

Ct value processing filters (in order of execution)

Primary filter:

- 1) For each gene,
 - a. For each gene, including controls, remove data with CtCall = FAILED and CtQuality < threshold (--Ct-quality-threshold , Default No Threshold)
 - b. For each gene, including controls, remove CtValues >= CtValueThreshold (--Ct-value-threshold, Default: 30.0) to filter out low expression genes (they will be not expressed)
 - c. Here: No more values that are FAILs.
 - d. For each gene, including controls, set all the CtCall to "INC" (inconsistent) if the difference between the maximum CtValue and the minimum CtValue > MaxCtRepDev (--max-Ct-deviation-between-replicates, Default: 2.0)

Sample filter:

- 2) For each control gene (in control gene list):
 - a. if it is not found, remove the whole sample row.
 - b. if that gene is marked as "INC" (in 2 of primary filter), remove the whole sample row
 - c. if no more CtValues are retained after primary filter or the number of CtValues < minValidReplicatesControl (--min-number-of-valid-data-point-per-control, Default: 1), remove the whole sample row.
 - d. If the mean of the CtValues > CtValueThresholdPerControl (--Ct-value-threshold-for-per-control-average, default: 25.0), remove the whole sample row.

Gene filter:

- 3) For each non-control gene:
 - a. if that gene is marked as "INC" (in 2 of primary filter) or has all Ctvalues removed, don't do anything here. Do not continue to next step.
 - b. If number of CtValues retained after primary filter is not zero but is < minValidReplicates (--min-number-of-valid-data-point-per-gene, default: 2), mark gene as "INC"
 - c. If the mean of the CtValues > CtValueThresholdPerData (--Ct-value-threshold-for-data-average, default: 30.0), remove gene (remove all CtValues)

ACx Output:

- 4) For each sample:

- a. If sample is invalidated by sample filter, don't continue to next step.
- b. For each non-control gene:
 - i. If gene not found for this sample, output NA
 - ii. If "INC", output NA
 - iii. If No CtValues (i.e., removed by primary filter or gene filter), output 0.0 (for genes that don't express, CtCall will be highly likely to FAIL in most/all replicates).
 - iv. Else output $AC_x(g,s) \times (-\text{offset-output, default: 20})$

Average Control at x (AC_x) values

$$AC_x(\text{gene } g, \text{ controls } c \in C, \text{ sample } s) = x + \overline{Ct(c,s)} - \overline{Ct(g,s)}$$

Property:

$$\text{When } \overline{Ct(c,s)} = \overline{Ct(g,s)}, AC_x(g,C,s) = x$$

Larger value => higher expression

To find fold change of the gene from sample1 (s1) to sample 2 (s2):

$$\frac{\text{expression of } g \text{ in sample 2}}{\text{expression of } g \text{ in sample 1}} = R = 2^{AC_x(g,C,s2) - AC_x(g,C,s1)}$$