NACHO QUALITY-CONTROL REPORT

Example study

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Samples	Cartridges	Flagged
48	**4**	**0**

Normalization	RCC version	NACHO
GLM	**1.6**	**3.0.0**



Generated by NACHO 3.0.0 on R 4.6.1

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1 Decision summary

48	0	0
samples pass every check	samples are flagged	metrics drive the flags

Every sample passes every check.

> **Warning: Instrument not in the RCC files**
> The RCC files do not name the nCounter instrument, so the MAX/FLEX/PRO binding density limits apply. If the data come from a SPRINT, set `instrument = "sprint"` in `load_rcc()`.

2 Settings and thresholds

These are the values NACHO used for this report. With the same RCC files and these settings, anyone can rerun the analysis and get the same result.

Table 1. Limits and settings

Parameter	Value	What it means	Source
Binding density	`0.05 to 2.25`	Optical features per square micron; too high means codes overlap.	MAX/FLEX/PRO (not in the RCC files)
Field of view	`at least 75%`	Share of imaged fields the scanner could count.	nSolver
Positive control linearity	`at least 0.95`	How well positive controls follow their known concentrations.	nSolver
Limit of detection	`at least 2`	How far the 0.5 fM positive control sits above background.	nSolver
Positive normalization factor	`0.3 to 3`	Scale applied to bring each sample's positive controls in line.	nSolver
Content normalization factor	`0.1 to 10`	Scale applied to bring each sample's housekeeping genes in line.	nSolver
Housekeeping genes above background	`at least 3`	Housekeeping genes that clear background in each sample.	nSolver
Normalization method	`GLM`	Positive controls, then housekeeping genes, scale each sample through a Poisson model.	your choice
Background	`none`	Counts are used as read, with no background from the negative controls.	default
Housekeeping genes	`ACTB, GUSB, MRPL19, PSMC4, PUM1, RPLP0, SF3A1, TFRC`	Genes used to correct for differences in sample input.	default
Housekeeping prediction	`no`	Whether NACHO picked the most stable genes as housekeeping genes.	default



Parameter	Value	What it means	Source
Housekeeping normalization	`yes`	Whether the housekeeping genes scale each sample.	default
Principal components	`10`	Components computed for the principal component plots.	default

> **How to read this**
> Each row is one limit or setting that NACHO used, with its value and what it means. The source says where the value comes from, such as nSolver, the instrument, NACHO, or a default. Rows tagged as your choice differ from the defaults, so mention them when you share the results.

3 Quality-control metrics

3.1 Binding density

The imaging unit only counts codes that it can tell apart. It does not count codes that overlap within an image. This gives you more confidence that the molecular counts come from clearly recognizable codes. Dropping the few barcodes that overlap rarely changes your data. Too many overlapping codes cause image saturation, and then data loss is possible, although serious loss from saturation is uncommon.

The nCounter instrument calculates the number of optical features per square micron for each lane while it processes the images. This is the **Binding Density** (**BD**). Use it to check whether image saturation compromised the data collection.

NACHO flags a lane when its **Binding Density** is outside the range of the preset:

- `0.05 - 2.25` for **MAX**, **FLEX** and **PRO** instruments (the legacy preset uses `0.1 - 2.25`).
- `0.1 - 1.8` for **SPRINT** instruments (the legacy preset uses `0.1 - 2.25`).

Within these ranges, few reporters on the slide surface overlap, so the instrument can count each reporter species accurately. A **Binding Density** above the upper limit means that reporters overlap on the slide surface. The instrument may have ignored many codes in such a lane, which can affect the quantification and the linearity of the assay.

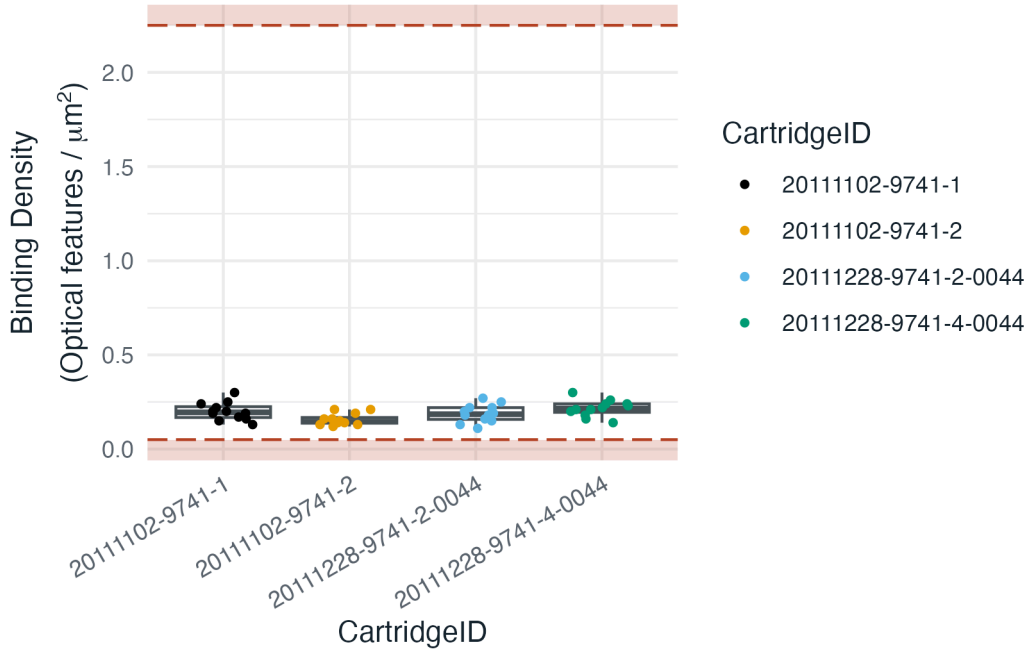


Figure 1. Binding density

How to read this
Each point is one sample, or one lane on PlexSet files, and each box covers one cartridge. Points between the dashed lines are fine, and the shaded bands above and below are outside the limits. A point in the upper band means that codes overlapped, so the instrument may have dropped some of them, and a point in the lower band often comes from low input or a poor hybridization. Triangles mark samples flagged by any check: find each one in the decision summary and check its input.

3.2 Field of view

The instrument divides each lane into a few hundred imaging sections called Fields of View (**FOV**). The exact number depends on the system (**MAX**/**FLEX** or **SPRINT**) and on the scanner settings. The system images each **FOV** separately. It sums the barcode counts of all **FOV**s of a lane to get the raw count of each barcode target. It then reports the number of **FOV**s that it imaged successfully as **FOV Counted**.

A large gap between the **FOV** that the instrument tried to image (**FOV Count**) and the **FOV** that it imaged successfully (**FOV Counted**) can point to a problem with imaging. The recommended share of registered **FOV**s (**FOV Counted** over **FOV Count**) is 75 %.

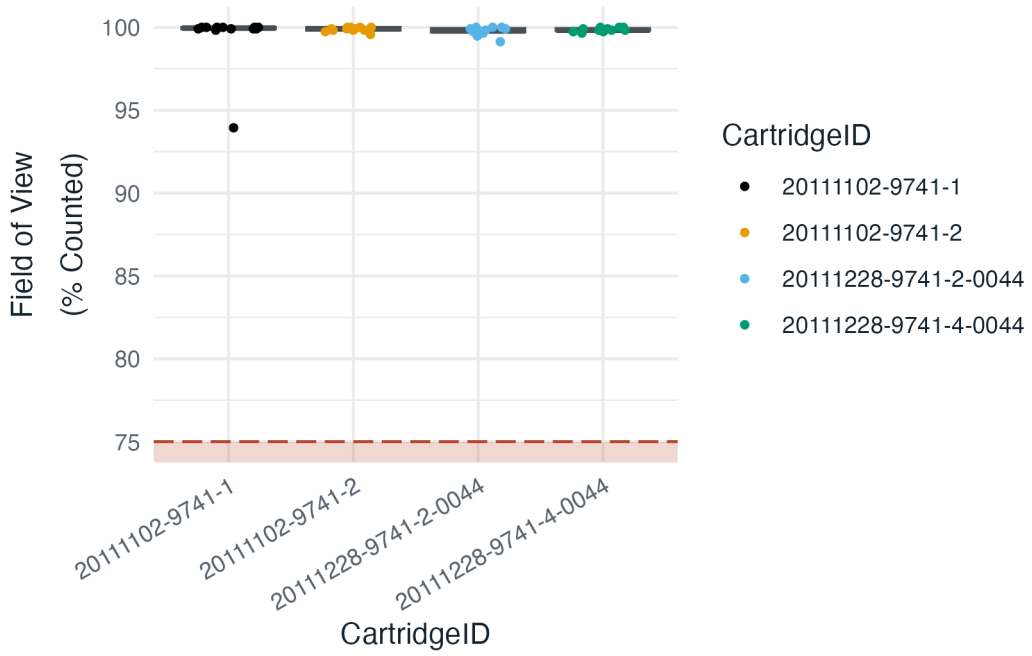


Figure 2. Field of view

> **How to read this**
> Each point is one sample, or one lane on PlexSet files, and each box covers one cartridge. Points above the dashed line are fine. A point in the shaded area below means that the scanner couldn't count many fields, often due to debris. If several samples from the same cartridge sit low, check that cartridge first.

3.3 Positive control linearity

Six synthetic DNA control targets come with every nCounter Gene Expression assay. In the codeset, their concentrations range linearly from `128 fM` to `0.125 fM`, and they are named **POS_A** to **POS_F**. These **Positive Controls** measure the efficiency of the hybridization reaction. Their step-wise concentrations also let you check the linearity of the assay.

The concentrations of the **Positive Controls** increase in a linear fashion, so the counts should do the same. The nSolver preset of NACHO computes the **Positive Control Linearity** (**PCL**) from **POS_A** to **POS_E**. The legacy preset also uses **POS_F**.

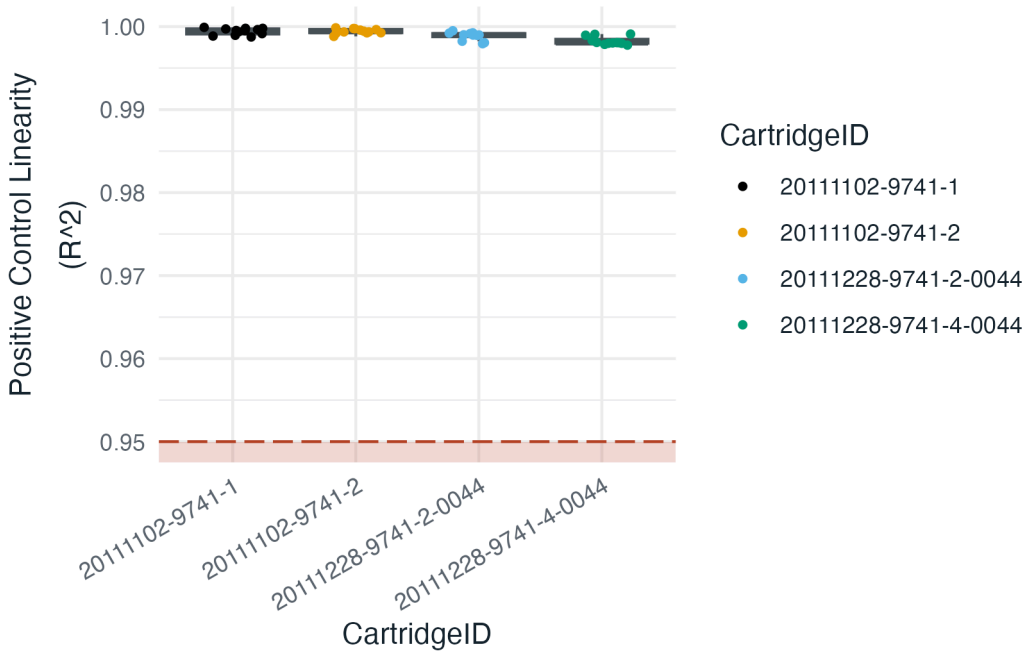


Figure 3. Positive control linearity

How to read this
Each point is one sample, and each box covers one cartridge. Points above the dashed line are fine: their positive controls rise in step with their concentrations. A point in the shaded area below means that the counts don't follow the dilution series. This can point to a hybridization problem, so treat the counts of such a sample with care or run it again.

3.4 Limit of detection

The limit of detection (**LoD**) comes from the detection of **POS_E**, the `0.5 fM` positive control probe. This probe corresponds to about 10,000 copies of the target in each sample tube.

On a **FLEX**/**MAX** system, the standard input of `100 ng` of total RNA corresponds to about 10,000 cell equivalents, assuming that one cell holds `10 pg` of total RNA on average. An assay on a **FLEX**/**MAX** system should therefore detect about one transcript copy per cell for each target, or 10,000 transcript copies in total. In most codeset assays, even the **POS_F** probe, which is equivalent to 0.25 copies per cell, is detectable above background.

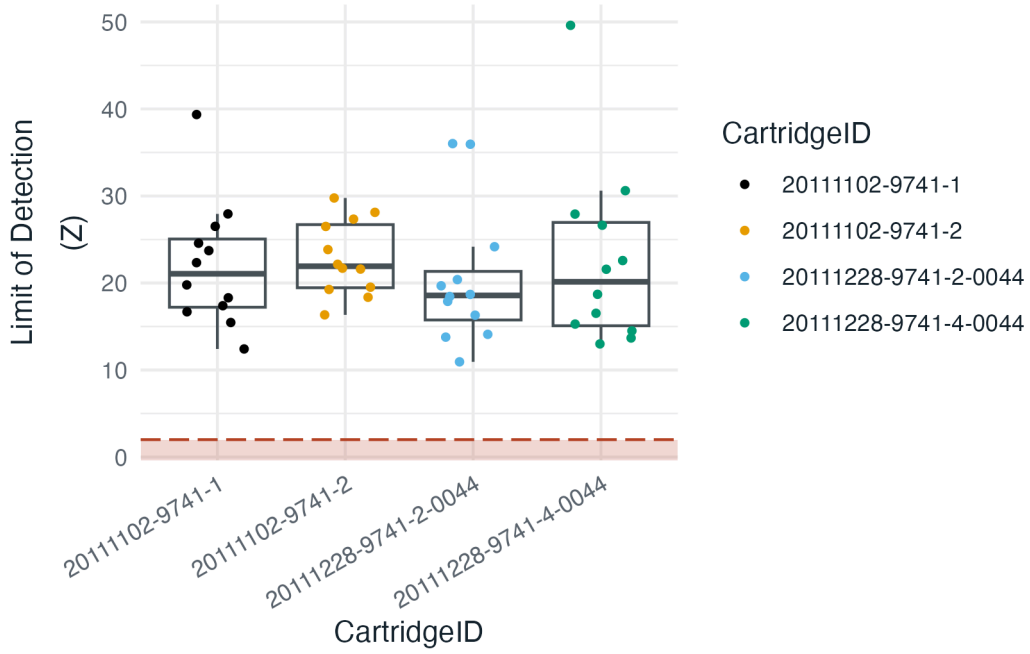


Figure 4. Limit of detection

How to read this
Each point is one sample, and each box covers one cartridge. Points above the dashed line are fine: the 0.5 fM positive control stands clear of the background. A point in the shaded area below means that weakly expressed genes can get lost in the noise. Check the negative controls and the input amount of that sample before you trust its low counts.

4 Control probes

4.1 Positive controls

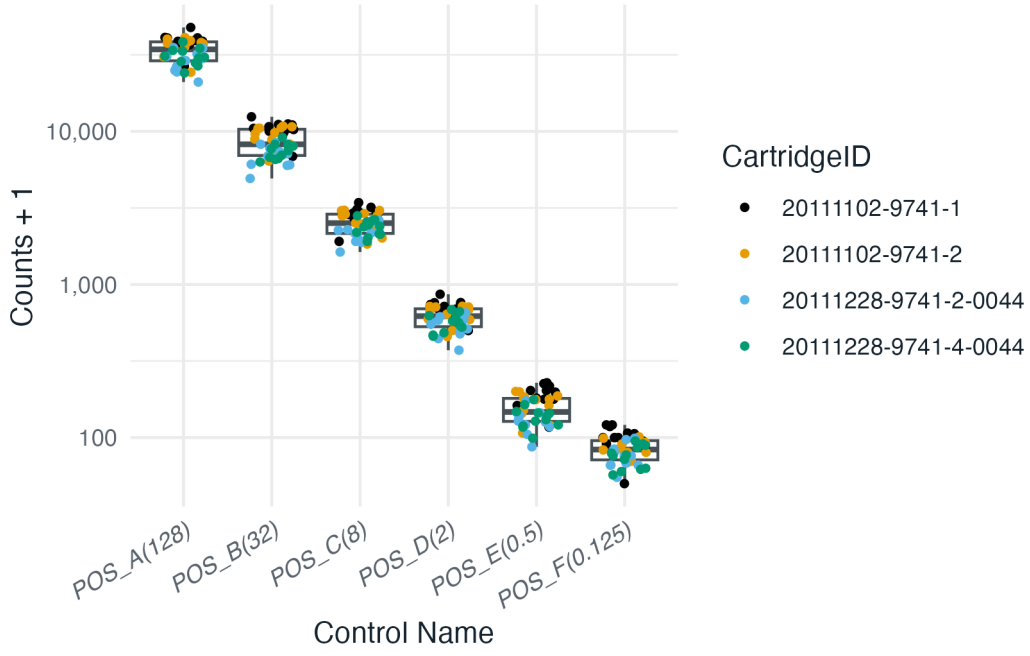


Figure 5. Positive controls

How to read this
Each box shows the counts of one positive control probe on a log scale, and each point is one sample, or one lane on PlexSet files. The boxes should step down evenly from POS_A to POS_F, and each box should be tight. A sample apart from the others on several probes had a weaker or stronger hybridization. Triangles mark samples flagged by any check, and the positive factor plot shows how much each was scaled.

4.2 Negative controls

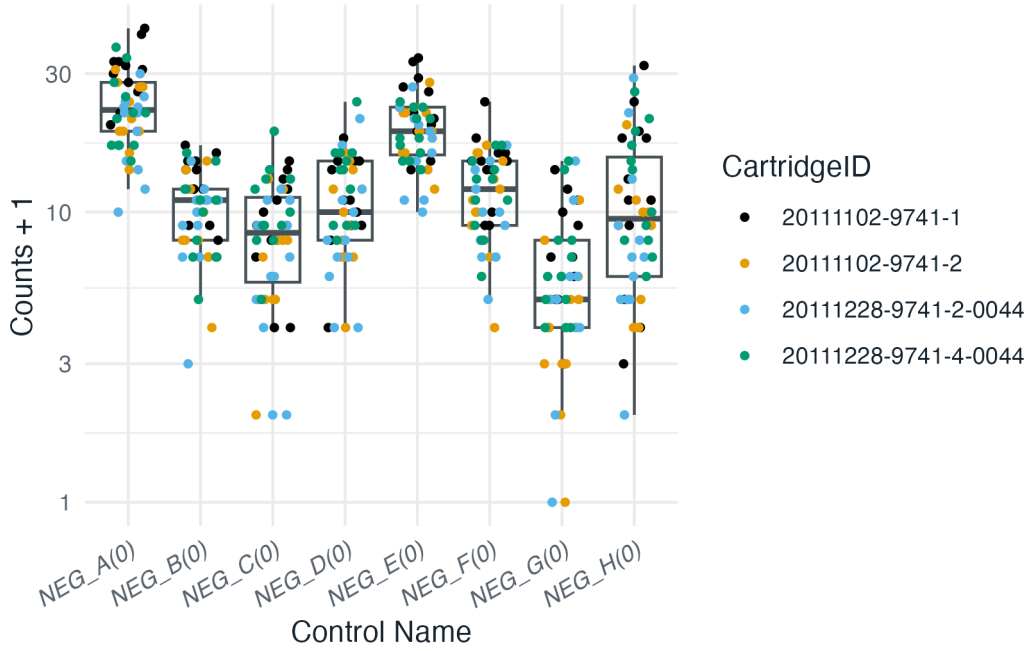


Figure 6. Negative controls

How to read this
Each box shows the counts of one negative control probe on a log scale, and each point is one sample, or one lane on PlexSet files. Good negative controls sit low and close together, because they only measure background. A probe well above the others may cross-hybridize, and a sample high on every probe has more background. If many samples sit high, look at the background setting in the settings table.

4.3 Housekeeping genes

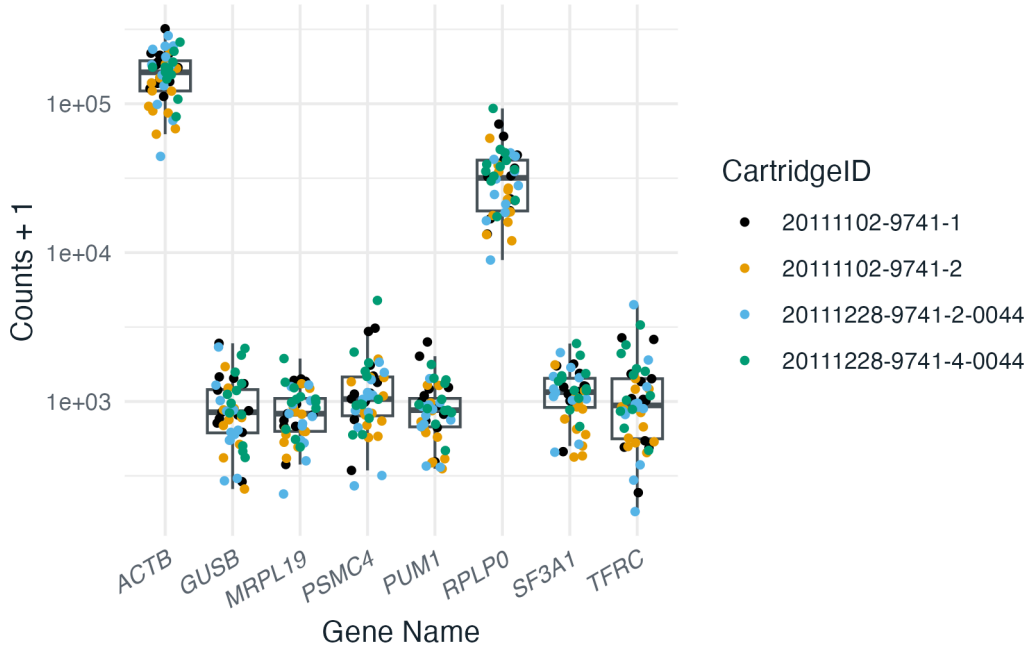


Figure 7. Housekeeping genes

How to read this
Each box shows the counts of one housekeeping gene on a log scale, and each point is one sample. Good housekeeping genes have high counts and tight boxes. A gene with a wide box or with counts near background is a poor choice for normalization. A sample low on every gene may have had less RNA input, and triangles mark samples flagged by any check.

4.4 Positive against negative controls

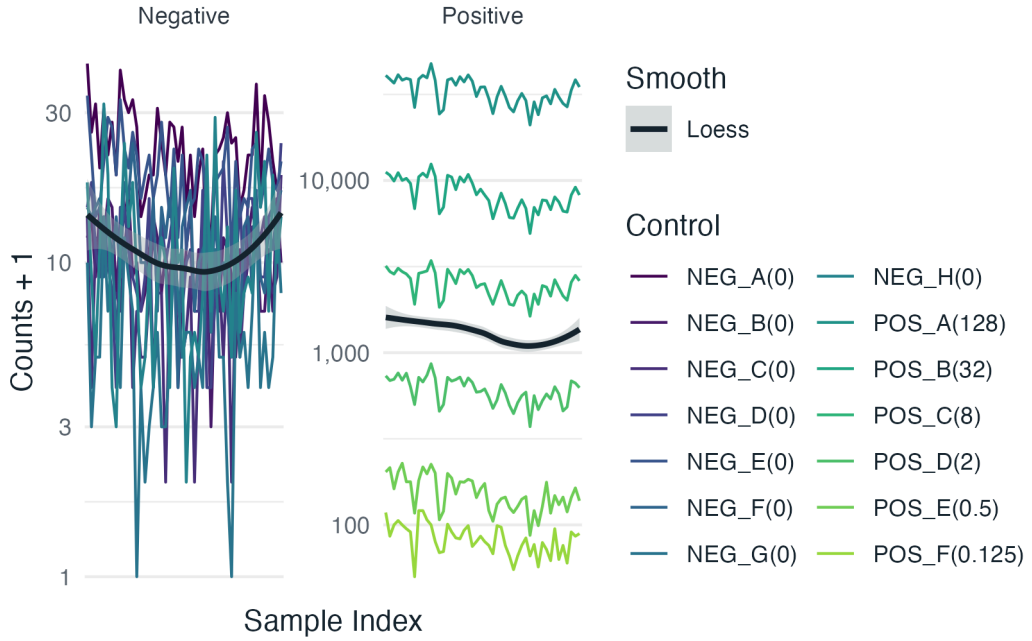


Figure 8. Positive against negative controls

How to read this
Each line follows one control probe across the samples, or the lanes on PlexSet files, on a log scale: negative controls left, positive right. Good lines run flat and parallel, and the smooth curve with the gray band stays level. A dip or a jump that many lines share points to a sample, or a run of samples, that behaved differently. Find those samples in the plots above to see which check they fail.

5 Counts

5.1 Average count against binding density

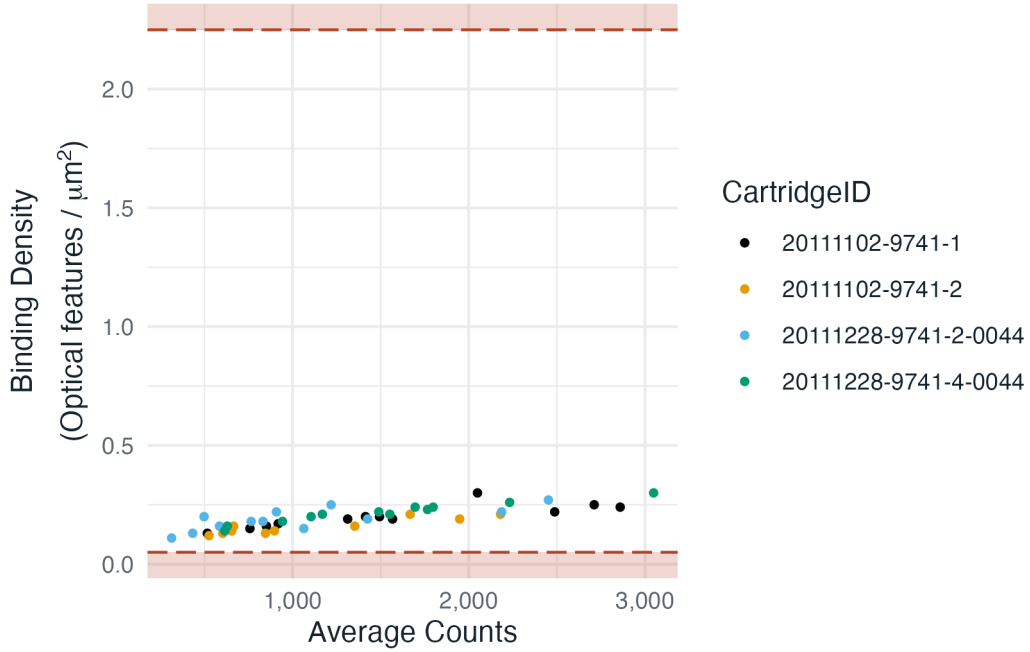


Figure 9. Average count against binding density

How to read this
Each point is one sample, with its average count across and its binding density up. The points should rise together along a rough line and stay out of the shaded out-of-limit bands. A point far from the others had an unusual signal for its density; triangles mark flagged samples. Look up such a sample in the binding density plot.

5.2 Average count against median count

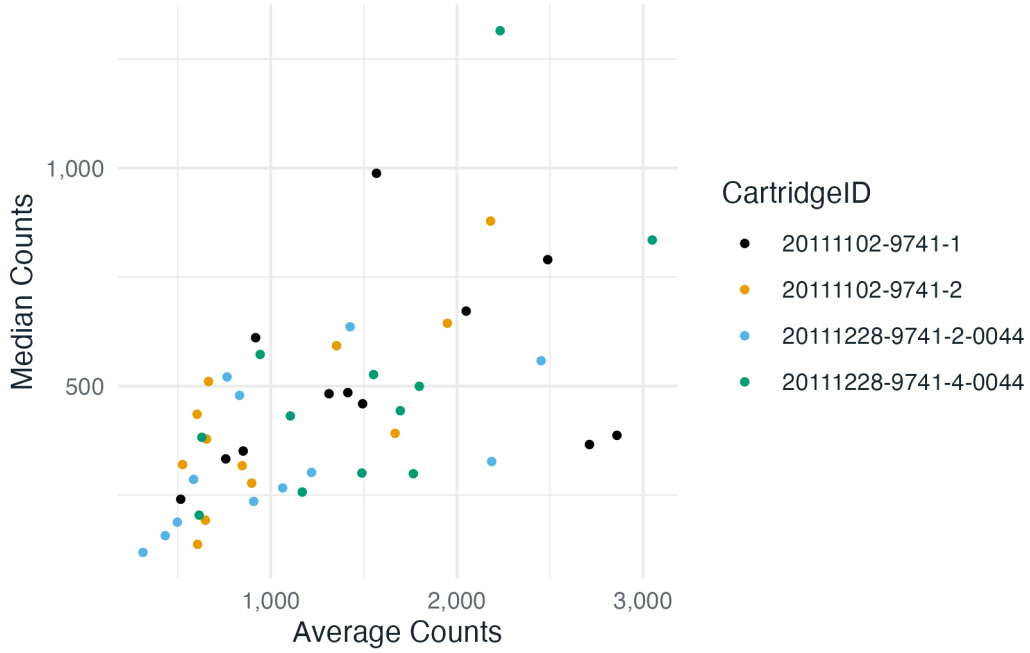


Figure 10. Average count against median count

How to read this
Each point is one sample, with its average count across and its median count up. The samples usually line up along a rough line. A point far from that line has skewed counts, often from a few very high genes or from a failed sample. Check the control plots of such a sample before you keep it.

6 Principal components

6.1 First two components

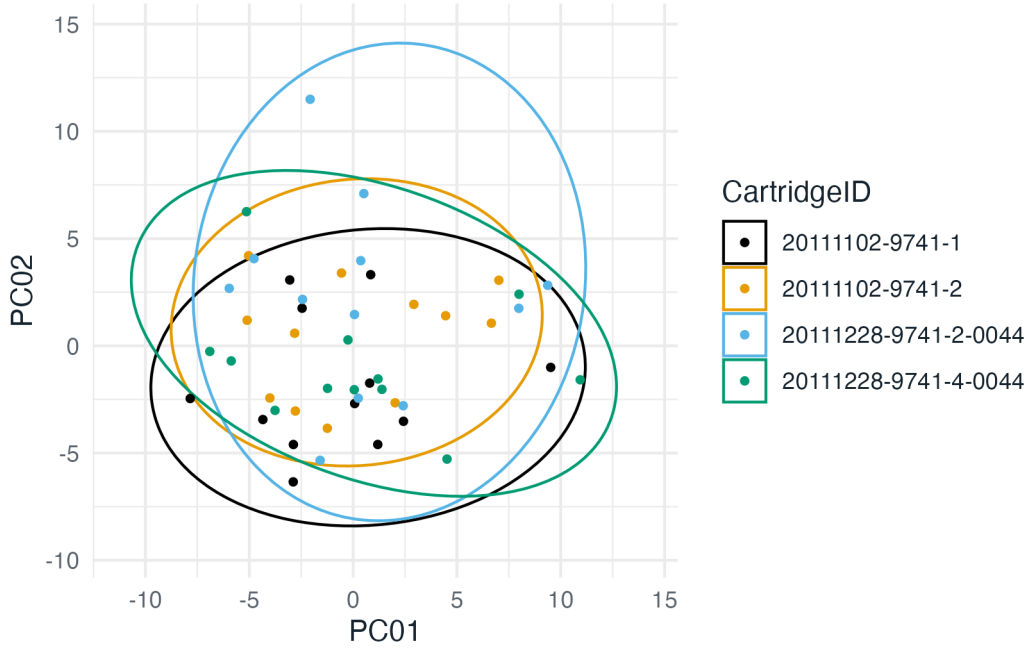


Figure 11. First two components

How to read this
Each point is one sample on the first two principal components, with an ellipse around each color group. Samples should group by biology, not by cartridge or date. A sample far from all the others may be an outlier, and groups that split by cartridge point to a batch effect. If the groups follow cartridges or dates, read the batch effects section.

6.2 Planes of the first components

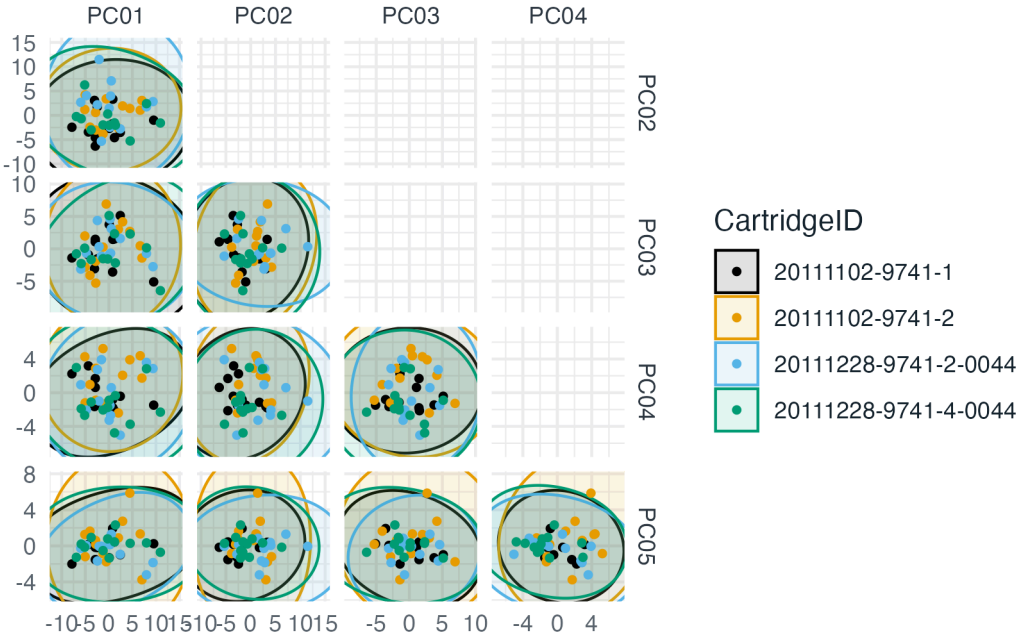


Figure 12. Planes of the first components

How to read this
Each panel shows the samples on one pair of the first principal components, with an ellipse per color group. Read each panel like the plot of the first two components. A sample that sits apart in several panels is a likely outlier. Groups that split by cartridge or date in any panel point to a batch effect.

6.3 Variance explained

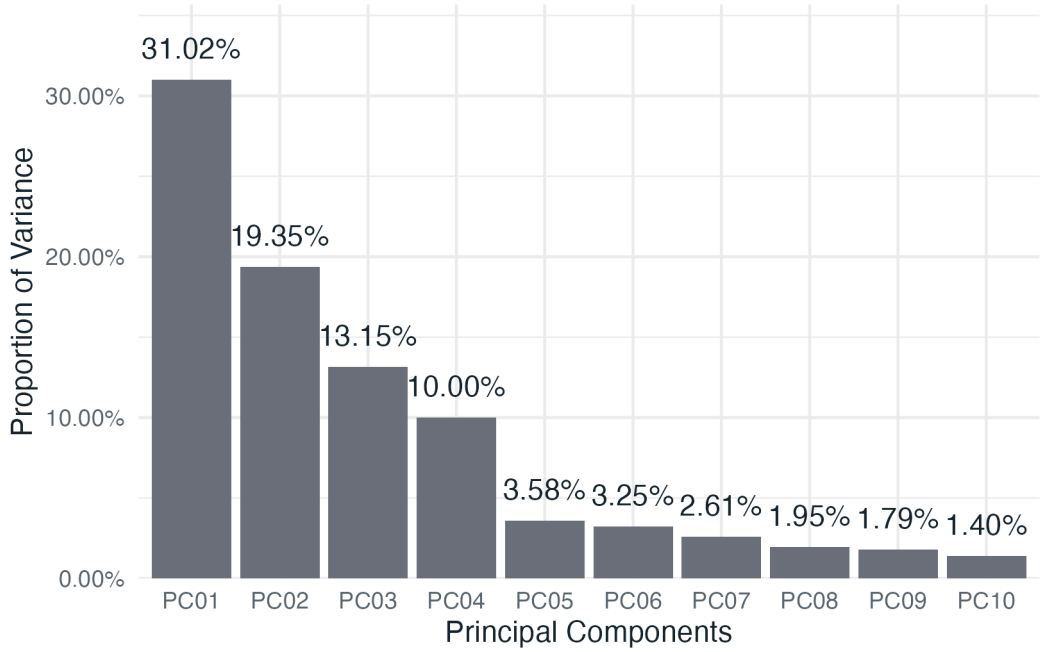


Figure 13. Variance explained

> **How to read this**
> Each bar is the share of the variance that one principal component explains. The first few bars usually carry most of the variance, and the rest fall off slowly. If one component carries a very large share, check whether it follows a batch or a single sample.

7 Normalization

7.1 Positive against negative factor

The **Positive Factor** (**PF**) is a normalization factor that NACHO computes from the positive probes **POS_X**.

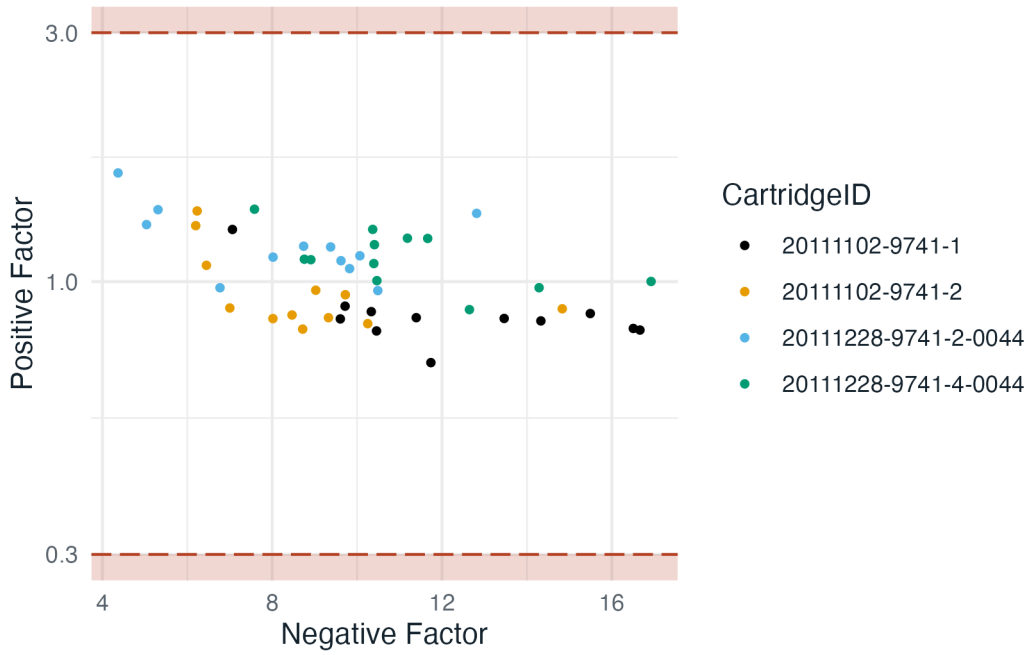


Figure 14. Positive against negative factor

How to read this
Each point is one sample, with its negative factor across and its positive factor up on a log scale. Points between the dashed lines are fine, and the shaded bands mark positive factors outside the limits. A point in a band needed a large correction, often after a poor hybridization or a pipetting error. Triangles mark flagged samples, so check them in the positive control plot before you keep them.

7.2 Content normalization factor

The **Housekeeping Genes Factor** (**HGF**) is a normalization factor that NACHO computes from the housekeeping gene probes, either provided or predicted.

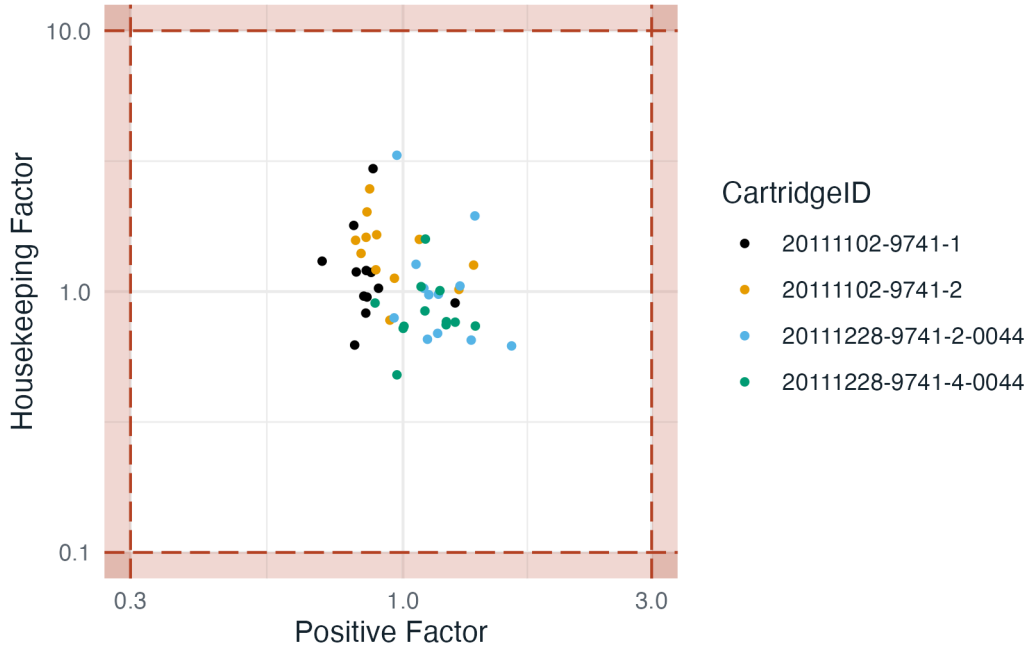


Figure 15. Content normalization factor

How to read this
Each point is one sample, with its positive factor across and its content factor up, both on a log scale. Points in the clear area between the dashed lines are fine. A point in the band above or below needed a large correction from the housekeeping genes, often because of too much or too little RNA input, and a point in the band left or right needed a large correction from the positive controls. Triangles mark samples flagged by any check, so look at those first.

7.3 Normalization result

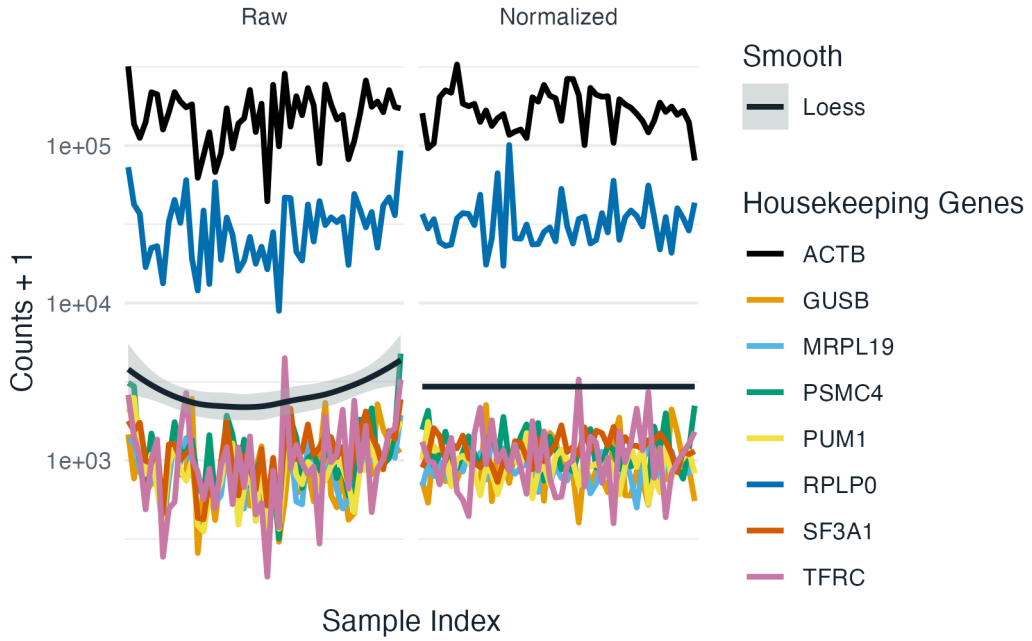


Figure 16. Normalization result

How to read this
Each line follows one housekeeping gene across the samples on a log scale: raw left, normalized right. Without housekeeping genes, the lines show the positive controls. After normalization, each line should zigzag less from sample to sample, and the smooth curve with the gray band should run level. A line that still zigzags points to a gene or a sample that the method couldn't correct.

7.4 Relative log expression

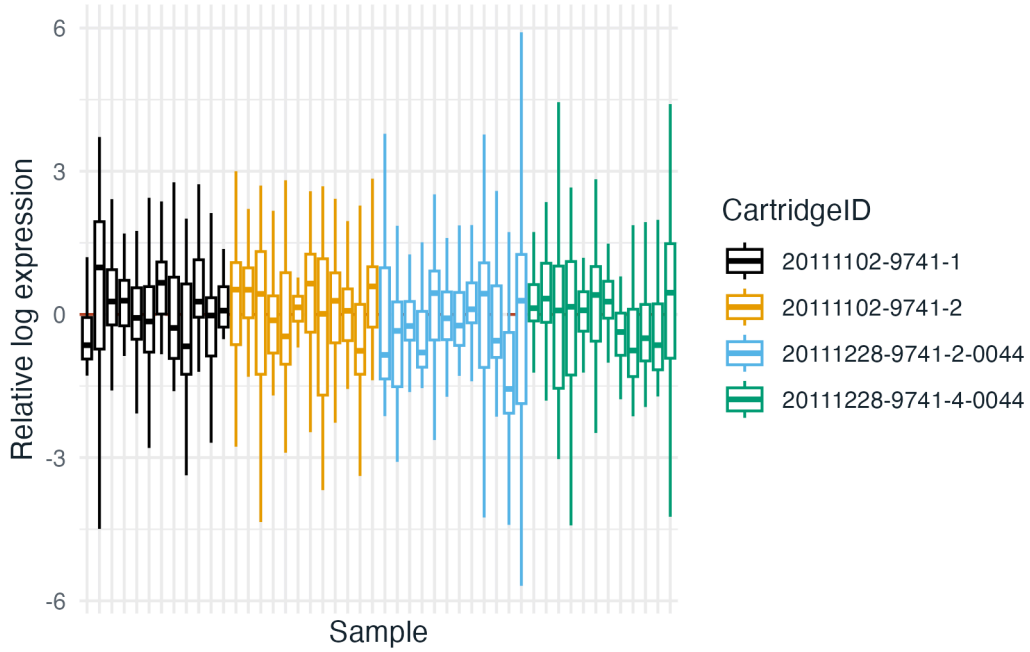


Figure 17. Relative log expression

How to read this
Each box shows how far the normalized genes of one sample sit from the median of each gene. Good boxes are narrow and centered on the dashed line at zero. A box shifted away from zero, or much wider than the others, marks a sample that normalization did not fully correct, or one that differs in its biology. Check the metrics of that sample, and if boxes of one color shift together, look for a batch effect.

7.5 Housekeeping gene stability

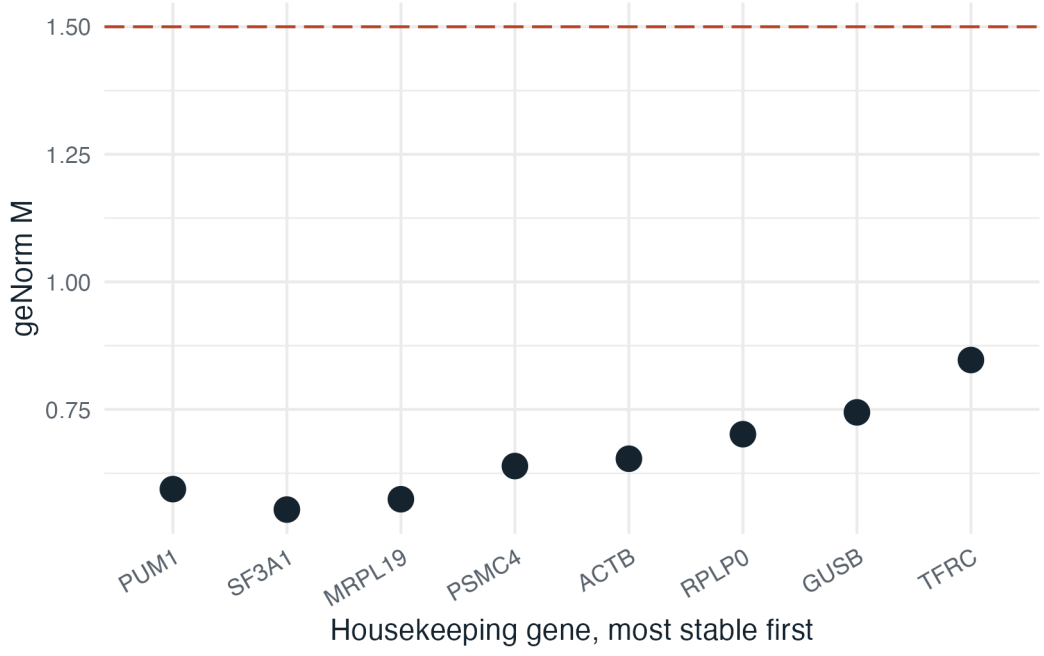


Figure 18. Housekeeping gene stability

How to read this
Each point is one housekeeping gene, from the most stable on the left to the least stable on the right. Genes below the dashed line at M = 1.5 are stable enough for samples of one kind. A gene above the line varies more than the others across the samples. Think about leaving it out of the housekeeping genes and normalizing again.

8 Batch effects

8.1 Normalization factors by cartridge

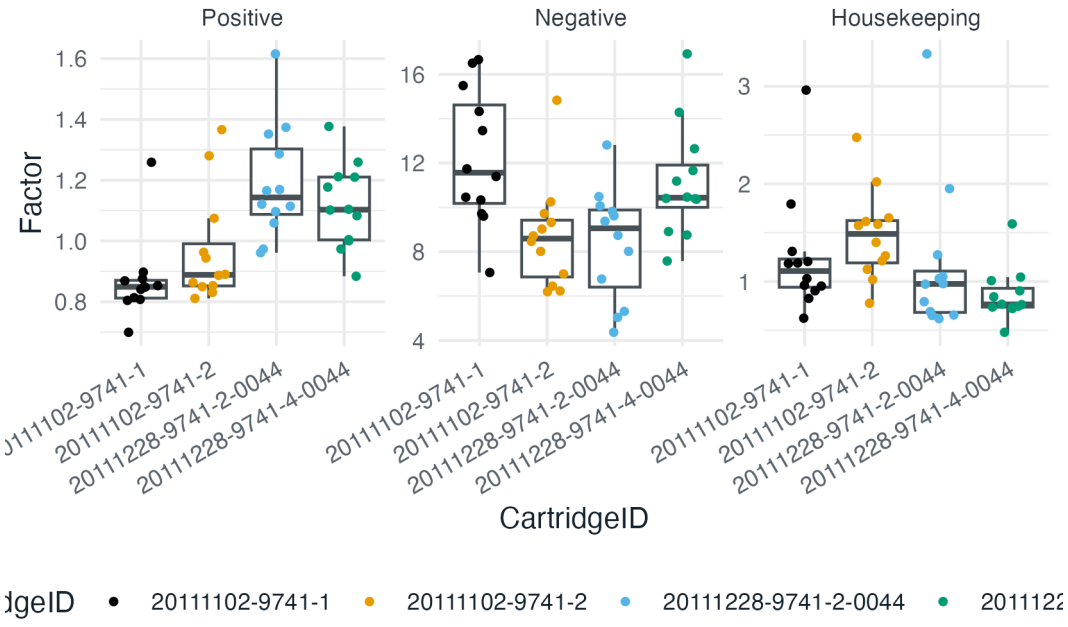


Figure 19. Normalization factors by cartridge

How to read this
Each panel shows one normalization factor, with one box per cartridge and one point per sample. The boxes should sit at about the same level across cartridges. A cartridge whose box sits higher or lower than the others needed a different correction. That hints at a batch effect, so check whether that cartridge holds only one kind of sample.

8.2 Principal components and batches

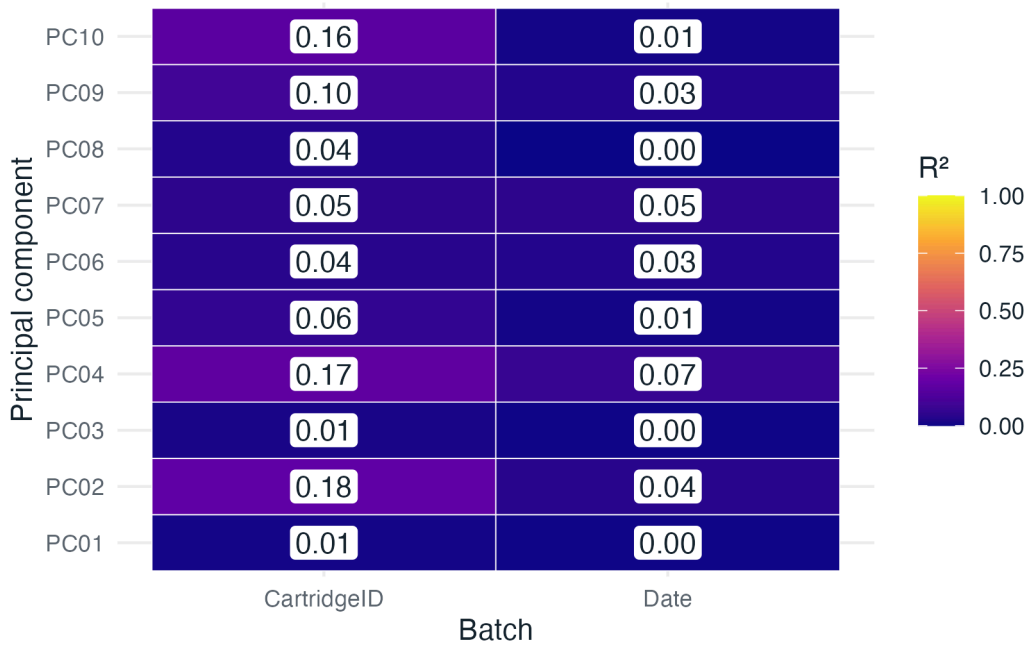


Figure 20. Principal components and batches

How to read this
Each tile shows the share of one principal component that cartridge or date explains, from 0 to 1. Low values in dark tiles are good, and gray tiles have no value. A bright tile with a high value on one of the first components means that the batch shapes the data. Then think about a batch correction, and check that batch and biology are not confounded.

Methods

NACHO checked each sample against 7 quality-control limits: binding density (0.05 to 2.25), field of view (at least 75%), positive control linearity (at least 0.95), limit of detection (at least 2), positive normalization factor (0.3 to 3), content normalization factor (0.1 to 10) and housekeeping genes above background (at least 3). A sample outside any limit is flagged, and the decision summary lists each one. These limits come from the nSolver preset, which follows the nCounter Gene Expression Data Analysis Guidelines from NanoString, now part of Bruker. The RCC files do not name the nCounter instrument, so binding density uses the MAX/FLEX/PRO limits. Positive control linearity uses POS_A to POS_E.

NACHO normalized the counts with the GLM method. Positive controls, then housekeeping genes, scale each sample through a Poisson model. Counts are used as read, with no background from the negative controls. It used 8 housekeeping genes to scale each sample: ACTB, GUSB, MRPL19, PSMC4, PUM1, RPLP0, SF3A1, TFRC.

NACHO 3.0.0 wrote this report with R 4.6.1. The RCC files are of version 1.6. The settings and thresholds section lists every value, so anyone with the same RCC files can run the same analysis again.

To cite NACHO in publications, use:

Canouil M, Bouland GA, Bonnefond A, Froguel P, 't Hart LM, Slieker RC (2020). “NACHO: an R package for quality control of NanoString nCounter data.” Bioinformatics, 36(3), 970-971. https://doi.org/10.1093/bioinformatics/btz647.

Session information

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R version 4.6.1 (2026-06-24)
Platform: aarch64-apple-darwin23
Running under: macOS Golden Gate 27.2
NACHO      3.0.0
data.table 1.18.6.1
ggplot2    4.0.3
ggforce    0.5.0
ggrepel    0.9.8
S7         0.2.2
```