User Bulletin
Applied Biosystems 1700 Chemiluminescent Microarray Analyzer

SUBJECT: Algorithm Processing and Image Import Utility

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Section 1 Algorithm Processing

This section covers:

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Introduction

Overview
This section of the user bulletin covers the automated algorithm processing of the Applied Biosystems 1700 Chemiluminescent Microarray Analyzer from the input image set to population of an output Feature Table that includes extracted feature signals, signal uncertainties, quality metrics and confidence values. The process divides into gridding and quantification:

- Gridding locates and identifies features in the image with respect to the known Microarray Design.
- During quantification, feature signals are extracted from the image set at these locations.

This section focuses on the feature quantification and a system error model, which is built throughout the steps in this process. The error model determines the estimated uncertainty on the final probe signal measurements.

This section also shows examples of how data from the Feature Table can be used to determine differences in gene expression level, presence of the gene expression product, and various ways to filter the data.

Image Processing
To encompass the entire array, the analyzer images the microarray in two positions. In each position, the camera captures a series of four images. The images from each position are processed independently, and the results from both positions are combined into a single output Feature Table.

For each position, the inputs to the algorithm are:

- A default 25-second chemiluminescent CL image
- A fixed 5-second short chemiluminescent image
- A fixed 25-second long fluorescent image
- A fixed 25-second long spectral correction image
- The array map that contains numeric codes for each feature that identify probes and different classes of controls

Output
The output of the algorithm is a Feature Table that contains the fully normalized feature intensities, confidence and quality metrics, and some intermediate quantification results. This section of the user bulletin describes the Feature Table contents in Table 1 on page 6, and details how you can use the confidence and quality metrics to mine data, assign gene Presence/Absence calls, and calculate fold change confidence between pairs of arrays.
Algorithm Workflow

Overview

Image processing comprises four major stages, outlined below:

- gridding
- image correction
- primary image analysis
- feature quantification refinement

The individual quantification stages are discussed in more detail later in this section of the user bulletin.

Gridding

Gridding is the automatic location and association of features as they appear in the image with the row/column coordinates of the array, as required for subsequent quantification processing.

If an insufficient number of features is found, or if the pattern of features does not match the expected array topology, gridding fails. Gridding failure occurs only as a result of data error. Otherwise, gridding results (i.e., locations and flags) are sent to quantification.

Image Correction

A number of corrections are applied to improve the raw CL and FL images prior to image analysis, where feature intensities are actually extracted. The steps are:

- **Image calibration** – Uses a series of calibration images associated with the detector to correct and normalize certain aspects of the CCD camera.
- **Spectral cross-talk correction of the FL channel** – Corrects for any spectral bleed-through of CL signal into the FL channel with a subtraction of the FX image.
- **CL-CLs merge** – Extends the dynamic range of the detector by replacing detector saturated pixels in the long CL image with the corresponding scaled unsaturated pixels from the CL short image.

Primary Image Analysis

The fully calibrated and registered CL and FL images, along with grid positions, are fed to the image analysis algorithm. Tasks that are accomplished during image analysis include:

- Background correction in both channels.
- Feature integration and feature normalization.
- Flagging of problematic features due to detector saturation and other issues.
- Rejection of outlying feature pixels associated with artifacts on the array.
- Estimation and propagation of uncertainties in background and signal at both pixel and integrated signal level into the extracted normalized signal uncertainty.
Further refinements of the output of primary image analysis are made to correct systematic trends and issues with the data. This step also finalizes quality assignments of quantification for each feature. Processing steps include:

- **Spatial normalization** – Removes any systematic spatial trends in feature ratios across the array.
- **Assay Background correction** – Subtracts a statistical bias in intensities due to any non-specific assay signal associated with each feature.
- **Error Model determination** – Tracks all known measurement uncertainties within the system to predict a signal CV for each feature based on its S/N. This is the signal CV that would be associated with technical replicates.
- **Further flagging of features** – Includes specific flags for quality metrics that fail internally defined thresholds.

### Table 1 Quantification output

<table>
<thead>
<tr>
<th>Applied Biosystems 1700 System Signal Table</th>
<th>2nd Analysis Export</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ICP Normalized Signal (Signal)</td>
<td>Gene Signal</td>
<td>This is the fully corrected signal associated with the probe or control, in counts. The CL signal is normalized by the FL signal divided by the feature integration aperture. To preserve the approximate feature CL counts, the CL/FL ratio is multiplied by the median FL signal (aperture integrated) for all features containing ICP (such as gene probes) quantified at a particular array position. <strong>IMPORTANT!</strong> In situations where the S/N &lt; 1, the signal is replaced with a 1 standard deviation upper limit, based on its probe signal SDEV, and the lowest bit of its FLAGS number is set to 1.</td>
</tr>
<tr>
<td>ICP Normalized Signal Error (SDEV)</td>
<td>—</td>
<td>Represents the estimated measurement uncertainty of Signal. Error estimates result primarily from propagation of background subtraction and signal integration uncertainties.</td>
</tr>
<tr>
<td>ICP Normalized S/N (S/N)</td>
<td>Gene S/N</td>
<td>Equals the ratio of Signal/SDEV. This metric expresses the confidence of feature “detectability.” A S/N of 3 represents a 3 SDEV confidence (99.9%) that the measurement is real (&gt;0).</td>
</tr>
</tbody>
</table>
Algorithm Workflow

Algorithm Processing and Image Import Utility

Table 1  Quantification output (continued)

<table>
<thead>
<tr>
<th>Applied Biosystems 1700 System Signal Table</th>
<th>2nd Analysis Export</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ICP Normalized CV (CV)</td>
<td>Gene CV</td>
<td>The Gene CV determines the individual feature reproducibility. The Gene CV is directly involved in a GEx Fold change confidence calculation (see “S/N, CV, Thresholds and Fold Confidence” on page 15). Since there are no replicates for a given gene, this is a predicted CV derived from an Error Model. Signal × CV represents the total probe signal variation that might otherwise be estimated analyzing a gene signal across multiple (globally normalized) technical replicate arrays.</td>
</tr>
<tr>
<td>Assay Normalizing Signal</td>
<td>Assay Normalizing Signal</td>
<td>The median of all valid probe signals in the same image area.</td>
</tr>
<tr>
<td>FLAGS</td>
<td>FLAGS</td>
<td>For each feature, a numeric code that identifies conditions given in Table 3, “Feature quantification flags,” on page 24.</td>
</tr>
<tr>
<td>QCmetric1</td>
<td>—</td>
<td>A feature quality metric given by the CL/FL feature pixel correlation.</td>
</tr>
<tr>
<td>QCmetric2</td>
<td>—</td>
<td>A feature quality metric that captures the fraction of usable pixels used in quantification.</td>
</tr>
<tr>
<td>CL Signal</td>
<td>—</td>
<td>Background-corrected integrated CL feature signal (with none of the feature corrections listed in “Corrections” on page 8).</td>
</tr>
<tr>
<td>CL Signal Error (CL Signal SDEV)</td>
<td>—</td>
<td>Background-corrected integrated CL feature signal uncertainty (with none of the feature corrections listed in “Corrections” on page 8).</td>
</tr>
<tr>
<td>FL Signal</td>
<td>—</td>
<td>Background-corrected integrated FL feature signal (with none of the feature corrections listed in “Corrections” on page 8).</td>
</tr>
<tr>
<td>FL Signal Error (FL Signal SDEV)</td>
<td>—</td>
<td>Background-corrected integrated FL feature signal uncertainty (with none of the feature corrections listed in “Corrections” on page 8).</td>
</tr>
<tr>
<td>CL Raw Signal (CLraw)</td>
<td>—</td>
<td>The integrated CL signal with no background correction (BG). The integrated CL background is given by CLraw – CL Signal.</td>
</tr>
</tbody>
</table>

Note: This BG, in addition to the CLraw, excludes the arbitrary CCD bias offset corrected for in the preprocessing step (see “Corrections” on page 8), which is not associated with actual BG signal.
Table 1  Quantification output (continued)

<table>
<thead>
<tr>
<th>Applied Biosystems 1700 System Signal Table</th>
<th>2nd Analysis Export</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>FL Raw Signal (FLraw)</td>
<td>—</td>
<td>The integrated FL signal with no background correction. The integrated FL background is given by FLraw – FL Signal. Note: This BG, in addition to the FLraw, excludes the arbitrary CCD bias offset corrected for in the preprocessing step (see “Corrections” on page 8), which is not associated with actual BG signal.</td>
</tr>
<tr>
<td>X</td>
<td>X position</td>
<td></td>
</tr>
<tr>
<td>Y</td>
<td>Y position</td>
<td></td>
</tr>
</tbody>
</table>

**Corrections**

**Image Corrections**

- CCD BIAS offset subtraction
- CCD BIAS image subtraction
- CCD DARK image subtraction
- FLAT-FIELD image division (CCD pixel-to-pixel gain variations)
- BADPIXEL masking
- FL spectral cross-talk correction (FL minus scaled FX image)
- CL-CL-short image combine to replace detector saturated pixel in the CL-long image with scaled unsaturated pixels from the CL-short image
- FL to CL subpixel image alignment

**Feature Corrections**

- CL and/or FL spatial cross-talk (SXT) correction to signals and signal errorbars.
- **Feature (ICP) Normalization** – The CL/FL ratio normalizes spotting variability, algorithmic issues (centroid variability, differences in integration apertures), as well as optical vignetting, which affects both channels equally.
- **Spatial Normalization (SPN) correction** – Controls are used to normalize out any large-scale trends in CL/FL ratios across the array.
- **Assay Background (ABG) correction** – Subtracts the median fully normalized signal CL signal of negative controls to remove the statistical bias of assay noise from the final CLinorm signal.

**IMPORTANT!** All CLinorm* table values are corrected for ABG by default.
Quantification Stages

The following describes the major stages of quantification in detail.

**Image Calibration**

After reading, all four input images are corrected for both artifacts associated with the CCD and the reader. There are five images in a calibration folder associated with the instrument:

- **SBIAS** – An offset image that is subtracted from all raw experimental images. This image represents residual structure in the CCD bias after correction for the typically floating CCD bias offset.

- **SDARK_100s** – An offset image that is subtracted from all raw experimental images after SBIAS correction. This image represents the CCD dark current as measured in 100s. This is scaled to the experimental image exposure time before the correction is made.

- **SPIXFLAT** – A divisor calibration image that is applied to all experimental images to correct the small scale pixel-to-pixel (typically 1 to 2%) CCD gain variations after SDARK correction.

- **CAMERA.csv** – A csv text file containing the parameters for the reader CCD. These parameters are used in quantification including noise calculations. The following are example values only:
  - GAIN, 2.0, electrons/count
  - READNOISE, 5.0, electrons
  - SATURATION, 39000, counts
  - CCDBIAS, 250, counts (nominal value for camera)

Typically, these image calibrations have minor impact on the data, but their value is in making quantification more robust.

**Note:** There is no calibration image to address optical vignetting, which divides out in the CL/FL ratio.
FX Correction

A spectral cross-talk correction is applied to the FL channel using the FX image, which captures the spectral cross-talk from the CL channel into the FL channel. This image is scaled to the FL exposure time and subtracted from the FL image.

**Note:** References to the “FL image” beyond this point refer to the spectral cross-talk corrected image.

![FL image before and after spectral cross-talk correction]

**Figure 1** A portion of an FL image (left) before and after (right) spectral cross-talk correction

Long-Short CL Merge

The purpose of taking a long (CL) and short (CLs) CL exposure is to extend the dynamic range of the detector. The intended purpose of the CLs exposure is to ensure all features are below detector saturation. The long CL exposure ensures faint features have adequate S/N, but the brightest features can potentially be saturated.

A combined CL image is made by taking all but unsaturated pixels from the long CL image and replacing saturated pixels with appropriately scaled values from the CLs image. This operation also obtains an accurate estimate for the CCD bias offset associated with each image. This offset (typically a few hundred counts) is intrinsic to the CCD detector, and it bears no relation to true background signal or noise on the image. This bias is subtracted from all images for subsequent analysis. The output columns that report background signals do not include this offset.

Background Correction

The background (BG) in the CL image is typified by diffuse chemical signal throughout the array. In addition, the high dynamic range of signals in this channel leads to spatial cross-talk signal from bright features contributing to the BG near adjacent probes. The BG is, therefore, estimated from a local annulus around each feature, and has the advantage of obtaining accuracy in BG estimates when the BG is changing on small scales. It also mitigates the spatial cross-talk errors, which especially impact weak signals close to bright ones. However, in these situations this introduces more uncertainty in the BG estimate. This uncertainty is reflected by higher signal uncertainty estimates for these features.

In addition, an error model of the BG for both channels captures the total uncertainty in the integrated signals, which includes measurements of correlated noise between adjacent pixels.
Feature Integration

After BG correction, feature pixel intensities are summed over a fixed aperture typically seven pixels in diameter. The algorithm improves on this simple integration in a number of ways, primarily by weighting each pixel in the sum:

\[
T = \frac{\sum w_i f_i}{\sum w_i}
\]

Equation 1

Estimating these weights requires tracking the noise associated with each pixel, as well as having a model for how the feature flux is distributed.

So, in addition to relying on the bright FL signal to identify the precise location of the CL feature flux to be integrated, the algorithm also takes advantage of the high correlation of the CL and FL pixel intensities within a feature and the high FL feature S/N to provide the model required to determine the optimal weights.

Figure 2 The integration algorithm takes advantage of the feature CL/FL pixel correlation to maximize the S/N of the final extracted intensity, as well as provide robust quantification in the case of outlier pixels that might be associated with system artifacts.

The noise model of the feature also allows for testing individual features for bad pixels by examining the consistency of pixel ratios using a z-score statistical test. Features with many bad pixels are flagged for rejection.
Data Normalizations and Corrections

There are three classes of normalization applied to the data followed by one bias correction.

**Feature Normalization**

Feature normalization of CL intensities by the FL channel has been shown to effectively compensate for:

- Spotting variations associated with the amount of probe deposited
- Sub-optimal feature centroids and pixilation effects of the finite quantification aperture (by virtue of the sub-pixel alignment of the CL and FL images and the fact that signals are quantified in exactly the same locations)
- Morphology differences between the same probe on different arrays
- Optical trends in the data

**Note:** There is a spatial sensitivity in the reader images associated with optical vignetting. Since this sensitivity affects CL and FL images equally, a normalized CL signal automatically compensates for this issue.

The CL/FL ratio is rescaled arbitrarily by the mean ICP probe FL intensity (hereafter, the Signal intensity). This ensures the array averaged normalized and un-normalized CL intensities are the same:

\[
I_{\text{SIGNAL}} = \left( \frac{I_{\text{CL}}}{I_{\text{FL}}} \right) \langle I_{\text{FL}} \rangle
\]

Equation 2

The measurement variance in the final Signal is given by:

\[
\sigma^2_{\text{SIGNAL}} = \left( \frac{\sigma_{\text{CL}}}{I_{\text{CL}}} \right)^2 + \left( \frac{\sigma_{\text{FL}}}{I_{\text{FL}}} \right)^2 I_{\text{SIGNAL}}^2
\]

Equation 3

where, generally, the high S/N in the FL signal ensures the fractional uncertainty in the FL signal is much smaller than the fractional uncertainty in the CL signal. These signal uncertainties are derived from propagating uncertainties in background subtraction and signal intensities.

Spotting variability may be apparent in FL images of the arrays. FL (ICP) normalization compensates effectively for this variability, ensuring that each probe CL/FL ratio will be the same.

**Spatial Normalization**

Spatial normalization compensates for remaining large scale trends in ratios within the image. Such trends might be associated with non-uniform LED FL illumination and residual optical and algorithm issues. This correction is made through spatial trends of measurements of CL normalized ratios identified in a series of high S/N controls with equal input ratios.
Specifically, control features called spatial normalization (SPN) controls are placed in a grid throughout the array at the same frequency as the subgrids. These control features have signal in both FL and CL and the ratio of signals on these features should be the same. To correct the Signal intensities for these trends, a coarse image is made from these SPN controls, normalized to 1, 2D median smoothed and interpolated under every feature, and then divided into the Signal intensities.

Figure 3  Spatial normalization correction map for $159 \times 119$ features derived from controls within an array. The amplitude of the correction is around 15%.

**Global Normalization**

On the Applied Biosystems 1700 system, the output column “Assay Normalized Signal” is the Signal column divided by the median Signal value for all probes in the same image area. This provides only a *rough* global normalization, which accounts for gross differences in sample, labelling, exposure time, hybridization conditions, and so forth. This rough global normalization may be useful if you wish to filter data based only on signal thresholds across arrays, instead of S/N or predicted CV. Applied Biosystems recommends that you pursue alternative normalization and downstream analysis methods for your data.

**Note:** Gene CV and Gene S/N are metrics that should NOT be scaled by any kind of global normalization of the data.

$$\text{Assay Normalized Signal} = \left( \frac{\text{Signal}}{\text{Assay Normalizing constant}} \right)$$

where:

- Signal = CL – INORM
- Assay Normalizing constant is the median of all CL – INORM probe signals on the array

**Equation 4**
This step statistically corrects signals for the assay background (ABG). This background, which lies above the optical background of the array addressed earlier, will be a combination of any non-specific signal and a possibly a sequence dependent signal due to cross-hybridization. An estimate of the assay background can be made from Assay BG controls that are made up of random oligo sequences on the array. These sequences are designed to not cross-hybridize against the genome.

The statistical significance of these measurements is given by the following S/N plot. See Figure 4.

![Assay BG Control S/N](image)

**Figure 4** The S/N of assay background controls

The plot shows a median S/N of 0.1. The median value is usually quite small with respect to the overall measurement errors in the system. High S/N points could be single bad measurements, or they could represent an ABG control that is susceptible to a large amount of cross-hybridization.

The median ABG distribution may be useful as a statistical correction to the average ABG bias of all probe signals.

**Note:** All CL normalized output columns (Signal, SDEV, S/N and CV) include the ABG correction.
S/N, CV, Thresholds and Fold Confidence

Overview

Noise sources ultimately determine the sensitivity and reproducibility of the system. The signal-to-noise ratio (S/N) allows you to express the detection of a gene in terms of a confidence probability. Similarly, when comparing measurements of a gene signal between one or more arrays, the gene coefficient of variation (CV) is used in calculating the confidence probability of fold change. The CV is determined by the gene S/N, as well as the intrinsic reproducibility of the system.

The system error budget that determines estimated standard deviation of the signal from each probe is built from propagating estimates of measurement uncertainties at different stages of the processing. The primary sources of uncertainty are pure counting (Shot) noise statistics on the background subtracted signals in both channels and uncertainties in the background estimate itself. The background noise has a component determined by Shot noise on signals associated with non-specific chemical signal in the CL channel and background fluorescence and scattered light in the FL channel, in addition to a minor component of “read-noise” from the CCD detector itself. However, overall background uncertainty tends to be dominated by structure in the images. In FL, this is primarily structure in the substrate revealed by the background fluorescence. In CL, it is limited mostly by spatial cross-talk between features compounding the local background estimate around each feature.

S/N and Detected Probes

Default exposure times for the two channels place images in a regime where these sources of background structure noise dominate. In this domain, both the noise and signal increase linearly with exposure (or number of images), which implies no further increase in signal-to-noise in a feature and, therefore, no increase in the number of detectable features. The feature S/N is the ratio of signal to signal standard deviation (SDEV) and the SDEV refers to the estimated measurement uncertainty of the signal on the array from propagating all these uncertainties associated with the final normalized signal estimate.

The S/N is a metric that captures the confidence of the measurement “detectability” above all known sources of noise. You can use this metric to bin genes or probes as “Present” or “Absent” at the desired level of confidence. Since the S/N expresses the number of standard deviations, the associated confidence can be looked up from a probability table for a normal distribution. For example, signals with S/N ≥ 3 (a recommended threshold) have > 99.9% confidence the measurement is real, while a S/N of 2 would have a higher false positive rate with a confidence of 97.7%.

Note: S/N represents confidence and although it correlates with signal intensity, it should not be used in quantitative analysis, except for assessing quantitative value.

Low S/N Probes

Probes which have a FLAGS value in which the lowest bit is set to 1 (i.e., FLAGS = 1 or an odd number) have measured S/N values < 1. These signals are replaced with a 1 standard deviation upper limit on the signal intensity based on their estimated signal standard deviation, not their actual measured intensity. These probes must be treated differently from probes with S/N > 1 in fold change calculations.

A situation common to all microarray platforms occurs when a gene that has zero (or undetectable) expression is frequently measured to have a negative intensity. Although such signals do not reflect physical meaning of gene expression, they are a natural consequence of a quantification step that involves background (optical and assay) subtraction against a small or zero signal where there are uncertainties in measurement in both signal and background. However, such probes contain useful
information in the reported signal uncertainty (signal SDEV), which can be used as an upper limit (for example, 1 SDEV, equivalent to 84% confidence) on the signal intensity (assuming its true value is zero). In turn, this upper limit can be used to calculate a statistically meaningful upper (or lower) limit on the fold change between measurements. An example is outlined below.

**Note:** The Applied Biosystems 1700 system platform leaves the S/N as it is actually measured by the algorithm. The product of the S/N and SDEV restores the signal to its measured value and should be done if you are aggregating signals from multiple arrays. Also, the replacement of signal with an upper limit is restricted to probes only, not controls.

**Gene CV and Fold Change Confidence**

Array experiments are often conducted to measure the differences in expression level between one or more samples. A common metric of these differences is the expression ratio where gene expression measurements from one array are divided by the measurement from another. The confidence value for the measured ratio-fold change, G, can be calculated based on the uncertainties associated with the gene signals. This calculation involves more than the measurement errors; it also involves accounting for the system precision (the intrinsic spread of the normalized signals independent of the measurement errors). This is estimated by measuring the coefficient of variation (CV; i.e., the fractional variation in signal) of high S/N replicate controls on the array. (The high S/N ensures that measurement uncertainties on these controls contribute minimally to the total variation seen.) The system precision is measured for each array and is typically around 5 to 7%. The reported gene CV is calculated from the following equation:

$$CV = \sqrt{\left(\frac{1}{S/N}\right)^2 + (CV_{prec})^2}$$

**Equation 5**

It should be emphasized that the gene CV here is a predicted CV derived from an Error Model and that gene signal multiplied by the gene CV represents the total probe signal variation that might otherwise be estimated analyzing a gene signal across multiple replicate (globally normalized) arrays, that is, technical replicates, not biological replicates. Applied Biosystems recommends that you estimate gene CVs from multiple replicate arrays. However, the predicted gene CV provided is useful for estimating a fold change confidence value between as little as a pair of arrays with no replicates. Because the predicted CV represents only the known uncertainties of the system (i.e., measurement variation instead of biological variation), fold change confidence estimates using this CV represent the maximum confidence the system can deliver in a situation with no replicates. This CV is, therefore, most useful in limiting what can be claimed in fold change confidence.
The scatter in the ratio measurements is close to log-normal (true of the ratio of two normal distributions with small CV uncertainties), so the analysis is made in LOGe (natural log) ratio space:

\[ R = \text{LOGe} \left( \frac{I_{\text{SIGNAL 1}}}{I_{\text{SIGNAL 2}}} \right) \]

**Equation 6**

which has the (LOGe standard deviation) uncertainty given by approximately:

\[ \sigma_R = \sqrt{CV^2_{\text{SIGNAL 1}} + CV^2_{\text{SIGNAL 2}}} \]

**Equation 7**

and is also tied to the uncertainty in the ratio distribution, which has a one standard deviation range of approximately:

\[ G/(1 + \sigma_R) \leftrightarrow G(1 + \sigma_R) \]

**Equation 8**

If the value is small, it approximates the fractional uncertainty (CV) of the ratio distribution.

**Note:** An estimate of the standard deviation of a distribution can be made with the following metric:

\[ rSD[R] = 1.4826 \times \text{median}[absc(R) - \text{median[R])] \approx SD[R] \]

**Equation 9**

This metric has the advantage of being insensitive to small numbers of outliers that typify real data, unlike the more traditional definition of standard deviation. The numerical constant is a scalar to align the median absolute deviation with the standard deviation estimate for a normal distribution. This ensures that for normal distributions with no outliers the rSD gives essentially the same result as SD. For ratio distributions built from signals with high CVs (for example, CV = 0.33, equivalent to S/N ≈ 3), **Equation 7** more closely predicts the rSD rather than the SD of the LOGe ratio distribution.

The fold change confidence (that G is not equal to 1) can be derived from the z-score:

\[ z = R/\sigma_R \]

**Equation 10**

where for small values of \( z (< 3) \) an approximate probability can be looked up from a normal distribution.
Example Fold Change Calculations

Example 1: Calculating a fold change confidence using output normalized signals and an independently determined global normalization

A gene on Array 1 has Signal1 = 2000 with a CV = 0.35, and on Array 2 has Signal2 = 3000 with a CV = 0.25. The user has separately determined a global normalization factor of 1.2 is required to normalize all signals from Array 2 to Array 1. (Note that this factor only applies to signals, not to S/N or CV.)

- \( R = \log_e(3000 \times 1.2/2000) = 0.59 \)
- \( \sigma_R = \sqrt{(0.35^2 + 0.25^2)} = 0.43 \)
- \( \frac{R}{\sigma_R} = 1.37 \) standard deviations
- Z-SCORE probability (from Probability tables) is 82.8%

The gene on Array 2 has a fold increase of 1.8× over Array 1 with a fold change probability of 82.8%.

Example 2: Calculating an upper limit on fold change confidence where a gene is Absent (low or negative S/N) on one array

A gene on Array 1 has a reported Signal1 = 250, a S/N ≈ -0.8 and, consequently, a FLAGS value of 1 (or an odd number) that indicates the signal is actually set to the 1 standard deviation upper limit value given by its SDEV. Array 2 has Signal2 = 3000, CV = 0.15. The user has separately determined a global normalization factor of 1.2 is required to normalize all signals from Array 2 to Array 1.

Array 1 will have a reported value of S/N of -0.8. However, a 2 SDEV (97.7% confidence) upper limit on this signal will be 2× Signal1 − SDEV = 500 counts (assuming the true value is actually zero).

Array 2 will have a 2 SDEV lower limit of 3000 × 1.2 × (1 − 2 × 0.15) = 2520 counts. You could, therefore, conclude that Array 2 has a 95.5% confidence (0.977^2*100) lower limit of fold change increase of 5.04× (2520/500).
The following plots compare the inter-array reproducibility and predictions of reproducibility based on the predicted CV:

**Array Performance**

**Figure 5** Inter-array reproducibility (for a Stratagene UHR sample) showing detected genes with at a S/N ≥ 3 threshold in red

An empirical measure of fold change confidence as a function of signal intensity can be made by determining the fold change that encompasses 95% of the points in an individual intensity bin (see **Figure 6**).

**Figure 6** Empirically measured fold change confidence. Each point represents 2% of genes on the array
The empirical measure of scatter can be compared against predictions from the system Error Model:

Figure 7  In this example, the Error Model (which determines the feature CV) agrees broadly with the empirical measure and slightly overestimates the scatter at a S/N of three

The limiting fold change measure of the system approaches $1.25 \times$ at 95% confidence for the highest S/N genes. At a detection limit of $S/N = 3$, the system is sensitive to fold changes of $\sim 3.3 \times$ at 95% confidence.

Note: This level of performance is a statistical certainty from the ratio of two features with a S/N of 3 that is independent of the system itself for systems of comparable precision.
In general, there is a direct relationship between feature S/N and signal CV given by Equation 5 at a certain system precision. In turn, the signal CVs for a pair of measurements predict their ratio CV (or more precisely the LOGe ratio standard deviation) given by Equation 7; roughly SQRT(2) times the signal CV for probe pairs with the same signal CV. Table 2 lists equivalences for a typical system precision of the AB1700 of 6% for each feature:

### Table 2  Equivalences for a typical system precision of the AB1700 of 6%

<table>
<thead>
<tr>
<th>Feature S/N</th>
<th>3.0</th>
<th>4.5</th>
<th>6.7</th>
<th>&gt; 30</th>
</tr>
</thead>
<tbody>
<tr>
<td>Predicted intra-array signal CV</td>
<td>0.34</td>
<td>0.23</td>
<td>0.16</td>
<td>0.06</td>
</tr>
<tr>
<td>Predicted interarray ratio CV</td>
<td>0.48‡</td>
<td>0.33‡</td>
<td>0.24</td>
<td>0.09</td>
</tr>
<tr>
<td>Predicted interarray LOGe ratio SDEV</td>
<td>0.48‡</td>
<td>0.33</td>
<td>0.24</td>
<td>0.09</td>
</tr>
<tr>
<td>Predicted interarray LOG2 ratio SDEV</td>
<td>0.70‡</td>
<td>0.47</td>
<td>0.34</td>
<td>0.12</td>
</tr>
<tr>
<td>Predicted interarray LOG10 ratio SDEV</td>
<td>0.21‡</td>
<td>0.14</td>
<td>0.10</td>
<td>0.04</td>
</tr>
<tr>
<td>GEX fold change at 95% confidence (p = 0.05)</td>
<td>3.3</td>
<td>2.0</td>
<td>1.6</td>
<td>1.2</td>
</tr>
<tr>
<td>p value for GEX &gt; 2X</td>
<td>0.184</td>
<td>0.050</td>
<td>0.006</td>
<td>&lt; 0.001</td>
</tr>
</tbody>
</table>

‡ At a S/N of 3, the ratio CV or LOG ratio SDEV is poorly defined. These values are calculated from the median absolute difference definition given by equation 9.

**Note:** With a S/N of ~30, values stay roughly the same approaching the numbers in the right-hand column.
Data Filtering of Problematic Measurements

**FLAGS** Values under 100 are considered valid quantification. Values in the range 100–1000000 have quality issues. Values exceeding 8191 are recommended to be rejected from analysis. These are based on quality issues captured by QCmetric2 below an internally defined threshold, as well as low S/N in the FL channel, low gridding confidence and strongly negative S/N values. Values above 1000000 represent failures or invalid quantification. You can filter data based on these FLAGS values.

**Feature Quality Metrics** Together with S/N and predicted CV, each feature has two quality metrics:
- QCmetric1
  You can filter out problematical data, based on your own preferred threshold. However, since low correlation can also reflect low S/N on the feature with otherwise valid quantification, Applied Biosystems recommends that you exercise caution with this potential filter. There is no internally defined threshold for triggering a FLAGS state for this metric.
- QCmetric2
  This is a feature quality metric that captures the fraction of usable pixels used in quantification. The selection criterion is based on a Chi2 test of CL/FL pixel ratios and can be used to filter out problematic measurements likely to be associated with artifacts on the array that are corrupting quantification. A FLAGS state is set for features falling below an internally defined threshold of 2/3.

**Flags** The algorithm automatically flags data for a number of quality issues, warnings, and failures. These states are captured in the FLAGS number and are listed in Table 2. The FLAGS number increases with the severity of issue and divides into the following classes:
- **0** – Feature has no issues with quantification
- **1** – Probe signal has S/N < 1 and is replaced with a 1 standard deviation upper limit based on it signal standard deviation estimate.
- **2–100** – Feature has issues that should not affect the quality of quantification. Generally, these issues include partial detector saturation on the feature or pixels that have been rejected during quantification. The algorithm is robust to these problems and these should be considered reliable quantification.
- **500–8191** – Refers to issues that will affect the quality of quantification (corrupted neighbors or features outside the optimal focus region of the array).
- **8192–1000000** – Refers to quality issues that should be considered failures. Specifically, these have been identified as low FL S/N (< 5), features which have had > 1/3 of feature pixels rejected from quantification, low gridding confidence, or strongly negative S/N (< −3) features (equivalent to QCmetric < 0.66).
- **> 1e6** – Refers to failures in quantification (for example, feature is heavily saturated or not identified during gridding).
Applied Biosystems recommends that you filter out signals with FLAGS values that exceed 8000 (or, specifically, 8191). You can also separately filter data based on your own chosen thresholds of low S/N, high CV, low QCmetric1 (low CL/FL feature pixel correlation, but note that this correlation can simply reflect low S/N) and low QCmetric2 (low fraction of available pixels used in quantification due high number of pixels rejected in fit). In such cases, you must first filter for features with FLAGS values > 1e6, which correspond to features with no valid quantification.

Figure 8  Data for Gene S/N > 3 on both arrays and filtered by FLAGS > 8000

Figure 9  Features on a single array flagged by a low QCmetric2, which tests the feature morphology integrity. Note that the quantification is robust in cases where artifacts affect a small number of pixels on the feature.
### Feature Quantification Flags

**Note:** Larger feature FLAGS values are associated warnings or errors of increasing severity. FLAGS = 0 indicates a measurement with no known errors or issues. FLAGS = 1 or an odd numbered FLAGS value indicates the probe has a S/N < 1 and the signal reported corresponds to a 1 SDEV upper limit, not a measured value. Multiple flags are additive, for example, FLAGS = 68 equals 64+4+0 (1000100 in binary) and decodes as “has replaced pixels from CLs image” and “has pixels rejected in fit.”

#### Table 3 Feature quantification flags

<table>
<thead>
<tr>
<th>Bit</th>
<th>Value</th>
<th>Assigned</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>1</td>
<td>Probe has S/N &lt; 1 and signal is replaced with 1 SDEV upper limit</td>
</tr>
<tr>
<td>1</td>
<td>2</td>
<td>Feature centroid is from interpolated grid position</td>
</tr>
<tr>
<td>2</td>
<td>4</td>
<td>Feature uses scaled pixels from CLs image</td>
</tr>
<tr>
<td>3</td>
<td>8</td>
<td>Partial saturation of feature CL quant pixels (&lt; 0.5)</td>
</tr>
<tr>
<td>4</td>
<td>16</td>
<td>Partial saturation of feature FL quant pixels (&lt; 0.5)</td>
</tr>
<tr>
<td>5</td>
<td>32</td>
<td>Partial saturation of pixels (1 or more) in local BG CL or FL annulus</td>
</tr>
<tr>
<td>6</td>
<td>64</td>
<td>Feature has pixels rejected in fit</td>
</tr>
<tr>
<td>7</td>
<td>128</td>
<td>Null</td>
</tr>
<tr>
<td>8</td>
<td>256</td>
<td>Null</td>
</tr>
<tr>
<td>9</td>
<td>512</td>
<td>Null</td>
</tr>
<tr>
<td>10</td>
<td>1024</td>
<td>Feature is outside optimal position limits ([350 1700])</td>
</tr>
<tr>
<td>11</td>
<td>2048</td>
<td>Feature has FL neighbor with quantification problem</td>
</tr>
<tr>
<td>12</td>
<td>4096</td>
<td>Feature has CL neighbor with quantification problem</td>
</tr>
<tr>
<td>13</td>
<td>8192</td>
<td>Null</td>
</tr>
<tr>
<td>14</td>
<td>16384</td>
<td>Feature has low FL S/N (&lt; 5)</td>
</tr>
<tr>
<td>15</td>
<td>32768</td>
<td>Feature has poor fit (QCmetric2 &lt; 0.66)</td>
</tr>
<tr>
<td>16</td>
<td>65536</td>
<td>Null</td>
</tr>
<tr>
<td>17</td>
<td>131072</td>
<td>Feature is strongly negative with S/N &lt; -3, suggesting quantification issue</td>
</tr>
<tr>
<td>18</td>
<td>262144</td>
<td>Probe centroid is from interpolated grid position</td>
</tr>
<tr>
<td>19</td>
<td>524288</td>
<td>Null</td>
</tr>
</tbody>
</table>
### Table 3  Feature quantification flags (continued)

<table>
<thead>
<tr>
<th>Bit</th>
<th>Value</th>
<th>Assigned</th>
</tr>
</thead>
<tbody>
<tr>
<td>20</td>
<td>1048576</td>
<td>Quantification returned a NaN value for a Signal field</td>
</tr>
<tr>
<td>21</td>
<td>2097152</td>
<td>All pixels in local BG (CL or FL) saturated</td>
</tr>
<tr>
<td>22</td>
<td>4194304</td>
<td>Full saturation of feature FL quant pixels (&gt; 0.5)</td>
</tr>
<tr>
<td>23</td>
<td>8388608</td>
<td>Full saturation of feature CL quant pixels (&gt; 0.5)</td>
</tr>
<tr>
<td>24</td>
<td>16777216</td>
<td>Feature is too close to edge of image for quantification</td>
</tr>
<tr>
<td>25</td>
<td>33554432</td>
<td>Feature has no Grid position</td>
</tr>
</tbody>
</table>

Bits 0–6 are informational

Bits 10–18 are quality issues

Bits 20–24 are failures in quantification

Features with FLAGS bits higher than 9 may have quality issues. Feature with FLAGS bits higher than 12 are recommended for rejection from analysis.
### Probability Table for Fold Change

Two-tail probability is the cumulative probability from 0 to $z$ (i.e., significance that the fold change is not equal to 1), one-tail probability is the cumulative probability from $-\infty$ to $+z$ for a normal distribution.

**Table 4  Fold change probability**

<table>
<thead>
<tr>
<th>$z$</th>
<th>P (two-tail)</th>
<th>P (one-tail)</th>
<th>$z$</th>
<th>P (two-tail)</th>
<th>P (one-tail)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>0.500000</td>
<td>2.1</td>
<td>0.964271</td>
<td>0.982136</td>
</tr>
<tr>
<td>0.1</td>
<td>0.079656</td>
<td>0.539828</td>
<td>2.2</td>
<td>0.972193</td>
<td>0.986097</td>
</tr>
<tr>
<td>0.2</td>
<td>0.158519</td>
<td>0.579260</td>
<td>2.3</td>
<td>0.978552</td>
<td>0.989276</td>
</tr>
<tr>
<td>0.3</td>
<td>0.235823</td>
<td>0.617911</td>
<td>2.4</td>
<td>0.983605</td>
<td>0.991802</td>
</tr>
<tr>
<td>0.4</td>
<td>0.310843</td>
<td>0.655422</td>
<td>2.5</td>
<td>0.987581</td>
<td>0.993790</td>
</tr>
<tr>
<td>0.5</td>
<td>0.382925</td>
<td>0.691462</td>
<td>2.6</td>
<td>0.990678</td>
<td>0.995339</td>
</tr>
<tr>
<td>0.6</td>
<td>0.451494</td>
<td>0.725747</td>
<td>2.7</td>
<td>0.993066</td>
<td>0.996533</td>
</tr>
<tr>
<td>0.7</td>
<td>0.516073</td>
<td>0.758036</td>
<td>2.8</td>
<td>0.994890</td>
<td>0.997445</td>
</tr>
<tr>
<td>0.8</td>
<td>0.576289</td>
<td>0.788145</td>
<td>2.9</td>
<td>0.996268</td>
<td>0.998134</td>
</tr>
<tr>
<td>0.9</td>
<td>0.631880</td>
<td>0.815940</td>
<td>3</td>
<td>0.997300</td>
<td>0.998650</td>
</tr>
<tr>
<td>1</td>
<td>0.682689</td>
<td>0.841345</td>
<td>3.1</td>
<td>0.998065</td>
<td>0.999032</td>
</tr>
<tr>
<td>1.1</td>
<td>0.728668</td>
<td>0.864334</td>
<td>3.2</td>
<td>0.998626</td>
<td>0.999313</td>
</tr>
<tr>
<td>1.2</td>
<td>0.769861</td>
<td>0.884930</td>
<td>3.3</td>
<td>0.999033</td>
<td>0.999517</td>
</tr>
<tr>
<td>1.3</td>
<td>0.806399</td>
<td>0.903200</td>
<td>3.4</td>
<td>0.999326</td>
<td>0.999663</td>
</tr>
<tr>
<td>1.4</td>
<td>0.838487</td>
<td>0.919243</td>
<td>3.5</td>
<td>0.999535</td>
<td>0.999767</td>
</tr>
<tr>
<td>1.5</td>
<td>0.866386</td>
<td>0.933193</td>
<td>3.6</td>
<td>0.999682</td>
<td>0.999841</td>
</tr>
<tr>
<td>1.6</td>
<td>0.890401</td>
<td>0.945201</td>
<td>3.7</td>
<td>0.999784</td>
<td>0.999892</td>
</tr>
<tr>
<td>1.7</td>
<td>0.910869</td>
<td>0.955435</td>
<td>3.8</td>
<td>0.999855</td>
<td>0.999928</td>
</tr>
<tr>
<td>1.8</td>
<td>0.928139</td>
<td>0.964070</td>
<td>3.9</td>
<td>0.999904</td>
<td>0.999952</td>
</tr>
<tr>
<td>1.9</td>
<td>0.942567</td>
<td>0.971283</td>
<td>4</td>
<td>0.999937</td>
<td>0.999968</td>
</tr>
<tr>
<td>2</td>
<td>0.954500</td>
<td>0.977250</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Section 2  Image Import Utility

This section covers:

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Installing the Utility ....................................................... 27
Using the Utility ............................................................. 28
Image File Name Specification .......................................... 32
Results ........................................................................... 32

Introduction

This section of the user bulletin describes how to use the image import utility to import image sets into the Expression Array System Software v1.1.1. You may need to import image sets if the original installation of the Expression Array System Software was damaged, or if you want to load data from another instrument on your own computer.

This utility makes it unnecessary to run image sets through the simulator, which no longer works with Expression Array System Software versions 1.1 and later.

IMPORTANT! Use the image import utility only to import images into a v1.1.1 installation.

Installing the Utility

The image import utility is installed automatically when you install the Expression Array System Software v1.1.1 or upgrade the software from an earlier version. The image import utility is installed in the <install path>\service\bin directory, where <install path> is the installation directory path, for example, D:\ABI\AB1700.
Using the Utility

1. Restart the computer to restart the AB1700 services.
   **Note:** If the AB1700 services remain running after reading with an instrument or the simulator, the system may generate duplicate QC reports, resulting in a slow-down of system performance.

2. Create a new folder outside of the installation directory, then put the images to be imported into the folder. For example:

   ![Folder with images](image)

   The folder in the example, above, contains images from two arrays (HA000FA and HA000FH).

   **Note:** The image importer will fail if there is more than one set of images for a particular microarray barcode. Either re-name one set or import each set into different directories.

   **Note:** The image importer may also fail if there are problems with the image file names. See “Image File Name Specification” on page 32.

3. Determine the directory for the calibration files. The default destination is:
   ```
   C:\Program Files\1700 Test Tools\Active\RSNxxxxxxx\Processed
   ```

   **IMPORTANT!** If the images were created using Expression Array v1.0.3 or earlier and were NOT upgraded, the calibration folder must contain the correct calibration files for these images (i.e., tifheader.txt and camera.csv). For images created with v1.1 or later, the calibration directory containing a set of calibration files must be provided, but the calibration files will not be used.
4. Obtain the instrument service name:
   a. Select **Start ▶ Programs ▶ Applied Biosystems ▶ Expression Array System Software ▶ Expression Array System Analyzer**.
   b. In the AB Navigator Dialog box type your Login Name and Password, then click **OK**.
   c. In the navigation pane, double-click **Expression Array System Software** to display the available applications.
   d. Expand the AB1700 Data Collection folder to show the instrument, then write down the name.
      
      **Note:** The default name is “1700-Reader.”

=capture

   ![AB Navigator](image)

   e. Exit the Expression Array System Software.

   **IMPORTANT!** You must exit the Expression Array System Software before running the image import utility.

5. Open a command window:
   a. In the desktop, select **Start ▶ Run**.
   b. Type **cmd**, then click **OK**.

   **Note:** The image import utility will write output to the command window, so keep it visible.
6. Change to the directory where the image import utility is installed:
   a. Change to the drive where the Expression Array System Software is installed by typing \"<drive letter>\:.\"
      For example:
      \d:
      \n      Note: The software is usually installed on D drive, but in some rare cases it is installed on C drive or E drive.
   
b. Press Enter.
   c. Change directories by typing \cd <install path>\service\bin, where <install path> is the installation directory path.
      For example:
      \cd abi\ab1700\service\bin
      
d. Press Enter.
      
   The following is an example of steps a through d:

   7. Run the image import utility by typing:
      \n      ImageImport.exe <directory of images> <calibration directory> <service name>
      \n   where:
   
   • <directory of images> is the full path from step 2
   • <calibration directory> is the full path from step 2
   • <service name> is the instrument service name from step 4

   Note: If the directory path contains any spaces, put it in quotes.
   For example, "C:\name with spaces\"

   Refer to the example below, which uses the correct syntax for the examples provided above:
8. Close any error dialog boxes to continue processing images if bad images cause the “MaskOperation” step to fail.

After the utility finishes upgrading all the images in the folder, the Expression Array analysis algorithms will launch automatically and run in the background. Each image set will take several minutes to analyze.

In the command window, the utility displays text similar to the following:

```
Use Default Images Directory C:/abi/AB1700/images
Starting new assay:
  HB0014LV DURHAMJG FL1_25S FEB162005 145319
  HB0014LV DURHAMJG FX1_25S FEB162005 145404
  HB0014LV DURHAMJG CL1_5S FEB162005 145428
  HB0014LV DURHAMJG CL1_25S FEB162005 145453
  HB0014LV DURHAMJG FL2_25S FEB162005 145516
  HB0014LV DURHAMJG FX2_25S FEB162005 145542
  HB0014LV DURHAMJG CL2_5S FEB162005 145609
  HB0014LV DURHAMJG CL2_25S FEB162005 145632

Before analysis
Success.Transforms:3 Signals:18921
Primary analysis for image area one of Microarray HB0014LV completed successfully.
Success.Transforms:3 Signals:18921
Primary analysis for image area two of Microarray HB0014LV completed successfully.

After analysis
Timing for: Analyze Assay Images, elapsed: 0:8:27
Wait a second.
Run QC Report
Timing for: Run QC Report, elapsed: 0:6:37
QC report completed for HB0014LV.
No more images to import
Timing for: Analyze Assay Images, elapsed: 0:0:0
```

9. Close the command window after the utility displays the message “No more images to import.”

**Note:** The utility will not return you to the command prompt.

10. After importing a group of images, put the next group of images in an empty folder, then run the utility again.

11. Start the Expression Array System Software and check the status of the arrays and assays in the AB Navigator. All imported assays will appear in the “Imported Folder.”

12. If you attempt to access a microarray, receive the message “can't lock - resource locked by another user,” then cannot access the array, exit the Expression Array System Software, then restart the AB1700 services.
Image File Name Specification

If the image importer fails, the failure may be caused by a problem with the image file names. Verify that the format of the file names meets the specification.

**Note:** If you edit the bar code field, make sure you follow the specification, below.

Image file names consist of six components, separated by underscore characters (_). The file names must conform to the following format:

```
Microarray(BarCode)_MachineName_ImageType_Exposure_Date_Time.TIF
```

where each component is further constrained:

- **Microarray(BarCode)** – Consists of two subcomponents: Microarray and (BarCode).
  - **Microarray** – Can include any combination of upper or lower case, but must contain 10 characters and be spelled correctly. No spaces are allowed, and this subcomponent must be followed directly by the (BarCode) subcomponent.
  - **(BarCode)** – Can include spaces or any letter or number, but must be enclosed in parentheses and must follow the Microarray subcomponent directly. The first two characters must be upper case and a valid cartridge type (for example, HA, HB, MA or RA).

**IMPORTANT!** Punctuation characters, including underscore characters, are not allowed in the (BarCode) subcomponent:

```
[_, ./<>?;'"\[]{}\|+-=!@#$%^&*]
```

- **MachineName** – Consists of all characters between the first and second underscore character (_), and must not contain embedded underscores.
- **ImageType** – Consists of all characters between the second and third underscore character (_). To run primary analysis, this component must be one of the following: CL1, CL2, FL1, FL2, FX1, FX2.
- **Exposure** – Consists of all characters between the third and forth underscore character (_). Typical values are 5S and 25S, and must also meet any primary analysis requirements.
- **Date** – Consists of all characters between the second-to-last and the last underscore character (_). The date must be in the format MMMddyyyy, where MMM is month abbreviation (for example, JAN, FEB, DEC).
- **Time** – Consists of all characters after the last underscore character (_) until the dot (.). The time must be in the format HHmmss.
- **TIF file extension** – Can be any combination of upper and lower case (for example, .tif, .TIF, .TiF). The TIF file extension must be a dot (.) followed by three characters.

Results

The utility creates a new set of assays each time it is invoked. Since original processing time and dates are preserved, this may result in what appears to be duplicates. The assay History tab lists the Date/Time of recovery.
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