- l2_to_g2(l2.sub)
g2 <- l2_to_g2(l2)
G2 <- TPM(g2)

libs$genes.r <- rarefy(t(g2), 3190)[rownames(libs)]
t.test(data=libs, genes.r ~ group)
```

### Welch Two Sample t-test

### data: genes.r by group
### t = 2.8877, df = 3.518, p-value = 0.05212
### alternative hypothesis: true difference in means is not equal to 0
### 95 percent confidence interval:
### -1.227913 157.191500
### sample estimates:
### mean in group PS_Hb mean in group RanN6
### 1491.744 1413.763

```r
G2mean <- function(TABLE) 
  data.frame( RanN6 = TPM(rowSums(TABLE[,RanN6])), PS_Hb = TPM(rowSums(TABLE[,PSHb])))

G2.sub.mean <- G2mean(g2.sub)
G2.mean <- G2mean(g2)
```
```r
head(G2.sub.mean[order(G2.sub.mean$PS_Hb, decreasing=TRUE),], 30)
```

<table>
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<tr>
<th></th>
<th>RanN6</th>
<th>PS_Hb</th>
</tr>
</thead>
<tbody>
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<td>96133.751</td>
<td>20271.6823</td>
</tr>
<tr>
<td>HBB</td>
<td>44096.134</td>
<td>940.4389</td>
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<td>J01415.2,J01415.24</td>
<td>33646.813</td>
<td>7836.9906</td>
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<tr>
<td>MALAT1</td>
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</tr>
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</table>

Gene list on normalized data (table l2.sub)

```r
RanN6_genelist.sub <- listSymbols(rownames(subset(G2.sub.mean, RanN6>0)))
PSHb_genelist.sub <- listSymbols(rownames(subset(G2.sub.mean, PS_Hb>0)))
genelist <- listSymbols(rownames(g2))
write.table(genelist, 'NC22.genelist.txt', sep = "\t", quote = FALSE, row.names = FALSE, col.names = FALSE)
```

Haemoglobin barplot
par(mar=c(2,2,2,2))
barplot(t(G2[grep('^HB[AB]', rownames(g2), value=T),]), beside=T, ylab='Normalised expression value (cpm).', col=c("gray50", "gray50", "gray50", "gray90", "gray90", "gray90"))
legend("topleft", legend=c("RanN6", "PS_Hb"), fill=c("gray90", "gray50"))
Figure S2
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