Data Analysis: Microarray Gene Expression

An overview of data processing using the NextBio platform for gene expression analysis.

Introduction

The Illumina NextBio library contains over 113,000 biosets obtained by mining the vast amounts of publicly available genomic data from sources such as the Gene Expression Omnibus, ArrayExpress, and other repositories. These data go through a systematic screening, curation, and analysis process (Figure 1).

Most genomic data within the NextBio platform are generated using the Affymetrix platform (Figure 2). Ideally, all Affymetrix data would be imported as CEL files, and processed using the same normalization method, such as Robust Multi-array Average (RMA)[1]; however, for pre-existing experiments, this is often impossible. In this case, probeset-level Microarray Suite version 5 (MAS5) intensities[2] or, in some cases, DNA-Chip Analyzer (dChip)-generated intensities[3] are imported.

The data pre-processing depends on the platform and format of the available data. This technical note outlines the analysis procedure used to create biosets from individual gene expression (RNA and miRNA expression) experiments. Standard statistical tests and analysis procedures are used throughout the analysis protocol.

Data Pre-Processing

Background Correction

Data pre-processed using MAS5 and RMA are corrected for background signal. For data obtained from other platforms, background signal intensities are subtracted when such information is available.

Expression Summarization

For Affymetrix data, CEL files are processed using RMA normalization, and the summarized data are analyzed using the statistical methods described in the following sections. When CEL files are unavailable, the existing summarized data are imported. These data may have been processed using MAS5, or some other procedure. For other platform providers, such as Agilent or Illumina, processed data files that contain all relevant statistical information for analysis (signal/control intensity, background, etc.) are typically provided by sources such as Gene Expression Omnibus (GEO).

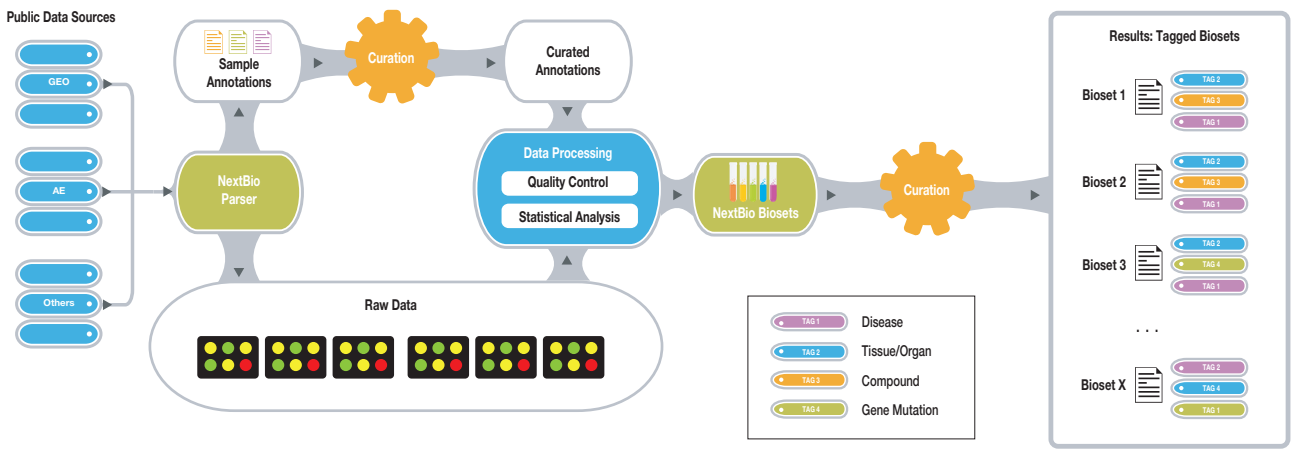
Normalization

As part of the NextBio analysis protocol, data are initially examined using diagnostic plots, such as box plots, for each study. In one-channel array experiments, if chip-to-chip variability is observed, data are marked for per-chip median normalization to be applied. In two-channel array experiments, the default normalization method employed for correcting intensity-dependent dye-bias is Lowess normalization. Box plots of post-normalized data are supplied with all public studies for user review.

Data that are not already in logarithimic scale are log-transformed (base 2). If technical replicates are present, they are averaged before statistical analysis.

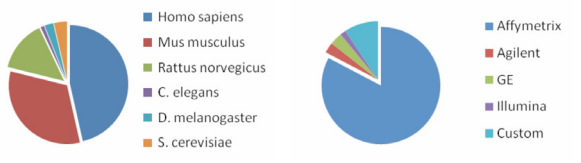
The choice of normalization procedures (RMA, MAS5, etc.) will affect the resulting comparison of p-values and fold-change values. However, the effect of this difference is minimized by focusing on the

Figure 1: Public Data Analysis



The workflow for turning public data sets into processed gene biosets includes raw data collection, sample annotation curation, data quality control, automated analysis, and manual tagging of resulting biosets with disease, tissue, and compound ontology terms (tags).

Figure 2: Bioset Compositions



Proportion of NextBio platform biosets from various platforms and species (as of June 2008).

resulting ranking of elements (which should be more invariant across different normalization procedures). That is, for a given experiment and treatment factor within that experiment, the elements that are most affected by the factor will rank fairly high, regardless of which underlying normalization was used. This information is used to construct the bioset. In addition, ranks enable comparison of data with different types of absolute values—e.g., protein abundance data with gene expression signatures or with sets of RNAi targets producing positive hits in phenotypic assays.

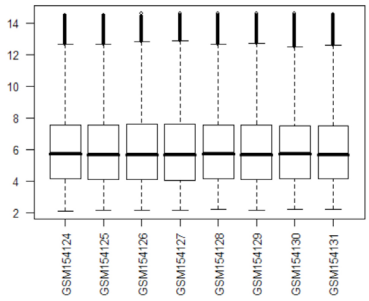
Data Visualization and Quality Control

To examine data quality and experimental design assumptions further, hierarchical clustering of samples is used to assess separation of samples visually, according to the treatment or test factors. If good separation is not seen for each factor of interest (i.e., factors that the experiment was designed to test), it is an indicator that the data may not be good enough to construct reliable biosets. These analyses, as well as the box plot analysis (Figure 3), are also used to identify "bad" or "outlier" samples. The determination on whether to exclude any samples is made on a case-by-case basis.

Several data quality metrics are reported for every data set to enable the assessment of data quality. These include:

a. The number of missing values per sample (flagged if it is > 2% of number of probes/genes in any sample). Missing values are not replaced.

Figure 3: Box Plot of Sample Intensities

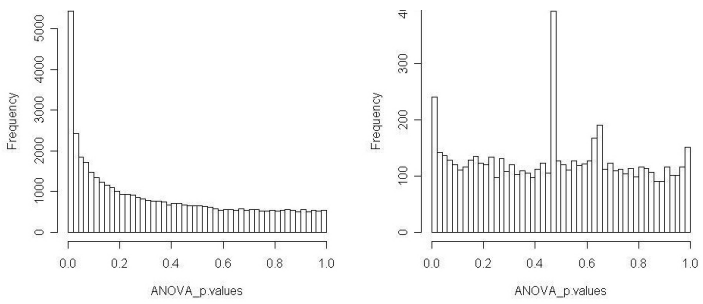


b. The number of negative datapoints (fails quality control if this number is > 10% of number of datapoints). Negative signal intensities in the data are corrected by either: i) setting all negative values to the smallest positive signal intensity (default); or ii) shifting signal intensities up by a constant so that the smallest value is positive.

c. The distribution of the signal intensities in terms of [0%, 25%, 50%, 75%, 100%]-quantiles.

In addition, an analysis of variance (ANOVA) F test is performed to compare the various factors (i.e., treatment or control groups) in the study. At a minimum, the number of genes showing significant changes must exceed the number expected by chance. A histogram of the ANOVA p-value distribution (Figure 4) is generated and examined for concordance with the expected distribution for a study with statistically significant results (i.e., disproportionately lower p-values). This figure is also associated with each public data set for users to validate the significance of the study results.

Figure 4: Data set Analysis Examples



Histograms of ANOVA F-test p-values for data sets of good quality (left panel) and poor quality (right panel). The p-values are grouped into 50 bins and plotted against the number of genes in each bin. The left panel graph indicates a subset of genes that show differential expression.

Data Filtering and Differential Expression Analyses

For each parameter of interest in the study, the following comparison procedure is used.

1. Signal quality: Genes are filtered to eliminate low-quality and less reliable measurements. The filtering is based on raw expression signal (after RMA or MAS5 processing for Affymetrix data). For each comparison (e.g., treated vs. control) the signal intensity must be above a certain threshold level of intensity in at least one of the two groups being compared. The threshold is calculated as the 20th percentile of all raw (probeset-level) measurements.
2. Comparisons: Samples are grouped by curators, depending on the parameter of interest. The following guidelines apply:
   a. In a time series study, all time points are compared against the zero time point.
   b. In studies with one or more treatments, all treatments are compared against the control.
   c. If the study involves investigating a disease vs. normal state, the disease samples are compared against normal samples. For studies investigating benign vs. primary vs. metastatic cancer, each cancer state is compared against benign, because benign is the closest to normal.
3. Statistical tests: When comparing independent samples, a two-sample t test is performed on each gene, comparing the treated group to the control group (a one-sample t test is applied when there are no control samples). The Welch t test is used by default, when variances cannot be assumed to be equal. When comparing paired samples, a paired t test is performed.
4. Gene filtering: Except for tissue atlas biosets where the full gene signature is presented to the user, genes in all other biosets are filtered using a p-value cutoff of 0.05, with no multiple-testing correction. The resulting set of genes is further filtered using a fold-change difference cutoff between the average intensity in test and control groups. Typically, the NextBio platform considers fold changes ≥ 1.2 based on microarray technology sensitivity, but the choice may also depend on the type of experiment, tissue source, and other factors. For example, changes in many cell lines, certain treatment types, or brain tissues can be a lot smaller than changes in blood cells of trauma patients. In this case, the cutoffs can be adjusted accordingly. Volcano plots can be used to evaluate fold-change cutoff thresholds using distribution of fold changes within a given comparison. For more "noisy" tissues (e.g., tumor, blood), cutoffs are more stringent relative to other types of samples. While the choice of fold-change cutoff is not an exact science, choices are limited to a small range that depends on the experimental context.

Constructing a Bioset

The resulting final list of genes is used to construct the bioset, as shown in Figure 5. The bioset consists of the list of genes, along with associated p-value, fold change, and average expression level in each treatment group, for each gene. In addition, a short description of the bioset and a summary of the analysis performed can also be included in the file to be uploaded. All biosets in the NextBio library contain this information, which can be viewed on the bioset information page through the web interface.

For rank-based meta-analyses, the NextBio platform uses standard fields in the column headers to rank features in the bioset. If more than

Figure 5: Gene List Example

	A	B	C	D	E	F	G	H
1	Bioset summary = GSE7035 - PPARgamma ligans and platinum-based drugs in cancer							
2	Comparison = Differentially-expressed genes between samples in A549 cells treated with rosiglitazone vs. non-treated samples							
3	Analysis summary = CEL files were normalized using RMA (R-package). Statistical t-test assuming variances are not equal (Welsh t-test							
4								
5								
6	Gene name	fold change	p-value	Custom field 1	Custom field 2	...		
7	200606_at	1.21	0.0107	259	213.4			
8	200762_at	1.29	0.0216	670.4	519			
9	200778_s_at	1.33	0.0291	1445.8	1084.8			
10	200789_at	1.53	0.00199	1145.7	749.5			
11	200798_x_at	1.25	0.0389	468.6	375.1			
12	200817_x_at	-1.24	0.0107	8177.9	10155.6			
13	200862_at	1.53	0.0236	329	214.6			
14	200878_at	1.48	0.0313	1112.9	753.2			
15	200903_s_at	-1.31	0.0474	1280.9	1677.6			
16	200906_s_at	-1.47	0.0178	386.9	570.1			
17	201015_s_at	-1.26	0.0424	214.2	270.6			
18	201029_s_at	-1.23	0.0495	1539.2	1894.5			
19	201060_x_at	1.39	0.0421	307.9	220.9			
20	201108_s_at	1.45	0.024	140.3	96.9			
21	201162_at	-1.22	0.0179	326.4	397.8			
22	201233_at	-1.21	0.0461	224.4	272.6			
23	201255_x_at	-1.24	0.031	393.4	486.5			
24	201369_s_at	-1.28	0.0219	60.1	77			
25	201398_s_at	-1.32	0.0186	1350.8	1785.7			
26	201432_at	1.57	0.00123	1263.1	806.4			
27	201461_s_at	1.38	0.0378	54.2	39.2			
28	201471_s_at	1.44	0.0267	5368.3	3740.8			
29	201480_s_at	-1.26	0.0369	425.6	536			
30	201578_at	1.75	0.0309	699.8	401			

A549 NSCLC cells + rosiglitazon

Sample bioset from drug vs. control microarray samples analysis from A549 cell lines treated with rosiglitazone. In this example, the "fold change" column is selected for ranking during bioset import into the NextBio platform. Supplemental information columns can be included as custom columns with user-defined titles (currently, a maximum of five columns) to provide average expression level information in treated and control groups for interested users.

one standard statistical column is present, the NextBio platform picks one of the columns for ranking in the following order:

- Fold change
- p-value
- Score (this can represent any scoring measure determined by the analyst)
- Rank
- Correlation

During data import, the ranking criterion column is thus selected, and consequent mapping of all probesets to gene references (NCBI Gene ID) takes place.

A multiple-testing correction is not used for the following reasons:

1. As stated in the introduction, a bioset within the NextBio analysis pipeline should include all potentially interesting results, and so the emphasis is on reducing false negatives (at the expense of including some false positives).
2. Meta-analysis is based only on rank, not level of significance, and p-value ranking is not affected by multiple testing corrections.

In case of data generated by the NextBio platform, most of the ranks are based on fold-change values. When comparing biosets generated from different experiments, fold change is used as the default ranking as it has been shown to give better concordance across platforms than p-values from statistical tests[4]. In certain types of analysis comparing multiple conditions, the p-value is the only choice of statistics that could be used for ranking. In this case, the p-value will be selected as the metric to be used for ranking during data import into the NextBio platform when the fold-change column is absent.

Tagging Biosets with Ontology Terms

The final step in preparing data for use in the NextBio platform is the tagging of biosets with standard ontology terms reflecting disease, tissue, treatment, experimental design, and sample source (Figure 1). This step is important and provides a semantic backbone to all of the analyzed data. This enables the NextBio computational engine to perform additional high-level correlations, and also makes it easier for users to find relevant data sets. Comprehensive ontologies cover anatomy (Foundation Model of Anatomy [FMA]/Medical Subject Headings [MeSH][5]), diseases (SNOMED Clinical Terms [CT][6]), and compounds (over 8 million compound clusters and associated structures).

References

1. Irizarry RA, Hobbs B, Collin F, Beazer-Barclay YD, Antonellis KJ, et al. (2003) Exploration, normalization, and summaries of high density oligonucleotide array probe level data. Biostatistics 4: 249–264.
2. www.affymetrix.com/support/technical/byproduct.affx?product=mas
3. Li C, Wong WH (2001) Model-based analysis of oligonucleotide arrays: expression index computation and outlier detection. Proc Natl Acad Sci USA 98: 31–36.
4. Shi L, Tong W, Fang H, Scherf U, Han J, et al. (2005) Cross-platform comparability of microarray technology: intra-platform consistency and appropriate data analysis procedures are essential. BMC Bioinformatics 15 (6 Suppl 2): S12.
5. sig.biostr.washington.edu/projects/fm/AboutFM.html
6. www.ihtsdo.org/snomed-ct/

Illumina • 1.800.809.4566 toll-free (U.S.) • +1.858.202.4566 tel • techsupport@illumina.com • www.illumina.com

FOR RESEARCH USE ONLY

Pub. No. 970-2014-010 Current as of 07 August 2014

illumina®