Technical Report

Preprocessing of Affymetrix Exon Expression Arrays

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Abstract

The activity of genes can be captured by measuring the amount of messenger RNAs transcribed from the genes, or from their subunits called exons. In our study, we use the Affymetrix Human Exon ST v1.0 microarrays to measure the activity of exons in Neuroblastoma cancer patients. The purpose is to discover a small number of genes or exons that play important roles in differentiating high-risk patients from low-risk counterparts. Although the technology has been improved for the past 15 years, array measurements still can be contaminated by various factors, including human error. Since the number of arrays is often only few hundreds, atypical errors can hardly be canceled by large numbers of normal arrays. In this article we describe how we filter out low-quality arrays in a principled way, so that we can obtain more reliable results in downstream analyses.

1 INTRODUCTION

This article describes how we preprocess the expression data acquired from our Neuroblastoma cancer cohort with the Affymetrix Human Exon ST 1.0 arrays[1]. The purpose is to deliver the details of our procedures, so that follow-up studies can prepare their data in a comparable manner. This will allow us integrating multiple data sets together, possibly leading to new discoveries for fighting with cancers.

Our preprocessing is composed of three main steps:

1. Quality assessment
2. RMA processing of each batch
3. Combining batches

In quality assessment, we examine the quality of individual arrays (i) via the visual inspection of arrays as images, and (ii) using the quality information provided by the Affymetrix Power Tools (APT). We will discuss each step in detail afterwards.

After filtering out low-quality arrays, we apply the robust multi-array algorithm (RMA) for the arrays in each batch. Here "batch" means a collection of arrays prepared in the same condition. We consider the arrays collected and processed in two different cities (Essen in Germany, Amsterdam in the Netherlands) as two batches, since this level of batches has shown significant differences.

The final step is making the RMA-processed data per batch comparable, so that we can combine batches for further analyses. We choose a very simple method, which can be applied independently to each batch.

In this article we use the terms "arrays" and "chips" interchangeably. Before preprocessing, the raw expression data of each chip is stored in a single file, with the extension ".CEL". So a CEL file amounts to an array or a chip.

We begin with the following chips in two batches:

Batch	No. of Chips	CEL Filename Prefix
Essen	113	GSM
Amsterdam	182	ITCC and NRC
Total	295	-

The Essen and Amsterdam batches contain all stages according to the International Neuroblastoma Staging System (INSS), and we process all stages together.

[1] For details, see http://www.affymetrix.com/support/technical/byproduct.affx?product=huexon-st

2 QUALITY ASSESSMENT

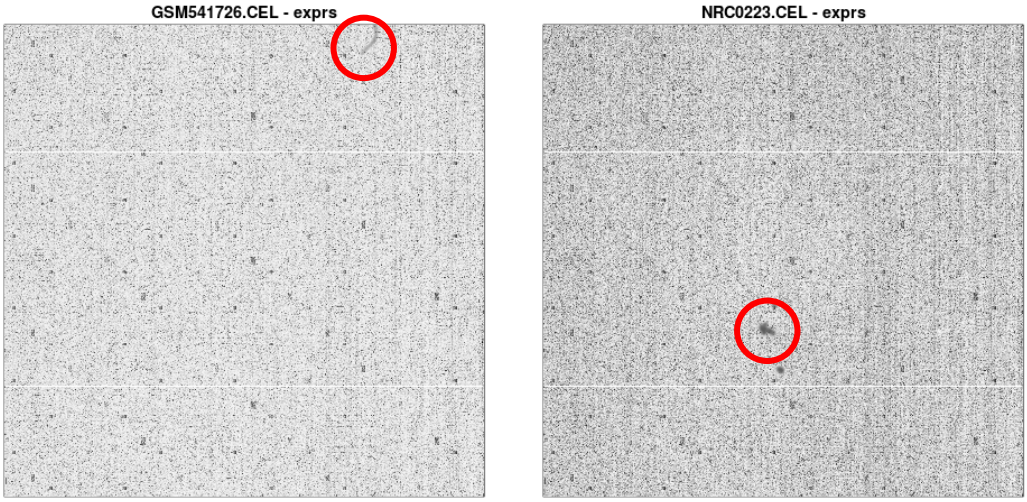
2.1 Visual Inspection of CEL Data as Images

The first step is to literally "see" the raw data in CEL files as images. For this we can conveniently use the "oligo" package[2] for R provided by the Bioconductor. Using this software, we draw each CEL file as a grayscale image, and look for any spurious artifact with our eyes.

Here is an R code snippet, to plot the image corresponds to the 3rd CEL file in /DATA:

```
library(oligo)
files <- list.celfiles("/DATA", full.names=TRUE)
celdata <- read.celfiles(files, pkgname="pd.huex.1.0.st.v2")
image(celdata, 3, col=gray((64:0)/64))
```

We present two images that contain unwanted artifacts, marked with red circles:



Here each small pixel corresponds to a "probe", and the amount of probe expression is represented as pixel intensity. The actual images are quite large in size, as each Affymetrix Human Exon ST array contains more than 1 million probes. Since the expression of each exon is detected by multiple probes, the identifier of an exon is often referred to as a "probe set ID" in Affymetrix terminology.

As we can see from the above pictures, artifacts can disguise the expression of certain probes to look quite differently from their actual amounts, leading to false positive / false negative discovery of important genes/exons.

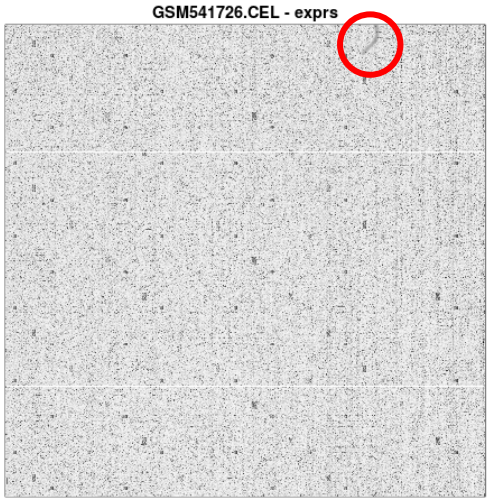
We present the list of chips with noticeable artifacts. These chips will be excluded from further consideration:

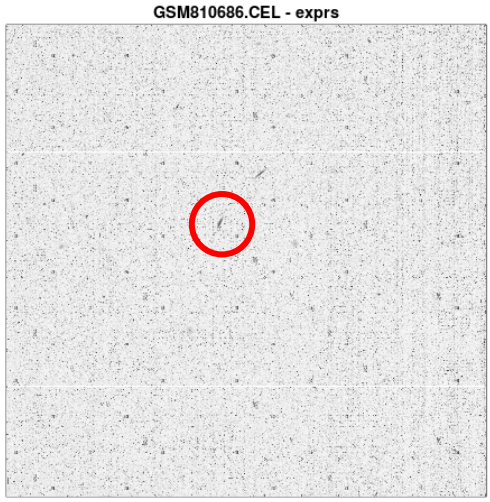
[2] http://www.bioconductor.org/packages/2.0/bioc/html/oligo.html

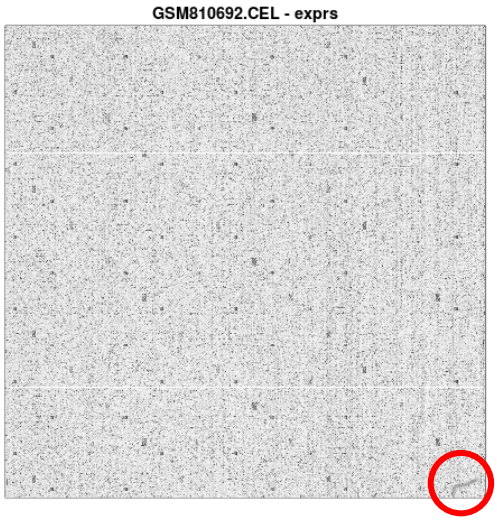
Batch	List of Affected Chips	No.
Essen	GSM541726, GSM810686, GSM810692, GSM810703, GSM810713, GSM810727, GSM810737, GSM810745, GSM810763	9
Amsterdam	NRC0223 (this chip does not have survival time annotation)	1

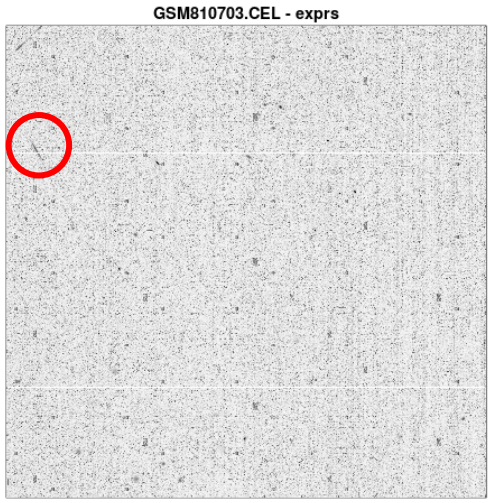
Since the overall signal intensity of Essen chips was much weaker than that of Amsterdam chips, we tried to remove all Essen chips that seemed to be affected by artifacts. About 1/3 of Amsterdam chips showed blurry circular artifacts in the center, but we did not filter them out since they were not distinct.

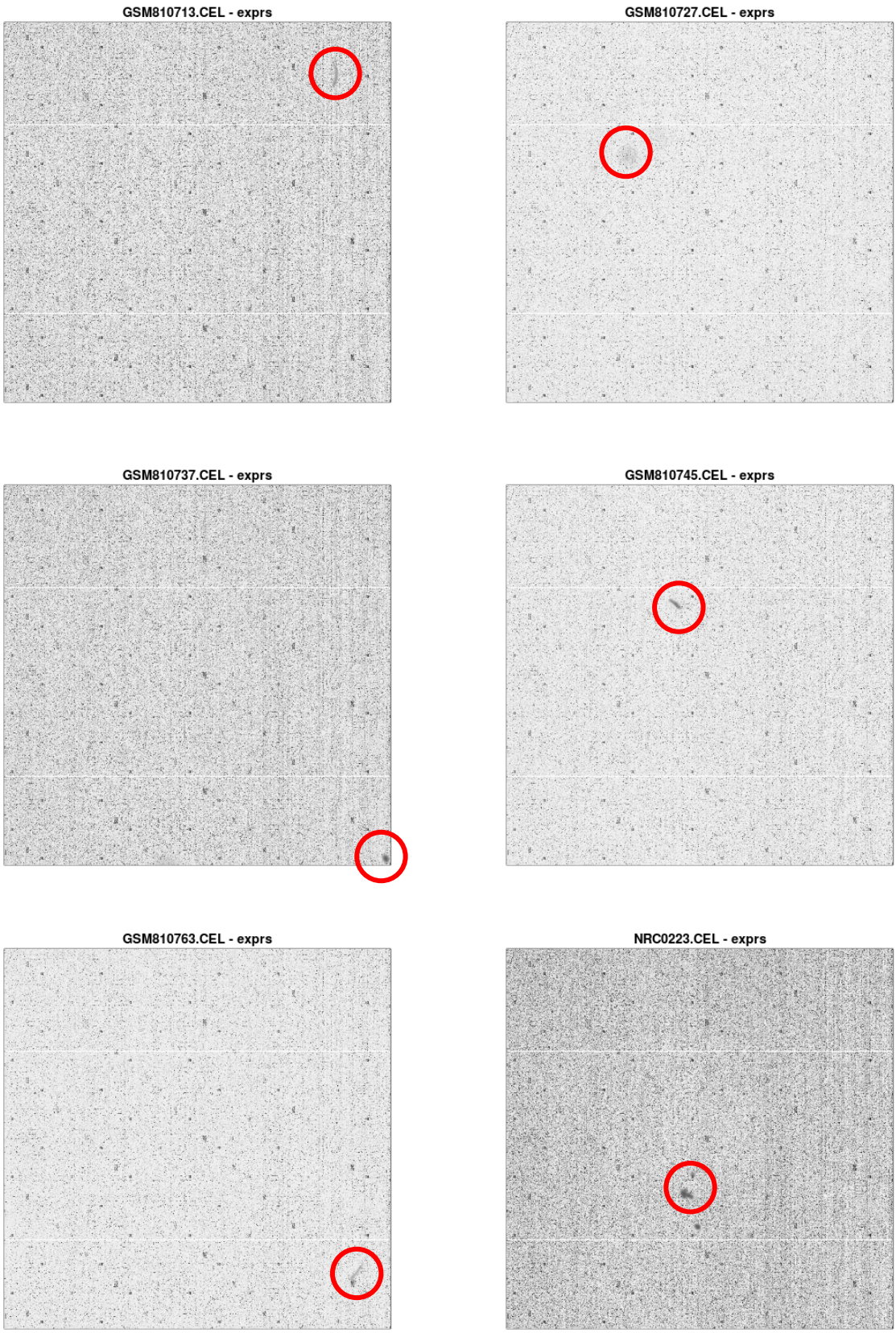
We show the images of all discards chips at this stage.











These chips with artifacts are discarded from any further processing, so that they would not affect RMA processing, for instance, in the following sections.

2.2 Quality Control Information from APT

Given a collection of CEL files, we can use the Affymetrix Power Tools[3] (APT) to create a summary of probes in the CEL files, as well as to obtain useful quality control information about the probes. Here a "summary" can be made either in exon-level or in transcript-level (interchangeably, gene-level), as each exon is detected by a collection of probes, and a transcript (or a gene) is again a collection of exons.

We begin with the following arrays, applying APT for each batch.

Batch	No. of Chips
Essen	113 – 9 (artifacts) = 104
Amsterdam	182 – 1 (artifacts) = 181
Total	285

For instance, we run APT as follows to obtain the exon-level summary of probes and the quality control information of Essen chips:

```
apt-probeset-summarize \
        -p HuEx-1_0-st-v2.r2.pgf \
        -c HuEx-1_0-st-v2.r2.clf \
        -b HuEx-1_0-st-v2.r2.antigenomic.bgp \
        --qc-probesets HuEx-1_0-st-v2.r2.qcc \
        -s HuEx-1_0-st-v2.r2.dt1.hg18.core.ps \
        -a rma \
        -o output_dir \
        ./ESSEN/*.CEL
```

Here the "-s" option specifies the category of exon-level summary we make about probes. The exons are categorized into "core", "extended", "full", "free", and "ambiguous", by the decreasing order of evidence we have about them. In this article we focus on the core exons. For gene-level summaries, the "-s" option is replaced by "-m". For example, to create core gene-level summary, we use

```
        -m HuEx-1_0-st-v2.r2.dt1.hg18.core.mps
```

We refer to an introductory article (Lockstone 2011) for details about the options and the Affymetrix library and meta probe set information files.

The above command generates two tab-separated text files in the output_dir:

- rma.summary.txt : the summary of probes in a specified category.

[3] APT version 1.15.0, the latest release at the time of writing, is available at http://www.affymetrix.com/partners_programs/programs/developer/tools/powertools.affx

- rma.report.txt : the quality control information of arrays

The quality control information is generated only if we specify the "--qc-probesets" option. In usual cases, the options above are all we need. More details about the options can be found on the APT online manual (Affymetrix 2007a).

In this section we describe how we use the quality control information. The output from APT provides many characteristics about the input arrays, and we focus a few of them that we consider significant. Note that after finding out low-quality arrays, we will run APT again to make summaries, excluding these low-quality arrays. We believe such exclusion is important since low-quality arrays are likely to have adverse effect on summary outcomes, especially for the popular robust multi-array analysis (RMA) algorithm (Irizarry 2003).

We follow a white paper from Affymetrix (Affymetrix 2007b) for quality assessment of arrays based upon the information from APT.

PM vs. Background

First of all, we examine the signal strength of probes. For this we use two sets of values from the quality information:

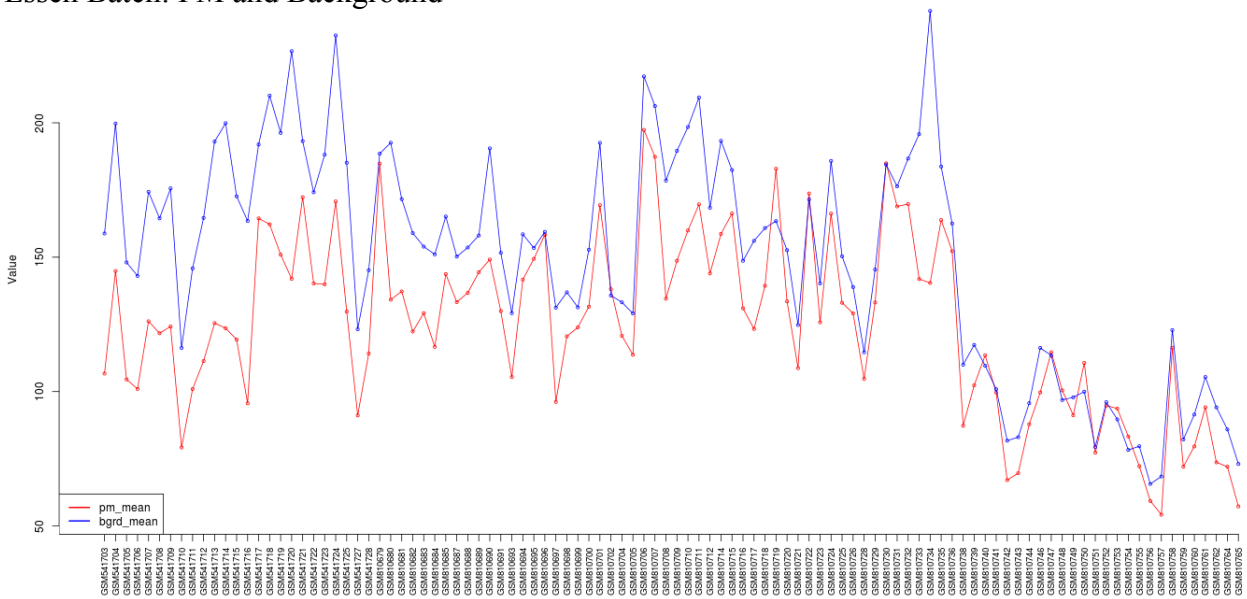
- pm_mean: the averaged raw intensity values of PM (perfect match) probes.
- bgrd_mean: the averaged raw intensity values of background probes.

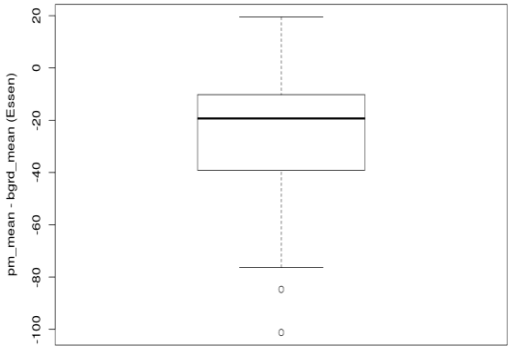
The "pm_mean" and "bgrd_mean" are also the corresponding column names of the quality information table in the rma.report.txt file. These values are about the raw intensity values, prior to any transformation.

The pm_mean values can be used to find chips that are too bright: being too bright is not a problem by itself because algorithms such as RMA can handle it to a certain degree, but it tells us that a closer look would be necessary. Also, in usual cases, the pm_mean and bgrd_mean values are positively correlated. So low pm_mean with high bgrd_mean values may indicate low quality. But as these are only indicative measures, we only use them to find arrays with possible issues.

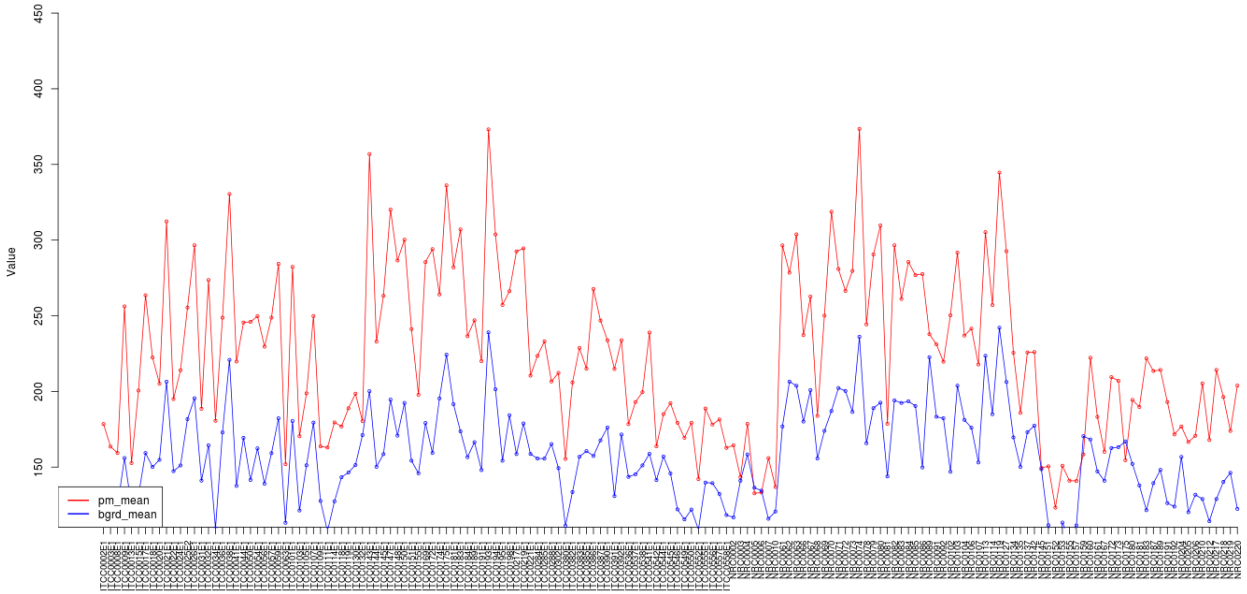
The next plots show pm_mean and bgrd_mean values for two batches, Essen and Amterdam. The pm_mean values range over 50~200 in the Essen batch, and over 100~400 in the Amsterdam batch. We mark the Essen chips with pm_mean < 100, as well as the two Essen chips with seemingly anti-correlated pm/bg values (outliers in the boxplots), for further inspection. All Amsterdam chips were fine with these criteria.

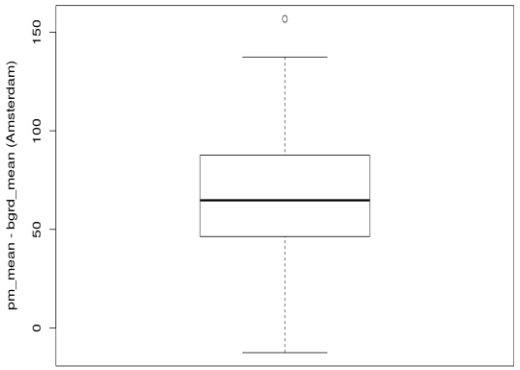
Essen Batch: PM and Background





Amsterdam Batch: PM and Background





The arrays marked for closer look is listed here:

Criteria	List of Arrays	No
pm_mean < 100	GSM541710, GSM541716, GSM541727, GSM810697, GSM810738, GSM810741, GSM810742, GSM810743, GSM810744, GSM810746, GSM810749, GSM810751, GSM810752, GSM810753, GSM810754, GSM810755, GSM810756, GSM810757, GSM810759, GSM810760, GSM810761, GSM810762, GSM810764, GSM810765	24
anti-correlation	GSM541720, GSM810734	2

AUC, MAD and RLE

Here we use three quality measures, pos_vs_neg_auc, all_probeset_mad_residual_mean, and all_probeset_rle_mean (we also call them as "auc", "mad", and "rle", respectively).

The pos_vs_neg_auc (auc) is the area under the ROC curve, comparing signal values for positive control to the negative control probe sets. The AUC value of 1.0 reflects perfect separation of two probe sets, whereas the value of 0.5 indicates no separation. Typical auc values in Affymetrix arrays are between 0.8 and 0.9.

The all_probeset_mad_residual_mean (mad) is the mean value of the absolute deviation of probes from the median. Here "all_probeset" stands for the probes in a specific category, which are "core exons" in our case. The mad values tell us the difference between the actual and the predicted values by a model used in the processing algorithm (e.g. RMA). So exceptionally high mad values would indicate problems in the corresponding chips.

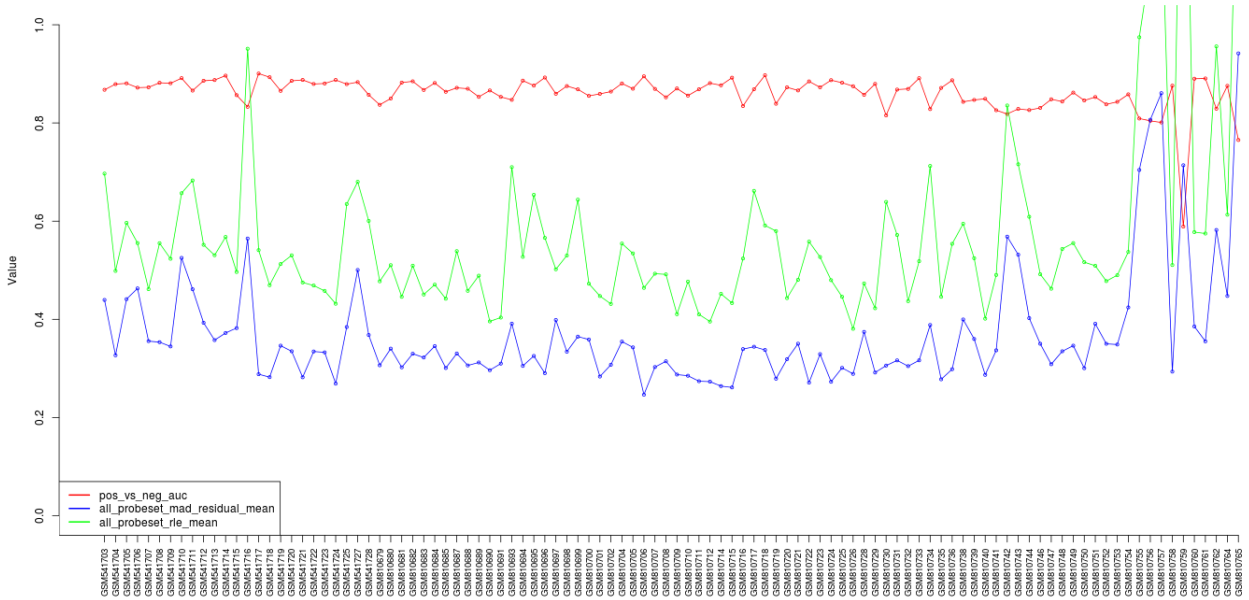
The all_probeset_rle_mean (rle) is the mean value of the absolute relative log expression (RLE) for all core exon probe sets, which is defined by

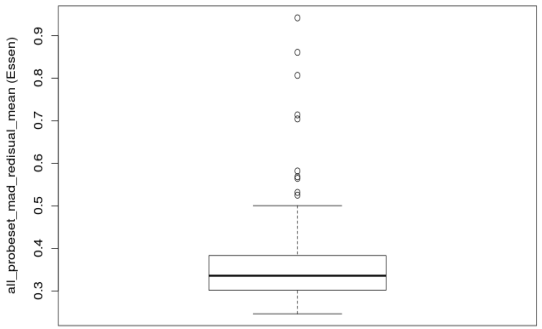
$$\text{RLE(a probe set p)} = \log_2 (| \text{signal(p)} - \text{median(p)} |).$$

The RLE values are averaged over probes in each array to produce the all_probeset_rle_mean value. This shows the variability in expression of each array, which could come from measurement error but also from sheer biological variation.

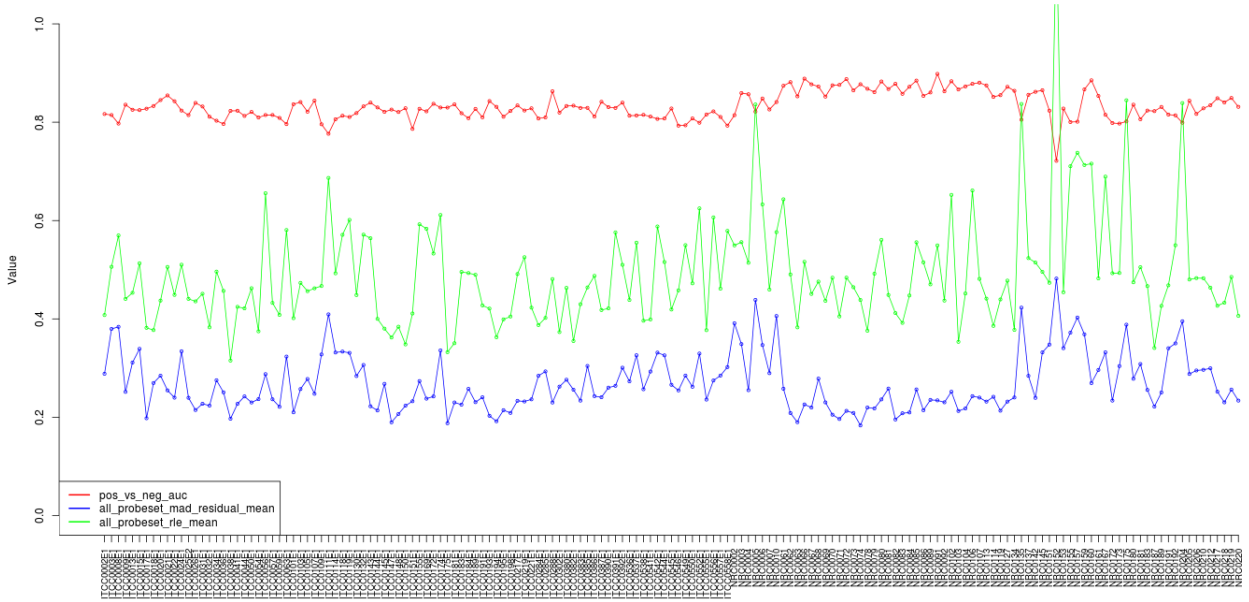
In the next page, we show the plots of the three quality measures.

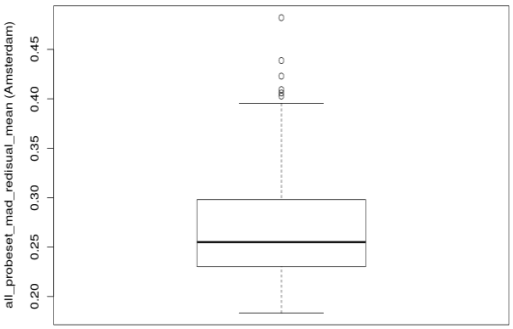
Essen Batch: AUC, MAD, and RLE





Amsterdam Batch: AUC, MAD, and RLE





Overall, the auc values (red curves) are higher in Essen chips (mean 0.86) than in Amsterdam chips (mean 0.83), although Essen chips look much brighter than Amsterdam chips. Considering the typical auc values in 0.8~0.9, we choose the threshold rule (auc < 0.8) to detect low-quality arrays. Here are the lists:

Batch (criterion)	List of Arrays	No.
Essen (auc < 0.8)	GSM810759, GSM810765 (both are in our bad-chip candidate due to pm_mean < 100)	2
Amsterdam (auc < 0.8)	ITCC0008E1, ITCC0036E1, ITCC0063E1, ITCC0109E1, ITCC0111E1, ITCC0151E1, ITCC0546E1, ITCC0549E1, ITCC0552E1, ITCC0558E1, NRC0152, NRC0172, NRC0173, NRC0204	14

Next, we investigate the mad values (blue curves). Seeing the outliers in the two boxplots of mad values above, we choose a reasonable threshold (mad > 0.5) for detection.

Batch (criterion)	List of Arrays	No.
Essen (mad > 0.5)	GSM541710, GSM541716, GSM541727, GSM810742, GSM810743, GSM810755, GSM810756, GSM810757, GSM810759, GSM810762, GSM810765	11
Amsterdam (mad > 0.5)	None	0

Finally, for the rle values, it is not as straightforward as the two previous quality measures to define a good threshold, since high rle values could be from meaningful biological differences. Therefore we do not use the rle values for filtering, but as we can see in the plots above, almost all chips with unusually high rle values are already removed by the mad > 0.5 criterion in the Essen batch, and by auc < 0.8 criterion in the Amsterdam batch.

Polyadenylated RNA Spike Controls

The polya_spike probes consist of four sets of polyadenylated RNA spike probes (Lys, Phe, Thr, and Dap). The RNA sequences of these probes do not appear in humans, and therefore we can use them for quality control. The polya_spike probes are designed to detect errors in the target preparation stage of microarray experiments.

In normal situation, the expression of polya_spike control probes should follow the following order:

- polya_spike : Lys < Phe < Thr < Dap.

The spike control probes typically show higher variability across arrays than other quality measures as they are shorter in length, but it is unimportant since we only examine the rank order of four probe sets in individual arrays.

We have found few arrays that do not preserve the correct order:

Batch (criterion)	List of Arrays	No.
Essen (polya_spike)	GSM810752, GSM810753, GSM810754, GSM810759, GSM810765	5
Amsterdam (polya_spike)	ITCC0544E1	1

In the next page we show the plots of polya_spike control probes for two batches.

Bacterial RNA Spike Controls

The bac_spike is another set of spike control probes, comprising of four subsets that hybridize to the pre-labeled bacterial RNA spike control probes (BioB, BioC, BioD, and Cre). This category is useful for detecting problems in the hybridization phase or in the arrays themselves.

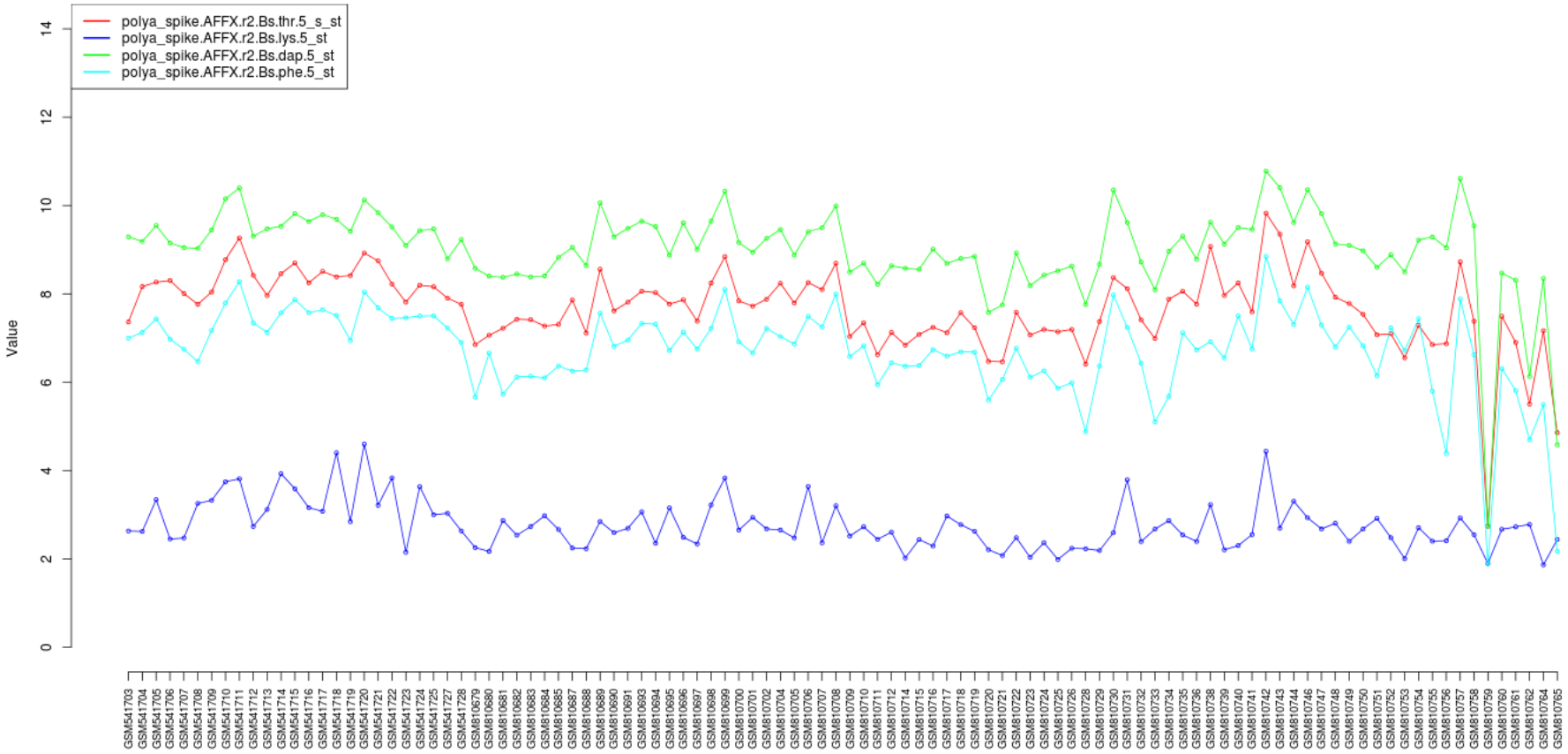
The usage of bac_spike control is similar to polya_spike. In normal situations, the expression of bac_spike probes should follow the predetermined order:

- bac_spike : BioB < BioC < BioD < Cre

There was no array in our data which showed incorrect bac_spike rank order.

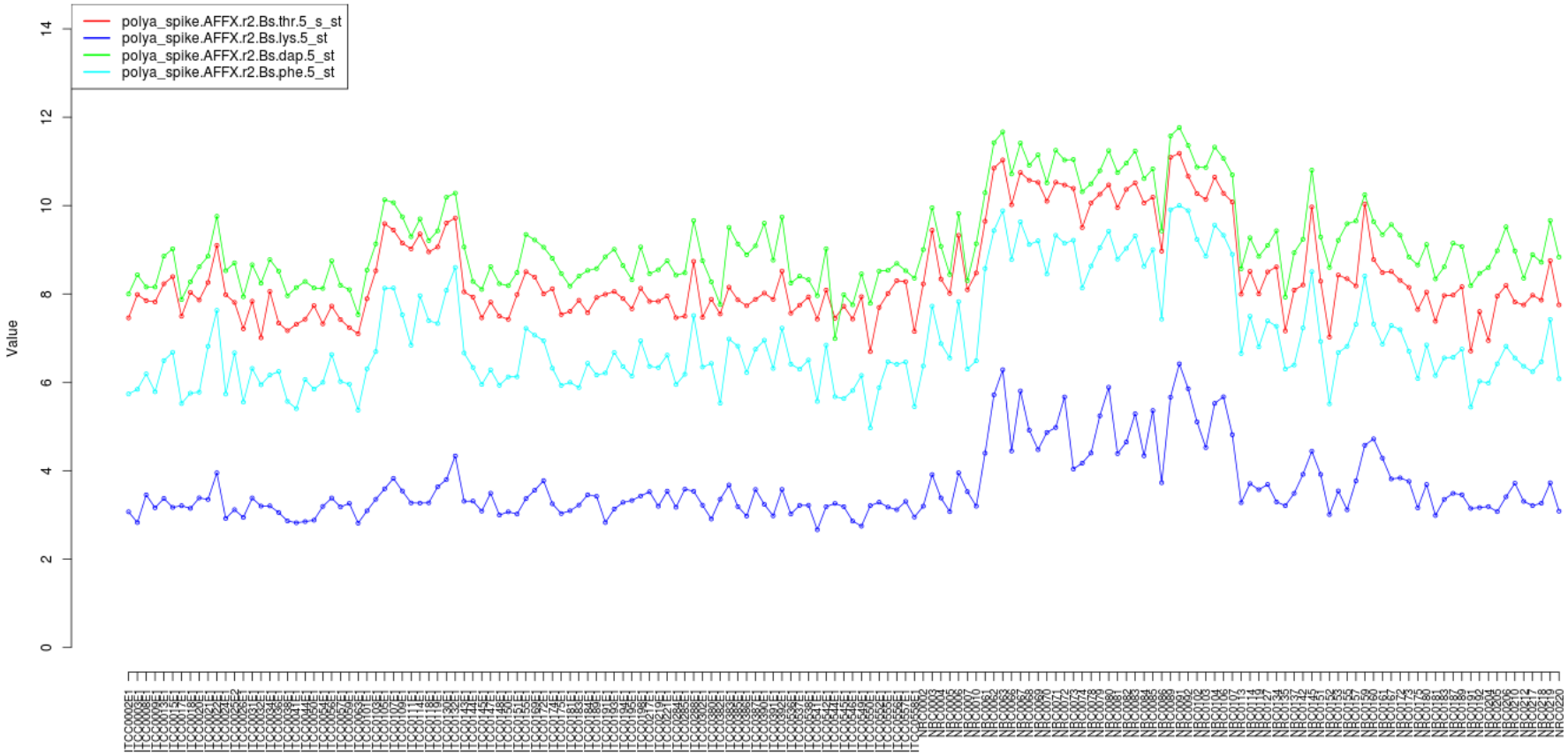
Essen Batch: Polyadenylated Spike Control

The correct order is: Lys (blue) < Phe (cyan) < Thr (red) < Dap (green)



Amsterdam Batch: Polyadenylated Spike Control

The correct order is: Lys (blue) < Phe (cyan) < Thr (red) < Dap (green)



2.3 Quality Assessment

Now we combine the information we have gathered in Section 2.2, and determine the arrays to exclude because of their inferior measurement quality.

For concise description, we name the two criteria for detecting possibly low-quality chips:

- CC1 (candidate criterion 1): pm_mean < 100, and
- CC2 (candidate criterion 2): pm/bgrd anti-correlation.

Similarly, the three more definitive quality criteria are called as follows:

- QC1 : auc < 0.8,
- QC2: mad > 0.5, and
- QC3: polya_spike has incorrect rank ordering.

Essen Batch

First, for the candidates due to CC1, we exclude the chips that are also detected by any one of the three quality criteria, Q1, Q2, and Q3.

Next, for the candidates due to CC2, we there was no outstanding problem in terms of QC1, QC2, or QC3. Then we investigated the CEL file images again, and found one of the two candidates had visible scratches. We thus excluded this chip, but did not exclude the other that showed no visible artifacts.

To conclude, we removed 15 chips from the Essen batch, in addition to the ones that have already removed by visual inspection.

Amsterdam Batch

Among the Amsterdam arrays, we only had the ones detected by QC1 and QC3. In fact, for QC1, the auc values were very close (about 0.79) to the threshold value of 0.80. Therefore we have removed only the chips that had auc values strictly less than 0.79. We had a single chip detected by QC3, having incorrect order in the polya_spike category. We removed this chip as well.

To conclude, we removed 4 chips from the Amsterdam batch, in addition to the ones that have removed by visual inspection.

The tables in the following pages summarize the situation.

Essen Batch

	CC1	CC2	QC1	QC2	QC3	**Decision**
CEL filename	pm < 100	pm/bg anticor	auc < 0.8	mad > 0.5	polya order	**Remove**
GSM541710	X			X		X
GSM541716	X			X		X
GSM541720		X (looks fine)				
GSM541727	X			X		X
GSM810697	X					
GSM810734		X (scratches)				X
GSM810738	X					
GSM810741	X					
GSM810742	X			X		X
GSM810743	X			X		X
GSM810744	X					
GSM810746	X					
GSM810749	X					
GSM810751	X					
GSM810752	X				X	X
GSM810753	X				X	X
GSM810754	X				X	X
GSM810755	X			X		X
GSM810756	X			X		X
GSM810757	X			X		X
GSM810759	X		X 0.5890	X	X	X
GSM810760	X					
GSM810761	X					
GSM810762	X			X		X
GSM810764	X					
GSM810765	X		X 0.7654	X	X	X

Amsterdam Chips

	Candidate	Candidate	Q1	Q2	Q3	**Decision**
CEL filename	pm < 100	pm/bg anticor	auc < 0.8	mad > 0.5	polya order	**Remove**
ITCC0008E1			X 0.7973			
ITCC0036E1			X 0.7965			
ITCC0063E1			X 0.7963			
ITCC0109E1			X 0.7959			
ITCC0111E1			X 0.7766			X
ITCC0151E1			X 0.7865			X
ITCC0544E1					X	X
ITCC0546E1			X 0.7931			
ITCC0549E1			X 0.7938			
ITCC0552E1			X 0.7990			
ITCC0558E1			X 0.7931			
NRC0152			X 0.7216			X
NRC0172			X 0.7985			
NRC0173			X 0.7973			
NRC0204			X 0.7991			

3 RMA PROCESSING

At this point we have discarded all arrays that have been classified as of low quality. We apply the RMA algorithm again for the remaining arrays, to make gene or exon level summaries for further analysis. The numbers of arrays look as follows:

Batch	No. of Chips
Essen	113 – 9 (artifacts) – 15 (quality) = 89
Amsterdam	182 – 1 (artifacts) – 4 (quality) = 177
Total	266

We run the robust multi-array analysis (RMA) algorithm (Irizarry 2003) for each batch separately, using the Affymetrix Power Tools (APT). Since the model used in the RMA algorithm assumes that the distribution of each probe is similar across all input arrays, which is often not true for arrays in different batches, it is desirable to apply RMA on each batch separately. In our case, the overall signal intensity of the Essen batch was much lower (dimmer images) than that of the Amsterdam batch, so we applied RMA separately for each batch.

3.1 The RMA Algorithm

For better understanding, we briefly introduce the RMA algorithm. RMA consists of (1) background correction, (2) quantile normalization, (3) $\log_2$ transformation, and (4) fitting a linear model to take advantage of "probe effect", that the variability between probes of a probe set in an array is typically larger than the variability of an individual probe across arrays. We focus on the steps (2) and (4) since others are straightforward.

Quantile Normalization

The goal of the step (2) is to make the distribution of probe intensities the same across, say, *n* arrays. This can be done by quantile normalization (Bolstad 2003). Let $\boldsymbol{q}_k = (q_{k1}, q_{k2}, \dots, q_{kn})$ be the vector of the *k*th quantiles for n arrays. To make the *k*th quantiles the same for all arrays, we project $\boldsymbol{q}_k$ on the *n*-dimensional diagonal vector $\boldsymbol{d} = \left(\frac{1}{\sqrt{n}}, \dots, \frac{1}{\sqrt{n}}\right)$, to obtain

$$proj_{\boldsymbol{d}}\boldsymbol{q}_k = \left(\frac{1}{n}\sum_{j=1}^{n} q_{kj}, \dots, \frac{1}{n}\sum_{j=1}^{n} q_{kj}\right).$$

That is, we can substitute the original values by their corresponding mean quantiles. We can write a simple algorithm for doing this:

1. Given *n* arrays with *p* features, construct a matrix X by putting arrays as columns.
2. Sort each column of *X*, producing X_{sort}.
3. For each row of X_{sort}, take the mean, and replace the row elements with the mean.
4. Rearrange X_{sort} so to have the same ordering as the *X*.

Since the original probe intensity values are replaced by mean values at each quantile, it would be undesirable to process arrays with genomic difference at the same time. For instance, if we have arrays for which the probes corresponding to gene A and B have their quantile orders exchanged, then the means can be taken mixing the expression of A and B.

Probe-Level Model Fit

After background correction, quantile normalization, and log2 transformation, the probe-level intensity values Y_{ijs} are used to find linear model fits (McCall 2010),

$$Y_{ijs} = \theta_{is} + \phi_{js} + \epsilon_{ijs}$$

for probes $j \in 1, \dots, J_s$ in probe sets $s \in 1, \dots, S$ on arrays $i \in 1, \dots, n$. Here θ_{is} represents the expression of a probe set *s* on an array *i*, ϕ_{js} is the probe effect for the *j*th probe of the probe set *s*. The last term represents measurement error. The purpose of this model is to capture the variability of probes in each probe set. After fitting the model, RMA reports the estimate $\widehat{\theta}_{is}$ as the final outcome.

To make the parameters identifiable, we assume that $\sum_{j=1}^{J_s} \phi_{js} = 0$ for each probe set *s*. This can be seen as assuming that on average, the probes measure the true expression. A popular way to estimate the parameters is the median polish method (Tukey 1977). To apply median polish, we rewrite the above model in a two-way layout for each probe set *s*,

$$\begin{matrix} e_{11} & \dots & e_{1J_S} & a_1 \\ \vdots & \ddots & \vdots & \vdots \\ e_{n1} & \dots & e_{nJ_S} & a_n \\ b_1 & \dots & b_{J_S} & m \end{matrix}$$

where $e_{ij} = Y_{ijs}$, and $a_i = b_j = m = 0$, for a fixed *s*. Then median polish produces $a_i \to \theta_{is}$ and $b_j \to \phi_{js}$ as we proceed, reducing residuals $e_{ij} \to 0$.

The median polish method alternates a row-sweep and a column-sweep, often up to two pairs of sweeps. In the row-sweep, for each row, we compute the median of the first J_s columns, subtract it from those columns, and add it to the last column. In the column-sweep, for each column, we compute the median of the first *n* rows, subtract it from those rows, and add it to the last row. We repeat this procedure until all residuals become close to zero. The estimates obtained from median polish are known to be more robust to outliers than least-squares estimates.

3.2 A Side Note

There are several arrays for which we do not have valid survival time annotation:

- Chips without survival time annotation (7 chips): ITCC0533E1, ITCC0551E1, NRC0009, NRC0099, NRC0100, NRC0109, NRC0110.
- Chips with too short (< 5 days) survival time without an event (11 chips): NRC0064, NRC0075, NRC0077, NRC0087, NRC0088, NRC0090, NRC0098, NRC0203, NRC0208, NRC0209, NRC0225.

Also, there are four arrays that pass all quality tests, but show unusually high all_probeset_rle_mean values: NRC0005, NRC0135, NRC0175, NRC0204.

We do not exclude these chips in the RMA processing.

4 COMBINING MULTIPLE BATCHES

Our final step of microarray preprocessing is to make multiple batches comparable to each other. This step is necessary because (i) we prefer to have large number of arrays in down-stream analyses whenever possible, but creating a single large batch is impractical, and (ii) if we apply RMA separately on multiple input array sets, then the resulting sets are not likely to be comparable since a local reference distribution is made from given data in each quantile normalization step of RMA. In addition, (iii) in order to classify new patients using a predictor learned from training data, we have to make the data from new patients comparable to the training data. Otherwise, the predictor would not work.

For convenience, we call the method that makes multiple (RMA-processed) batches comparable as a data combining (or a data merging) method. We prefer to choose a data merging method that is applicable for each batch independently, especially for the batches from new patients.

We should mention that there is a possible alternative for multi-array summarizing, called the frozen RMA (McCall 2010), which produces comparable outcomes even if we apply it per batch or per array. The difference of fRMA to RMA is that fRMA makes use of a global reference created from publicly available expression arrays, instead of a local reference from input arrays. Therefore no data merging is required after applying fRMA. We found that fRMA worked fine for gene chips, but unfortunately not for exon arrays. We expect that the performance of fRMA will improve with more exon arrays available in public.

It is a common practice is to apply RMA for all arrays in a study. However, this may not be preferable in several aspects: (i) if there are arrays with permuted probe distributions, then quantile normalization in RMA could make averages over probes corresponding to different genes/exons, (ii) lumping arrays together in order to cancel out batch effects is not a good idea, since RMA does not explicitly deal with any batch effect[4], and (iii) in principle, test data should be prepared separately.

In a strict sense, the arrays that are processed at the exactly same condition should define a batch. However, the size of such batches is quite small (24~96) due to technical limitations, and we often do not keep the corresponding batch information. In this article, we consider batches in terms of two processing locations, Essen and Amsterdam, since our arrays look most significantly different at this level of separation. So, strictly speaking, our batch stands for an experiment, a collection of batches.

In the following we make a short survey of the data merging methods that look the most plausible for our purpose

[4] A simple 3-D projection of data using PCA often reveals this fact.

4.1 Data Merging Methods

There are only handful approaches for making data from multiple batches comparable. A collection of them can be found, for example, in an R package called "inSilicoMerging"[5].

We introduce three methods, SIMPLE, BMC and COMBAT, using their names in the inSilicoMerging package. The SIMPLE method just combines batches together without any transformation.

BMC (Mean Shifting)

This method makes each exon expression centered at the zero value, by subtracting the mean expression of each exon over arrays from the expression value of the exon, per batch. This method does not perform any scaling (by standard deviation, for example). No scaling might be preferable in our case, since (i) RMA-processed data usually have similar scale, and (ii) scaling may conceal biological variability. It is reported that this method can cancel multiplicative systematic bias (Sims 2008).

The merit of this method is that we can apply it completely independently on each batch.

COMBAT

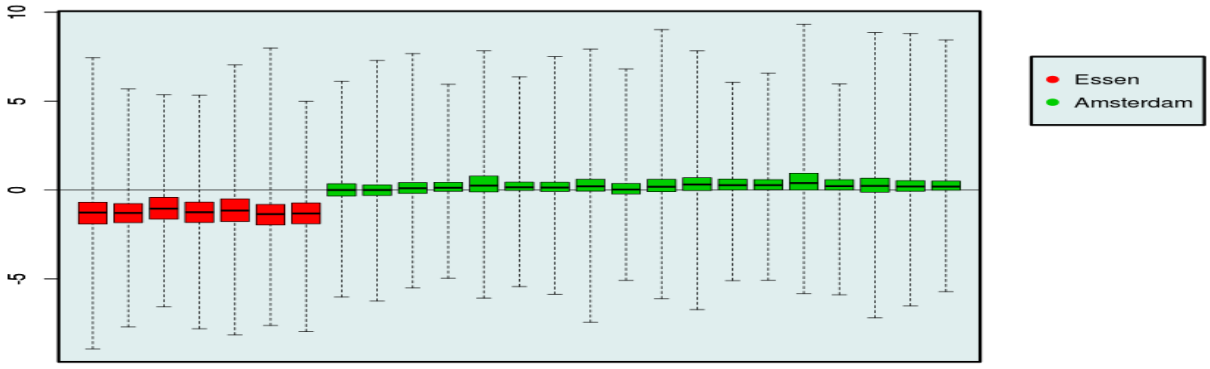
COMBAT is an empirical Bayesian approach that tries to estimate and cancel batch effects (Johnson 2007). This method is known to work better than other merging methods when the sizes of batches are small (< 25). In our case, we cannot find any significant difference to the simpler alternative, BMC. Also, COMBAT requires to "see" all batches at once, and thus it cannot be applied separately for each batch.

In the next page we show the mean RLE (relative log expression) values of 25 arrays randomly selection from the Essen and Amsterdam batches, after merging the data by SIMPLE, BMC, and COMBAT, respectively. Clearly, BMC and COMBAT make the intensity values more similar across batches, compared to SIMPLE. Comparison between BMC and COMBAT is less obvious.

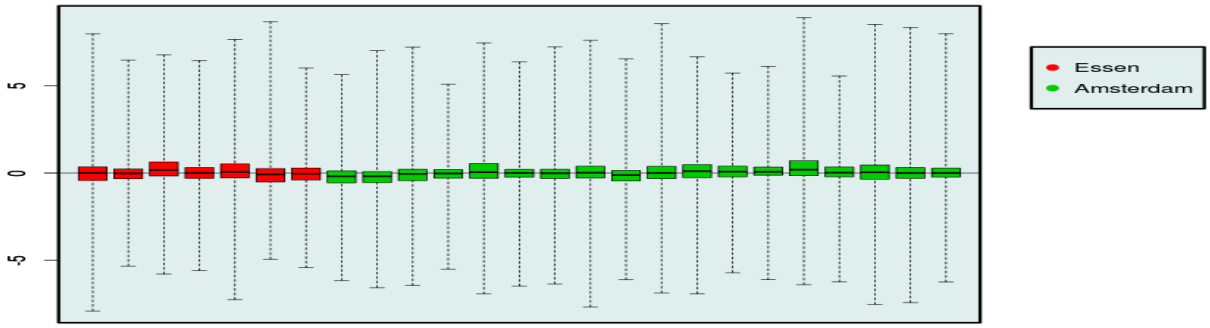
In conclusion, we choose the BMC (mean shifting) method for combining our batches.

[5] http://www.bioconductor.org/packages/2.11/bioc/html/inSilicoMerging.html

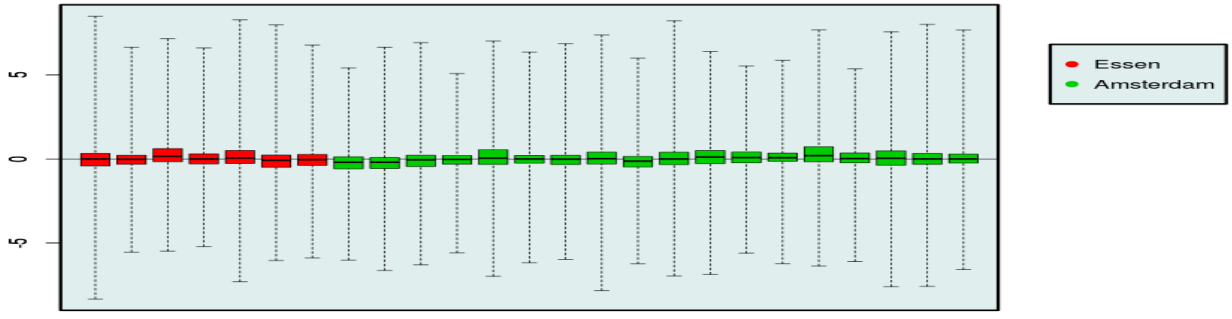
SIMPLE:



BMC:



COMBAT:



5 PER ARRAY PROCESSING – FROZEN RMA

The preprocessing approach we have discussed so far has several caveats: (1) it requires to apply the algorithm again for the entire arrays whenever new arrays arrive, and (2) it is not straightforward which algorithm to choose for merging RMA-processed batches of data.

An alternative of RMA is the frozen RMA (McCall 2010). In addition to the probe effects, fRMA is designed to handle probe-specific random effects. Comparing to RMA, fRMA makes use of a global reference, instead of a reference distribution estimated from input arrays. The global reference is platform specific, and obtained from a large number of arrays available in public (GEO, for example).

In this report, we use the fRMA package in Bioconductor (version 1.11.0) with the global reference package for human exon arrays, huex.1.0.st.v2frmavecs (version 0.0.4).

There is a useful addendum to fRMA, called GNUSE (global normalized unscaled standard error), which allows us to assess the quality of individual arrays (M. McCall 2011). Here we use only GNUSE scores to access array quality, without performing visual inspection and filtering by quality measures provided by the APT (Affymetrix Power Tools).

The GNUSE score is computed for each probe in an individual array, and the median value > 1.0 and large IQR indicate that the array is of poor quality. As some recent papers make use of looser threshold value of 1.2, we try 1.0, 1.1, and 1.2 and see their characteristics.

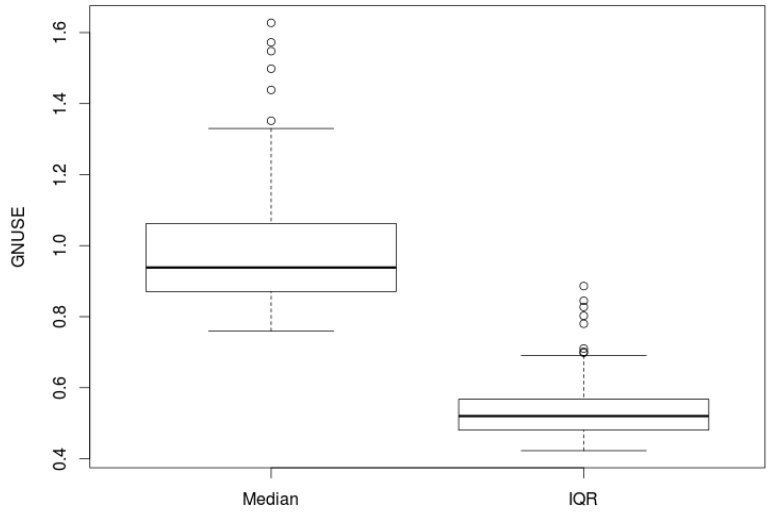
We begin with the following arrays:

Batch	No. of Chips	CEL Filename Prefix
Essen	113	GSM
Amsterdam	182	ITCC and NRC
Total	295	-

5.1 GNUSE Values of Arrays

We show the boxplot of median and IQR of GNUSE values for 295 arrays. In fact many arrays from Essen had low GNUSE values.

In an ideal situation, fRMA will allow us to use the processed data as they are, without further processing for batch effect cancelation. We investigate if our case is so, for different threshold values of GNUSE median, 1.0, 1.1, and 1.2, by computing the correlation between batch annotation and clustering of fRMA-processed data. If batch effect is not resident after fRMA, the correlation between the two should be negligible. Otherwise, we may need extra work to deal with batch effect.



5.2 Filtering by GNUSE of fRMA-Processed Arrays

Here we summarize the arrays to be filtered for three threshold values.

Threshold for median GNUSE values	No. of arrays to be removed	Arrays
1.2	21	GSM541703, GSM541705, GSM541706, GSM541710, GSM541711, GSM541712, GSM541716, GSM541727, GSM810686, GSM810727, GSM810742, GSM810743, GSM810754, GSM810755, GSM810756, GSM810757, GSM810759, GSM810760, GSM810762, GSM810764, GSM810765
1.1	53	GSM541703, GSM541705, GSM541706, GSM541707, GSM541708, GSM541709, GSM541710, GSM541711, GSM541712, GSM541713, GSM541714, GSM541715, GSM541716, GSM541720, GSM541725, GSM541727, GSM541728, GSM810682, GSM810683, GSM810684, GSM810686, GSM810693, GSM810697, GSM810698, GSM810699, GSM810700, GSM810704, GSM810705, GSM810718, GSM810721, GSM810727, GSM810728, GSM810734, GSM810738, GSM810742, GSM810743, GSM810744, GSM810746, GSM810749, GSM810751, GSM810752, GSM810753, GSM810754, GSM810755, GSM810756, GSM810757, GSM810759, GSM810760, GSM810761, GSM810762, GSM810763, GSM810764, GSM810765
1.0	105	GSM541703, GSM541704, GSM541705, GSM541706, GSM541707, GSM541708, GSM541709, GSM541710, GSM541711, GSM541712, GSM541713, GSM541714, GSM541715, GSM541716, GSM541717, GSM541718, GSM541719, GSM541720, GSM541721, GSM541722, GSM541723, GSM541724, GSM541725, GSM541726, GSM541727, GSM541728, GSM810680, GSM810681, GSM810682, GSM810683, GSM810684, GSM810685, GSM810686, GSM810687, GSM810688, GSM810689, GSM810690, GSM810691, GSM810693, GSM810694, GSM810695, GSM810696, GSM810697, GSM810698, GSM810699, GSM810700, GSM810702, GSM810703, GSM810704, GSM810705, GSM810708, GSM810709, GSM810711, GSM810712, GSM810716, GSM810717, GSM810718, GSM810720, GSM810721, GSM810723, GSM810725, GSM810726, GSM810727, GSM810728, GSM810729, GSM810731, GSM810733, GSM810734, GSM810736, GSM810738, GSM810739, GSM810740, GSM810741, GSM810742, GSM810743, GSM810744, GSM810745, GSM810746, GSM810747, GSM810748, GSM810749, GSM810750, GSM810751, GSM810752, GSM810753, GSM810754, GSM810755, GSM810756, GSM810757, GSM810758,

		GSM810759, GSM810760, GSM810761, GSM810762, GSM810763, GSM810764, GSM810765, ITCC0003E1, ITCC0111E1, ITCC0114E1, NRC0145, NRC0151, NRC0152, NRC0155, NRC0157

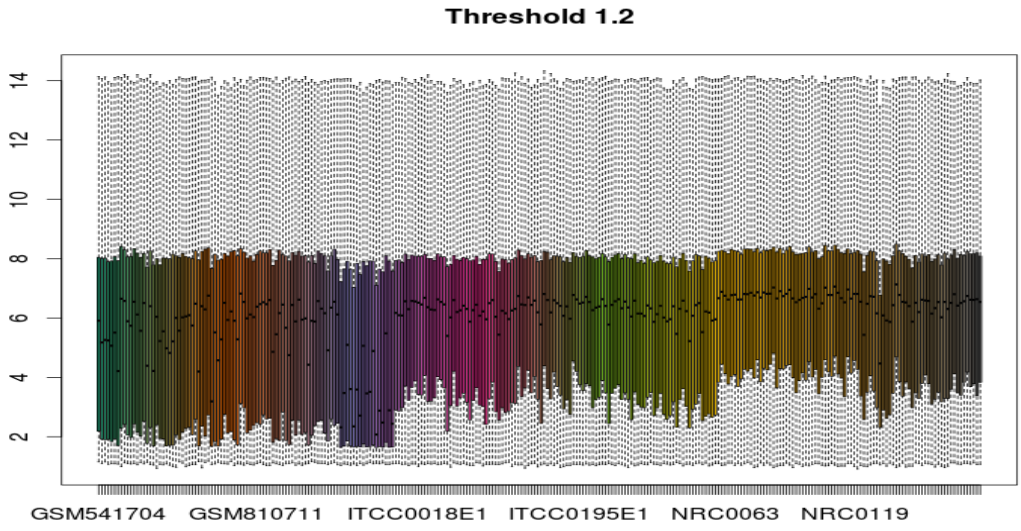
We show the plots of exon expression after fRMA processing, for the three threshold values of median GNUSE in the next page. As we can see, there are few arrays still remaining after filtering with the threshold values 1.2 and 1.1, that have quite different median expression compared to the others. For the threshold value 1.0 however, median expression values seem alike.

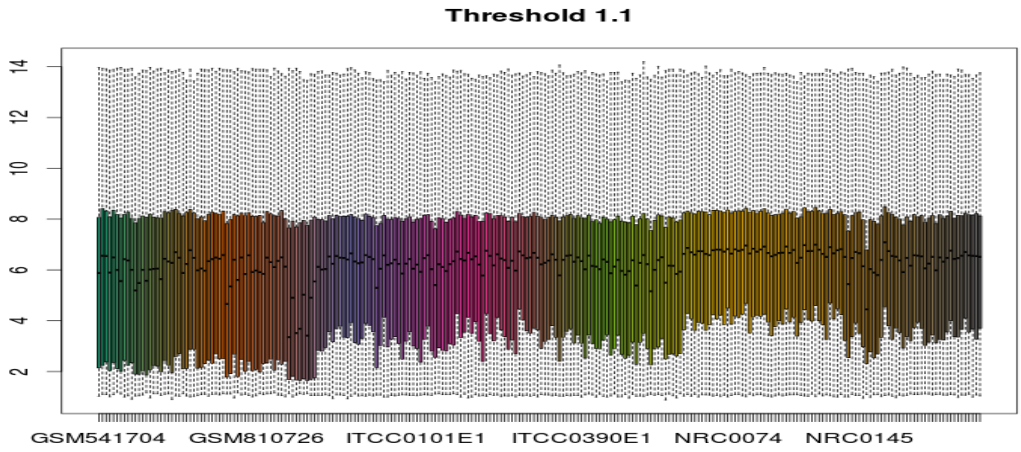
We compute the correlation between the batch annotation (a vector of 1, 2, and 3 corresponding to "GSM", "ITC" and "NRC" arrays) and the kmeans clustering result (a vector of cluster assignment, in our case mostly 1 to "GSM", 2 to "ITC" and 3 to "NRC"). High correlation between the two vectors suggests that the fRMA-processed data still has high correlation to the batches.

Threshold	1.2	1.1	1.0
Correlation	0.81	0.58	0.23

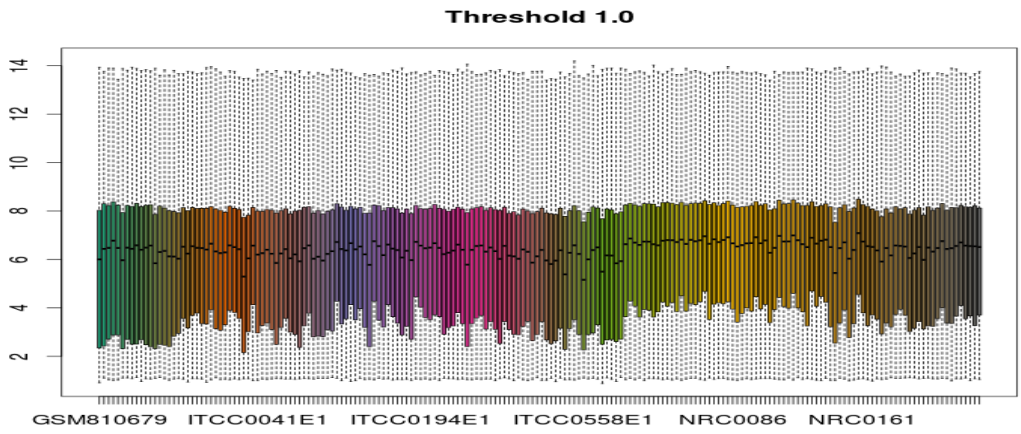
In this respect, the threshold value of 1.0 appears to be the best choice, except for the fact that it removes almost all arrays from the Essen batch.

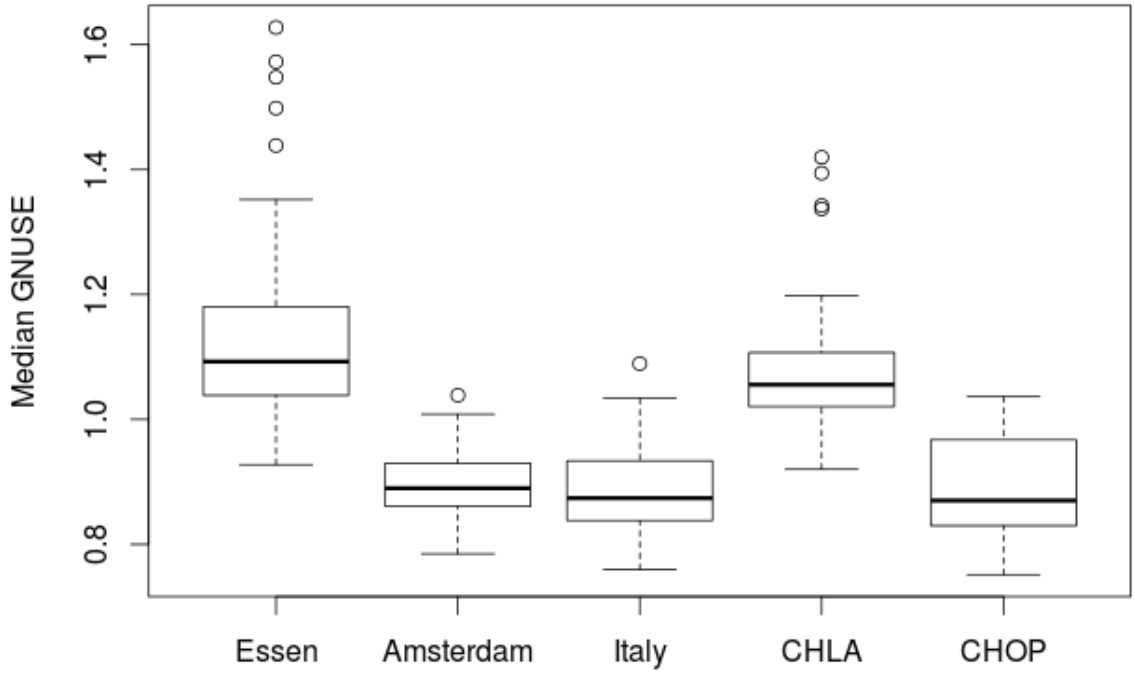
No. of Arrays with Prefixes	GSM	ITCC	NRC
Before Filtering	113	92	90
After Filtering (threshold = 1.0)	16	89	85





5.3 GNUSE Per Batch




Median GNUSE
0.8
1.0
1.2
1.4
1.6
Essen
Amsterdam
Italy
CHLA
CHOP

6 DISCUSSION

We present our data preprocessing steps for microarrays experiments. Our goal is to obtain as clean as possible data for further analyses, since the number of arrays is often not large enough to make atypical cases negligible. The importance of careful preprocessing is emphasized in lectures and textbooks, but it appears to be ignored in practice surprisingly often.

Many steps in preprocessing require human intervention and experience, which might have contributed to insufficient attention to preprocessing. Developing computer software for some of the tasks would help the community therefore, for instance, to automatize visual inspection of arrays based on computer vision or machine learning technology.

There is also room for improvement in the algorithms creating probe summaries, such as RMA or fRMA. For example, the quantile normalization step in RMA has an underlying assumption that the rank order of probe intensity values would not change across input arrays. However, it is often the case we have a collection of arrays consists of unknown genotype subgroups, where different subgroups are expected to have different rank orders of probe expression.

We hope that researchers and students may find this article helpful for preparing their microarray data.

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