User Manual

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Chapter 1
Introduction to GENEVESTIGATOR®

1.1 What is GENEVESTIGATOR®?

GENEVESTIGATOR® is an innovative search engine to investigate in a single analysis gene transcriptional regulation across thousands of experimental conditions. It summarizes data by condition types such as tissues, cancers, diseases, genetic modifications, external stimuli, or development (see Chapter 3) based on the concept of meta-profiles as described below (Chapter 1.1.1). GENEVESTIGATOR® integrates manually curated and quality-controlled gene expression data from public repositories (Figure 1.1) but can also integrate proprietary data (Hruz et al., [1]).

![Figure 1.1: GENEVESTIGATOR® is a user-friendly search engine to explore thousands of manually curated and quality-controlled public and private gene expression experiments.](image)

More advanced analyses are also possible. For instance, GENEVESTIGATOR® can search for genes specifically expressed under certain conditions, e.g., in certain tissues, in certain cancer types or in a specific disease (see Chapter 4). It can also search for genes sharing similar expression regulation with a target gene or group genes with similar expression by means of clustering and biclustering (see Chapter 5).

GENEVESTIGATOR® contains data from a variety of species including human, rat, mouse, monkey, dog, drosophila, pig, model and crop plants, and microorganisms. More information on the database content is available at:

https://genevestigator.com/gv/doc/content.jsp

1.1.1 The concept of meta-profiles

Meta-profiles summarize expression levels from many samples according to their biological context. Thus, in a meta-profile, each signal value corresponds to the average expression level of one gene over a set of samples sharing the same biological context, e.g., samples from the same tissue type. In contrast, in “standard expression profiles” each signal value corresponds to the expression level of one gene in one sample. GENEVESTIGATOR® uses five types of meta-profiles which group samples...
according to the following aspects: anatomical parts, cell lines, cancers types, developmental stages, and perturbations types. The “Perturbations” meta-profile comprises responses to various experimental conditions (drugs, chemicals, hormones, etc.), diseases, and genotypes. All samples in the database are annotated according to these biological dimensions. For each meta-profile, the summarization leads to a matrix of genes versus categories as shown below (Figure 1.2).

Figure 1.2: Schematic representation of the meta-profile concept. Expression values from many samples (top) are summarized into meta-profiles (bottom) according to their annotations.

Most categories are populated with several samples, thereby providing a robust estimate for the expression of genes within these categories. An analysis based on meta-profiles requires that sample signal values are comparable between the different experiments and, generally, between laboratories. Several lines of evidence support the validity of the summarization into meta-profiles as performed in GENEVESTIGATOR®. For example, Prasad et al. [2] demonstrated that transcriptome variations due to the tissue of origin are much larger than the variations due to perturbations or lab effects. Therefore, summarizing tissue-specific expression by grouping data from various experiments provides a relatively good estimate for the expression level of a gene in a given tissue. Likewise, good representative expression meta-profiles can be obtained for development, cell lines and cancers. For the “Perturbations” meta-profile, however, results are created by comparing groups of samples from individual experiments. Data from multiple experiments are not mixed to create a single value. As a result, this tool contains large compendia of response types collected from many experiments.
1.1.2 Software components

GENEVESTIGATOR® comprises three main components (Figure 1.3):

- **The website** to start the analysis tool or to register for a user account. It also contains additional information such as the user manual, publications, video tutorials, training modules, etc.

- **The analysis tool** or the “client application”, with which the analyses are performed. It communicates with the server to get all necessary data for the analyses.

- **The GENEVESTIGATOR® search engine** containing all data content and the software necessary for answering the requests of the analysis tool. Access to the GENEVESTIGATOR® server and to its data is provided via the “client application” and additionally via programmatic access for Enterprise customers.

![Diagram of the three main GENEVESTIGATOR® components: website, analysis tool, and search engine (server cluster).](image)

**Figure 1.3: Overview of the three main GENEVESTIGATOR® components: website, analysis tool, and search engine (server cluster).**

1.1.3 Requirements

The GENEVESTIGATOR® analysis tool is an independent application available for Windows, Mac and Linux. It can be installed as a standard user. On each start-up, it checks for a new version and, if necessary, auto-updates. Working with GENEVESTIGATOR® requires an internet access.

Minimal requirements to work with GENEVESTIGATOR®:

- Screen resolution: 1024 x 768
- Memory: 2 GB RAM
- Operating system: 64 bit
1.2 Types of analysis

In GENEVESTIGATOR®, analyses can be performed at two “data levels”:

- **SINGLE EXPERIMENT ANALYSIS**: to visualise the expression of genes across individual experiments (Samples tool see Chapter 2.1) or to quickly and easily analyse a particular experiment (Differential Expression tool, see Chapter 2.2)

- **COMPENDIUM-WIDE ANALYSIS**: to visualise the expression across thousands of experiments in a single analysis (see Chapters 3, 4 and 5)

The COMPENDIUM-WIDE ANALYSIS consists of three toolsets allowing specific types of queries (Figure 1.4):

- **CONDITION SEARCH TOOLS** to find out which conditions regulate up to 400 genes of interest (see Chapter 3)

- **GENE SEARCH TOOLS** to search the entire content for genes that are specifically expressed in a chosen set of conditions (a specific tissue type, cell line, cancer type, or perturbation) (see Chapter 4). It also contains the RefGenes tool to find the best reference genes for normalizing RT-qPCR experiments (see Chapter 4.4) and the Ortholog Search tool to quickly find the most likely functional ortholog across species (see Chapter 4.5)

- **SIMILARITY SEARCH TOOLS** to find relationships between genes. This toolset groups various tools for Hierarchical Clustering and Biclustering, Co-Expression and Gene Set Enrichment analyses and Signature search. It focuses on the identification of groups of genes having similar expression profiles within a dataset, or conditions having similar expression signatures (see Chapter 5)

![Figure 1.4: The three toolsets of the COMPENDIUM-WIDE ANALYSIS in GENEVESTIGATOR®. A: the CONDITION SEARCH TOOLS help you find conditions regulating your genes of interest. B: the GENE SEARCH TOOLS help you find genes having specific expression profiles, such as biomarkers. C: the SIMILARITY SEARCH TOOLS help you interpret your results and infer regulatory networks.](image-url)
1.3 User interface of the analysis tool

The interface of the GENEVESTIGATOR® analysis tool has the following main components (Figure 1.5):

1. “Home” button and field indicating the toolset currently in use, e.g., SINGLE EXPERIMENT TOOLS
2. “Data Selection” panel holding folders containing the selected biological samples
3. “Gene Selection” panel holding all folders containing the genes of interest that you entered
4. Tabs to select analysis tools within a toolset
5. Tool overview and results panel where the result of an analysis is displayed

Figure 1.5: Main components of the user interface. 1) “Home” button, 2) “Data Selection” panel, 3) “Gene Selection” panel, 4) Tabs for tools, and 5) Tool overview and results panel. Note: The Cell Lines and Cancers tools are specific for the biopharma community.

1.4 Analysis workflow

The entire curated gene expression data content of GENEVESTIGATOR® can be explored all at once or only a subset of it can be used. Typically, an analysis runs on a selection of data and genes consisting of \( n \) samples and \( m \) genes. This \([m \times n]\) matrix defining experimental conditions and genes is the basis for most types of analyses. The results are produced from the combination of data from this matrix and sample annotation information (meta-data). The choice of working with the entire content or only a subset depends on the type of biological question to be answered (Figure 1.6). For example, to check how five genes respond to the complete compendium of drugs, two selections must be created:

1. All samples (n)
2. The five genes (m) of interest

The following sections provide more details about each step.
1.4.1 Get Started

By clicking on the “GET STARTED” button a dialogue listing the main use cases will open (Figure 1.7). On the right panel instructions on how to perform the selected analysis (highlighted in blue in the left panel) can easily be followed.

Figure 1.7: “GET STARTED” button and dialogue. The left panel displays a list of the main use cases. The right panel contains some information and instruction on how to perform the selected analysis.
1.4.2 Organism and data selection

To select a set of samples, click on “New” in the “Data Selection” panel (Figure 1.8, upper image). A dialogue box will open (Figure 1.8, lower images), allowing you to choose an organism and a platform. By default, the platform containing the most samples for the chosen organism is selected.

The “Data Selection” defines which samples will be used for an analysis. For some tools based on meta-profiles, such as the CONDITION SEARCH TOOLS, the more samples are selected, the more conditions will be displayed and the more robust the results will be (averages are then calculated from a higher number of single measurements). Therefore, to identify conditions regulating the expression of a gene, it is preferable to start with a maximum number of conditions. For such analyses, just choose the organism you want to work with and click on “OK” without further filtering.

Figure 1.8: Selecting data for analysis. Upper image: the "New" button allows you to create a new data selection, while the "Add" button allows you to add samples to an existing selection. Lower images: “Data Selection” dialogue boxes where you can choose an organism, the technology platform. The number of samples available for the selection is indicated. Specific filters can be applied. Left image: dialogue specific for the biopharma community. Right image: dialogue specific for the plant community.

In other types of analyses, you may want to include only a portion of the database, e.g., a subset of experiments related to a specific disease, or to exclude some experiments or samples that may bias the analysis. To work with only a subset of samples, the general filters or/and the specific filters can be used (Figure 1.8, lower images) as described below.

Select specific experiments and studies

1. “Search by keyword, accession number, author”

This search option (Figure 1.8, lower images) allows you to search experiments by keywords present in any of the sample or experiment descriptions, by accession number either from GENEVESTIGATOR® or from the public repository, or by author name. The search will be performed across all organisms and
platforms by default, but it is possible to limit the search to selected organisms and platforms or to certain types of information (Figure 1.9). Actively select the experiments of interest to include them in the data selection.

Figure 1.9: Experiments / Studies search by keyword, accession number or author. The search is made across all organisms and platforms and allows you to quickly find all the experiments containing a certain keyword. In this example, a search for the keyword COPD within the biopharma content resulted in 40 experiments from 3 different species on 11 different platforms.

2. “Search by research area /study design” (available only for the biopharma community)

This search option (Figure 1.8, lower left image) allows you to search experiments related to a research area and/or based on their study design. The search will be performed across all organisms and platforms by default, but it is possible to limit the search to selected organisms and platforms and/or based on the study type and design (Figure 1.10).
Figure 1.10: Search by research area/study design. This search option allows you to search for all the experiments related to a specific research area or having a specific experimental design. In this example, the entire content was searched for experiments related to idiopathic pulmonary fibrosis (IPF) without further filtering. 28 different experiments from 3 different species and on 9 different platforms were found. By resting the mouse on an experiment, additional information on this experiment will appear in a tooltip.

Select data based on annotations

1. General filters

These filters allow you to quickly select all samples (across multiple studies) having a specific annotation or property (e.g., all Cell Lines samples or only samples with a wild-type genetic background) (Figure 1.8, lower images).

2. “Refine selection by conditions”

The “Refine selection by conditions” button (Figure 1.8, lower images) allows you to search subsets of samples associated with specific biological contexts, e.g., all samples from a tissue type.

For each category (Anatomy, Development, Genotype, Perturbations, etc.) you can browse through the corresponding ontology (controlled vocabulary) and select the desired conditions, e.g., liver in the Anatomy ontology (Figure 1.11).
3. Filter data by experimental/clinical parameters

Once a data selection has been created, the samples from this selection can be filtered based on experimental/clinical parameters by clicking on “Filter” in the “Data Selection” panel (Figure 1.12). The samples (from the selection in focus) containing the deselected parameters will be removed from the analysis, e.g., if you deselect “diseased”, all samples from diseased patients will be deselected. This filter is specific for the biopharma data.

Figure 1.12: Experimental / clinical parameters filter. This filter allows you to quickly select subsets of samples based on the experimental or clinical parameter across the whole “Data Selection”, e.g., only samples from healthy patients.
A note about the platforms:

For some organisms, GENEVESTIGATOR® contains expression data from multiple types of platforms, e.g., different generations of Affymetrix GeneChip® arrays. On these arrays, individual genes are frequently represented by a different set of probes (see Chapter 1.4.3 for the definition of a probe). Expression measurements for a given gene may be targeting different transcript regions or splice variants and are not necessarily comparable between platforms. Therefore, to keep the analysis results easily interpretable, data from different array types are not mixed. Thus, a sample selection always contains only datasets from the same platform.

A note about the replicates:

Experiments are often repeated to increase the robustness of the results (biological replicates) or the same samples from a given experiment are measured multiple times (technical replicates). In GENEVESTIGATOR®, almost all replicates are biological replicates. Replicates have the same sample name up to the suffix, e.g. “_1-1”, “_1-2”, “_1-3” for replicates 1 to 3 of sample 1.

1.4.3 Selecting genes of interest

The Samples tool, the CONDITION SEARCH TOOLS and the SIMILARITY SEARCH TOOLS help you prioritize or interpret a list of genes by visualizing these genes against various biological contexts or grouping them according to their similarity of expression. Therefore, a list of genes must be entered prior to the analysis. In contrast, no list of genes is required for the GENE SEARCH TOOLS as these tools aim at identifying novel genes with particular properties.

The “Gene Selection” panel (Figure 1.5, number 3) holds all created gene selections from the current analysis session. To create a new selection of genes, click "New" in the “Gene Selection” panel. The below “Gene Selection” dialogue will open (Figure 1.13).

Figure 1.13: The “Gene Selection” dialogue. Genes can be entered in different formats, e.g., probe IDs, Entrez gene, UniGene, gene symbol, or identifiers from other gene models. All recognized models are listed on the right side of the window (marked in red) upon clicking “HELP”. Additional search options such as “Exact names or probe IDs” or “Names starting with…” are available.
With microarrays, expression levels are measured by probes targeting particular transcript regions. For this reason, the results displayed in GENEVESTIGATOR® correspond to the expression values of these probes (see below for the definition of a probe). The GENEVESTIGATOR® database contains a mapping of these probes to gene identifiers (gene model) such as Entrez, ENSEMBL, UniProt, or gene symbol, so you can enter gene identifiers in any of these supported formats. Lists of identifier formats available for the selected organism are available in the “Gene Selection” dialogue under “HELP”.

A note about the probe definition:

In some microarray technologies, the expression level of a gene is measured by a single probe. In others, multiple probes are measured and summarized into a single value per gene. Furthermore, different terms are used for groups of multiple probes, e.g., “probe set” for Affymetrix. As GENEVESTIGATOR® contains data from several types of microarray platforms, we are using the term “probe” to define a probe or a probe set representing a given gene. Whenever a piece of information is specific to Affymetrix, we use the Affymetrix “probe set” terminology. By extension, the term “probe” is used in GENEVESTIGATOR® in the sense of measurement unit for other technologies like RNA-Seq and proteomics.

Additional options to enter gene IDs are provided:

1. Exact names or probe IDs
2. Names starting with…: to search all genes of a family, e.g., COX
3. Synonyms or descriptions containing…: to create a list of genes with similar functions, e.g., “receptor kinase”

Enter your list of genes. By default, the best probe for the entered genes will be chosen automatically (see below for the definition of best probe). By automatically selecting the best probes for a gene, the selected genes are linked to an organism but not to a specific platform. Consequently, the same gene selection can be used with data selections from different platforms. Nevertheless, as some platforms do not measure all genes and as all gene models are not mapped on each platform, it can happen that some genes of the selection will not be found on all platforms (Figure 1.14). In this case, a warning will be displayed on top of the results panel.

![Figure 1.14: Gene Selection used for analyses on different platforms. As some genes are not measured on all platforms, it may happen that genes from a “Gene Selection” are not measured on certain platforms.](image)

The best probe selected for a gene is indicated in parentheses next to the gene name. If a probe maps to more than one gene, the name of the other measured genes will be displayed (Figure 1.15)
A note about the best probe definition:

Every platform supported in GENEVESTIGATOR® makes use of specific measurement probes to target genes, transcripts, or proteins in a sample. GENEVESTIGATOR® allows users to input any desired gene/protein/transcript identifier (ID) of interest for which we have an entry. This ID may or may not directly map to a measurement probe of a supported platform. To facilitate data exploration, GENEVESTIGATOR® will try to automatically determine the most specific measurement probe that can best represent the relative expression rate of the entered ID. The probe which is returned as the "best" probe for a given ID may only approximate the actual expression rate as each technology is limited to a subset of all measurable content in a sample.
For example, a user may enter a protein ID using the UniProt gene model and search for relevant probes on the Affymetrix Human Genome U133 Plus 2.0 Array. The Affymetrix array does not directly measure the quantity of protein in the sample but rather the relative mRNA content. A pre-calculated mapping of UniProt IDs to Affymetrix probe sets is used to determine the best measurement probe for representing the relative expression rate of the protein. There is no guarantee that this mapping is always accurate or direct. The mapping can only be as accurate as the underlying genome and protein information on which it is based. In this regard, GENEVESTIGATOR® attempts to provide the best effort mapping of a given ID to a measurement probe; the user is free to choose (a) different probe(s) by switching to the “Probeset” information level (Figure 1.16).

Limits on gene selection

Depending on your license type, the number of genes (or of probes if a gene is mapped by several probes) per “Gene Selection” is limited to 1 or 400. Please visit the GENEVESTIGATOR® website (www.genevestigator.com) to find out how many genes can be analyzed in parallel for each type of license.

Creation of a list of orthologs from an existing gene selection: It is possible to translate a gene selection from one species to another, using a mapping by OMA (see Chapter 4.5). To create a new selection with orthologs from another species, simply right click on the gene selection of interest, go on “Create Orthologs” and select the desired organism (Figure 1.17). A new gene selection containing the corresponding gene orthologs will appear as new folder in the “Gene Selection” panel.

Select genes from predefined gene sets

In addition to the abovementioned methods, genes can also be selected from predefined gene sets such as Reactome pathways or Gene Ontology categories. To do this, click “Select gene set from gene set collection” in the “Gene Selection” dialogue (Figure 1.13) and search for gene sets of interest, e.g. by keyword (Figure 1.18).
1.4.4 Tool selection

Once a “Data Selection” and eventually a “Gene Selection” (mandatory for the Samples tools, the CONDITION SEARCH TOOLS and the SIMILARITY SEARCH TOOLS) have been defined, several types of analyses can be performed. As mentioned above (see Chapter 1.2), four main toolsets exist and each toolset comprises several individual tools (Figure 1.18). To start working with a tool, click on the corresponding icon. You can switch to another tool by going on the “Home” button (Figure 1.5, number 1) and selecting a new tool. Alternatively, you can change the tool within a toolset by selecting a different Tab (Figure 1.5, number 4). The different types of queries are described in the next chapters (see Chapters 2, 3, 4 and 5).
1.5 Viewing results

For the **Samples** tool (see [Chapter 2.1](#)) and the **CONDITION SEARCH TOOLS** (see [Chapter 3](#)), the results are immediately displayed based on the data and gene selections. The **Differential Expression** tool (see [Chapter 2.2](#)), the **GENE SEARCH TOOLS** (see [Chapter 4](#)) and the **SIMILARITY SEARCH TOOLS** (see [Chapter 5](#)) require additional choice and therefore have to be triggered using the “Run” button.

The results can be displayed in various formats, the choice of which mainly depends on the number of genes you would like to visualize.

1.5.1 The different type of plots

- **Boxplots** consist of boxes, whiskers and outliers. The box delimits the upper and lower quartiles (IQR), while whiskers represent the lowest datum still within 1.5 IQR from the lower quartile, and the highest datum still within 1.5 IQR from the upper quartile. Outliers, represented as stars, are values outside this range. Only one gene can be represented at the time. For boxplots of absolute expression values (see [Chapter 1.5.4](#) for the meaning of absolute expression values), a bar above the plot indicates which expression values can be considered “LOW”, “MEDIUM” or “HIGH”. These ranges are determined by looking at all expression values of all genes over all samples for the platform in use. “LOW” corresponds to the first quartile, “MEDIUM” to the interquartile range and “HIGH” to the fourth quartile (see [Chapter 1.5.4](#), Percentiles). This type of representation is only available in some of the **CONDITION SEARCH TOOLS** and in the **RefGenes** tool, where data from several samples are aggregated per category (Figure 1.19).
Figure 1.19: In the boxplot representation, the aggregated measurement value for each category is displayed as a box, whiskers and eventually outliers.

- **Scatterplots** consist of a dot for the mean expression level and error bars showing the standard error of the mean. Depending on the context, signals are either shown as “ratios” (experimental versus control values) or as “absolute” expression values. For scatterplots of absolute expression values, a bar above the plot indicates which expression values can be considered "LOW", "MEDIUM" or "HIGH" as described above for the boxplots.
  
  - Scatterplots can display up to 10 genes simultaneously using 10 easily distinguishable colors. Colors can be assigned to a gene by dragging a color from the color palette on the selected gene ("Change color" option in the "Gene Selection" panel).

- **Heatmaps** are of two types in GENEVESTIGATOR®, differing by the kind of expression values.
  
  - **Absolute values** (blue-white or burgundy-white color-coding for linear or log scales, respectively) (Figure 1.20, A). They are normalized to the expression potential of each gene (see below). The darkest blue or burgundy color represents the “maximum” level of expression for a given probe across all measurements available in the database for this probe. Therefore, **color intensities can only be compared between elements from the same probe but not with those from other probes**. In other words, a light color only means that a gene is weakly expressed compared to its expression potential. Another gene can have a much darker color with a lower expression value, if it has a lower expression potential. This scaling with the expression potential allows you to compare patterns between genes and ensures that most expression values are in a useful color-range. If you are interested in comparing the absolute expression values between genes, switch from the heatmap view to the scatterplot view.

  - **Relative values** (green-red color-coding) (Figure 1.20, B) represent ratios of experimental versus control values. Green stands for “down-regulated”, red stands for “up-regulated”, while black stands for “unchanged”. Relative values are found in the **Perturbations** tools or other tools working with the **Perturbations meta-profile**. Color-blind users can change the green-red scale to a yellow-blue scale ("View" option in the toolbar) (Figure 1.20 C). The log2-ratio and the fold-change indicate how big the difference is between the average of the expression in the experimental samples and the average in the control samples.
Figure 1.20: Color-coding used in GENEVESTIGATOR®. A: absolute values are displayed in blue-white (linear scale) or burgundy-white (log scale). In this case, the colors (values) are normalized to the expression potential of a gene. B: relative values are displayed in green-red. C. Color scheme can be changed from green-red to blue-yellow.

View results as a List or as a Tree.
- A tree is a structured representation of an ontology. For example, the anatomy tree shows the hierarchy of organ systems, organs, tissues and cell types. The purpose of a tree is to show the categories in an organized way and enable you to browse across categories of interest. The expression values indicated for each category is the average expression of all samples annotated to it and to its subcategories.
- A list shows the leaf categories of the tree and sorts them by expression level or log-ratio (for relative data) starting with the category where the expression is the highest. The purpose is to rapidly identify the most relevant conditions. Conditions are not seen according to their classification in a larger system, therefore additional information is needed to indicate this. For example, in anatomy the category “epithelium” can be found in different organs. In the list view, you will see multiple categories called “epithelium” ranked by the level of expression of a given gene, but the corresponding tissue context will be provided in the tooltip.

1.5.2 Visualization of the expression in log or linear scales

Expression values are given either in linear scale or in a logarithmic scale. Linear values are intended to be proportional to gene expression, while logarithmic values are obtained from the linear values by the formula $\log_2 (v+1)$ where $v$ is the linear value. We use $\log_2 (v+1)$ instead of $\log_2 v$ to avoid negative expression values and an excessive sensitivity to small changes in low expression values.

In the Samples tool and in the CONDITION SEARCH TOOLS, the values can be visualized and the analyses carried out either in the linear or in the logarithmic scale.

In the logarithmic scale and when working with the average expression level of a gene (like in the CONDITION SEARCH TOOLS), the displayed result is the average of the logarithmic values and not the logarithm of the average value. Consequently, the ranking of averaged gene expression values can be different in logarithmic and linear scales, e.g., in which tissue a gene is most expressed can be different between logarithmic and linear scale.
1.5.3 Expression potential and signal background

The expression potential of a gene (probe) is a robust indicator for the maximal expression level of this gene. It represents the top percentile (= 99th percentile) of all expression values for this gene. The signal background is calculated as the first percentile of all expression values for this gene.

When exporting data from the Condition search tools, one can select to export 'Measure attributes: Signal Background and Expression Potential', and 'Data values: Ratio of Expression Potential'. The exported value 'Signal Background' is defined as the lower 1 percentile of all mean values for each gene/probe. The exported value 'Expression Potential' reports the length of the range between the 1st and the 99th percentile of mean values for each gene/probe. The exported value 'Ratio of expression potential' is defined as (mean value − signal background) / (expression potential − signal background + epsilon). A small epsilon with a value of 1 is added to the denominator to avoid division by zero in cases where the expression potential and signal background are (almost) the same. Since the expression potential is a range between the 1st and the 99th percentile of mean values, any mean value below the first 1% will result in a negative ratio of expression potential, and any mean value above the higher 1% will result in a ratio of expression potential bigger than 1.

Getting additional information

Additional information, such as the actual expression values, the list of experiments included in the calculation of a mean, etc. is available in the tooltips that appear when the mouse pointer rests on an element (see Chapter 1.5.7).

1.5.4 Meaning of "absolute" expression values in GENEVESTIGATOR®

The expression values in GENEVESTIGATOR® are calculated using standard normalization methods for the different microarray platforms and scaled between experiments to make the expression values comparable, (see Chapter 7). The "absolute" expression values shown are called "absolute" because they represent expression values of a gene in a sample (or group of samples) as opposed to a ratio or relative value that compares the expression in treatment samples to the expression in control samples. However, the "absolute" expression values are not really absolute as it is practically impossible to determine an absolute quantity like the number of mRNA transcripts per cell. Most methods for quantifying expression therefore report the expression value compared to some "average" expression of all genes in the sample (or experiment). For comparability, the microarray expression values in GENEVESTIGATOR® are scaled such that the average (trimmed mean) is equal to 1000. This gives a rough indication of the strength of expression. Which expression values are "HIGH" or "LOW" is based on the calculation of percentiles.

Percentiles

The Xth percentile is calculated by pooling all expression values in question, sorting them and then picking the expression value for which X percent of the values are smaller than this value. Typical values include the lower quartile (25th percentile) ("LOW"), median (50th percentile) ("MEDIUM"), upper quartile (75th percentile) ("HIGH"). The interquartile range (IQR) is defined as the range between the 25th percentile and the 75th percentile. Consequently, half of the expression values lies within the IQR.
1.5.5 Working with multiple selections

Multiple selections for genes and data can be created. Switching between them is done by a simple click on the corresponding folder. The analysis tools will then display data based on the new combination of samples and genes defined by the selections in focus highlighted in light blue. For each panel, only one selection can be in focus at the same time. The data and the gene selections in focus must be compatible, i.e., they must be based on the same organism and eventually platform if the “automatically choose best probe” option was not used to create the gene selection.

1.5.6 Editing, copying or deleting an existing selection

An existing data or gene selection can be edited, copied or deleted in the context menu that appears upon right-clicking on the folder in focus (Figure 1.21).

![Figure 1.21: Editing, copying or removing a folder from the “Data Selection” or the “Gene Selection”. The genes contained in a folder can also be copied to the clipboard, with or without descriptions and either as “Gene Identifiers” or as “Probe”.

1.5.7 Additional information about experiments or genes

Additional information about individual experiments, samples or genes is available in the tooltips that appear when resting the mouse over the elements (Figure 1.22). Many of these tooltips contain links to other sources of information, e.g., to the repository containing the original raw expression data. To click on such a link, freeze the tooltip by pressing “F2” on the keyboard. The links will open in your browser (not in the GENEVESTIGATOR® application). Make sure that your browser allows pop-up windows for www.genevestigator.com.
1.5.8 Displaying different gene models

Various gene identifier formats are recognised and available in GENEVESTIGATOR®. To select another format, click on “View” in the toolbar and select “Gene Labels” (Figure 1.23). A “Gene Label Preferences” dialogue will open and allow you to set up your preferences per organism. The preferred format will be used in the results displayed in GENEVESTIGATOR®.

Figure 1.22: Gene identifier format. For each organism, several gene identifier formats are available. A drop-down menu allows you to select one of them per organism. These preferences will be used to display the results obtained for an analysis.
Chapter 2
SINGLE EXPERIMENT ANALYSIS

2.1 The Samples tool

The Samples tool displays the expression values of selected genes across selected samples (Figure 2.1).

2.1.1 Getting started

1. Create a “Data Selection” (see Chapter 1.4.2)
2. Create a “Gene Selection” (see Chapter 1.4.3)

The plot will automatically be generated (see Figure 2.1). Several options to display the results are available and described in the next section.

Figure 2.1: Screenshot of the results obtained with the Samples tool for the CA3 and AMPD1 genes on studies from the Affymetrix Human 133 Plus2 platform. The expression levels of the selected genes are displayed across selected samples. Experiments are sorted according to experiment number and the default order of samples within experiments is based on the experimental design. Samples within experiments can also be sorted according to the highest average expression signal (“avg. sign.”) of one or multiple genes simply by (CTRL/-SHIFT-) clicking the gene(s) of interest. Users of the Enterprise version can see all sample annotations displayed on the right of the plot and can sort samples by these annotations.
2.1.2 Features

The plot has the following features:

- In the scatterplot view, up to 10 genes can be displayed using a palette of 10 distinct colors. In the heatmap view, up to 400 genes can be viewed in parallel.
- Expression intensities are represented by dots as obtained from quantile normalization (e.g., RMA for Affymetrix data) or TPM (for RNA-Seq data).
- The default “unsorted” order of the samples in the plot is defined by the curators and reflects the logic design of the experiment.
- Within each individual experiment, the samples can be sorted based on the (average) expression values for the gene(s) in focus (from highest expression to lowest).
- By resting the mouse on a “dot” or on a sample, additional information will appear in a tooltip. This tooltip can be frozen / closed by pressing ‘F2’

Creation of gene signatures for use in other tools

It is possible to export the list of genes together with the corresponding expression values (“signatures”) from a chosen sample (or group). To create such a “signature”, select the sample (group) of interest and click in the toolbar on the Signature: “Copy”, “New” or “Add” buttons.

The “signature” will appear in the “Gene Selection” panel with a “S” tag.

A “signature” can be opened directly in the Signature tool (see Chapter 5.3) and allows you to compare it with data from other studies. Doing such a comparison is useful to find other studies giving similar or opposite results, or to verify the nature of a given sample. This export feature exists for most tools.

Visualize expression by group

For each level of annotation, it is possible to plot the expression by groups. This can be achieved by clicking on the “Plot groups...” button and then selecting the levels for which groups must be created (e.g., Anatomical part), or by clicking on the header of the annotation table that is on the right side of the plot. In both cases, a “detailed view” panel will appear showing the group-level expression plots. Care must be taken on what is selected and actually being compared, since the tool allows cross-study comparisons without limitations, but not all comparisons necessarily make biological sense. Also, batch or study effects must be considered when selecting data for such a group-level visualization.

2.1.3 Statistics

All microarray data in GENEVESTIGATOR® are normalized at two levels: RMA within experiments and trimmed mean adjustment to a target for normalization between batches or experiments (see Chapter 7). This combination makes data highly comparable between different experiments. The microarray data that you see in the Samples tool are normalized with this scheme, and the resulting signal values are indicated in the tooltips. RNA-Seq do not require such normalization (see Chapter 8).
2.2 The Differential Expression tool

The Differential Expression tool allows you to find the genes that are significantly differentially expressed between two conditions within an experiment from the GENEVESTIGATOR® database, e.g., a treatment versus a control condition.

A video tutorial for this tool is available on the following link or directly from the tool (button with camera icon) (Figure 2.2 A): https://genevestigator.com/support/

2.2.1 Getting started

1. Select an experiment of interest

Go to “Select individual experiments” in the “Data Selection” dialogue box (see Chapter 1.4.2). If you already have a “Data Selection” comprising several experiments, the first one in the list will be selected by default and displayed in this dialogue. However, it is possible to select another experiment using the drop-down menu in the “Define Comparison” dialogue box (Figure 2.2 B).

2. Define the groups you want to compare

Click on “Define Comparison” or on “Edit” (Figure 2.2 A). In the opening dialogue box (Figure 2.2 B), select the samples for groups A & B. Some predefined comparisons may be available under “Comparison” (Figure 2.2 B). These comparisons have been manually defined by our curators and can be used as such or edited. Various parameters describing the different samples are listed in the right panel (Figure 2.2 B) or in the samples tooltip (see Chapter 1.5.7).

Figure 2.2: The Differential Expression tool’s toolbar (A) and the “Define Comparison” dialogue box (B). In this dialogue box, a drop-down menu of “Experiment” allows you to select an experiment of interest (from your “Data Selection” in focus) and define the two groups of samples to be compared. Some predefined groups may be available in the drop-down menu of “Comparison” (green rectangle). Groups can also be defined manually by checking the boxes next to the samples or by SHIFT-/CTRL-
clicking in combination with the “Add to…” and “Remove” buttons (red rectangle). Experimental parameters listed on the right side of the corresponding samples can be helpful for manual selection of the two groups. A click on the camera icon in the toolbar will open a page in your browser containing a video tutorial explaining how to use the tool.

3. Run the analysis

Once a comparison is defined, click on the “Run” button to start the differential expression analysis. Several parameters can be adjusted (False-Discovery Rate, visualization plots, log-ratio, only up- or down-regulated genes) (Figure 2.3, framed in red and see Chapter 2.2.2). The resulting genes are represented in a scatterplot (Figure 2.3 A) or in a volcanoplot (Figure 2.3 B) and listed in the adjacent table. The resulting genes, selected subsets of genes or a signature (a list of genes with their corresponding expression ratios for the displayed comparison) (see Chapter 2.1.2) can be copied to the clipboard, used to create a new “Gene Selection” or added to an existing selection (Genes: “Copy”, “New” or “Add” buttons or Signature: “Copy”, “New” or “Add” buttons in the toolbar) (Figure 2.3 A, framed in blue). Genes can also be copied to the clipboard, together with the displayed values and descriptions, using the usual keyboard shortcuts, e.g., CTRL+C.

Figure 2.3: Screenshot displaying the genes that are differentially expressed in the experiment GSE5390. The adjustable parameters are framed in red. In A the results are displayed as scatterplot and in B using a volcanoplot.

2.2.2 Features

- The “Define Comparison” button allows you to modify the samples for both groups. Note that you need to restart the analysis after having made some changes
- All differentially expressed genes can be displayed
- It is possible to visualize only a subset of genes (up-regulated or down-regulated) or all dys-regulated genes
- Changing FDR and Ilog-ratio by moving the corresponding sliding bars will trigger an on-the-fly recalculation and GENEVESTIGATOR® will display the new results
Subsets of genes for further analyses can be selected in the plot with a lasso tool or in the table by CTRL/CMD-clicking.

As for the Samples tool, it is possible to create and export gene signatures from this tool (see Chapter 2.1.2).

### 2.2.3 Statistics

The genes are filtered using 3 different methods:

1. The genes are filtered by their log fold-change.
2. Computation of p-values:
   - For microarray data, the p-values are computed according to the Limma algorithm (Smyth, [3]). This algorithm is essentially an extension of the classical t-test that uses an improved variance estimate computed from the expression data of all genes measured in an experiment.
   - For RNA sequencing data, the p-values are computed by the Voom algorithm (Law et al., [4]) for genes with enough read counts and by a simplified version of the edgeR algorithm for small counts. The Voom algorithm is a refinement of Limma that uses the dependence between the expression of a gene and the precision of the measure of this expression to better estimate the error of the expression for each gene. The Voom algorithm performs badly for genes with very low read counts. We thus use a simplified version of the procedure used in edgeR to compute p-values for genes with low counts. Essentially, this procedure is a variant of Fisher's exact test using a negative binomial distribution whose parameters are estimated by the method of moments.
3. The false discovery rate (FDR) (Benjamini and Hochberg, [5]) is controlled by applying the Benjamin-Hochberg procedure to compute the threshold under which the p-values are considered sufficiently small. This threshold is used to do a second round of filtering.

### 2.3 The Dimension Reduction tool

The Dimension Reduction tool helps you to visualize and explore multi-dimensional data in a two-dimensional plot, and thereby to identify clusters of samples that are similar within an experiment. In GENEVESTIGATOR®, the visualization is integrated with customizable labelling from a rich choice of metadata.

Reduction of dimensionality while retaining important information is achieved using the t-SNE algorithm as described below in Section 2.3.1.

### 2.3.1 Getting started

1. **Select an experiment of interest**

   Go to the “Data Selection” dialogue box and select the species, platform and experiment you are interested in (see Chapter 1.4.2). If you have an active “Data Selection” containing more than one experiment, the first experiment in the list will be selected by default. Note that the experiments containing more than 2000 samples cannot be analyzed.
2. Start the Dimension Reduction tool

Click the icon of the Dimension Reduction tool on the home screen.

3. Select a perplexity value

Select a perplexity value from the drop-down menu in the toolbar of the Dimension Reduction tool (Figure 2.4). By default, the upper limit of the perplexity is set based on the sample size (the bigger the number of data points, the higher the predefined upper limit of perplexity). The chosen perplexity should be empirically explored and requires fine-tuning. The appropriate value depends on the density of the selected data. Typical values for the perplexity range between 5 and 50. The perplexity value should be smaller than the number of data points.

![Figure 2.4: The drop-down menu in the toolbar of the Dimension Reduction tool is used to select the perplexity value.](image)

4. Run the analysis

The result will be displayed in a two-dimensional scatterplot upon clicking “Run” (Figure 2.5). As mentioned above, empirically explore different perplexity values to find out which one gives the best result. This Dimension Reduction tool is based on the t-SNE (t-distributed stochastic neighbor embedding) algorithm first described by Van der Maaten and Hinton [16]. To learn more about the perplexity and how to use it effectively for the Dimension Reduction tool, we recommend looking up this original research paper and, particularly, the remarks by Wattenberg et al. [17].
Figure 2.5: The **Dimension Reduction** tool used to visualize multi-dimensional data in a two-dimensional plot. The data was selected using the keyword search for “HS-02941”. The perplexity value was explored, and the value 30 was chosen as giving the best representation. The sample level annotation “Stimulus” was chosen, resulting in a list of annotations for this category appearing in a new table on the right. All cases of this B-cell lymphoma receiving CHOP treatment were colored in red, whereas those samples from patients receiving CHOP together with the monoclonal antibody Rituximab (R-CHOP) treatment were colored in blue, and the two groups of CD19+ and CD19− samples were colored in grey. The color of each annotation was adjusted manually.

### 2.3.2 Features

- The **Dimension Reduction** tool is a powerful yet simple tool to visualize and explore multi-dimensional data in a two-dimensional plot.
- The results are displayed in a scatterplot (Figure 2.5). Detailed information on a sample is available in a tooltip upon resting the mouse on a data point.
- A table listing existing sub-categories of meta-data is displayed next to the plot. Selecting one of these sub-categories creates a second table containing the sample level annotations available in the current sample selection and sub-category. You can assign individual colors to these annotations by dragging-and-dropping from the color palette on top of the table to visualize clusters of data points.
- As in many other tools, it is also possible in the **Dimension Reduction** tool to select groups of samples with the lasso tool and/or by CTRL-clicking and save new or add to existing sample selections (e.g., [Chapter 1.4.2](#)).
2.3.3 Methodology

The t-SNE dimensionality reduction method was developed by Van der Maaten and Hinton [16]. Starting with a set of points in high dimension, it constructs a low-dimensional embedding by optimizing a measure of the similarity between the neighborhoods of the original points and the neighborhoods of their low-dimension images using the gradient descent technique.

The output of t-SNE is non-deterministic and the gradient descent might not always converge even to a merely local minimum of the similarity measure in the number of iterations we allow the algorithm (1000 iteration steps). For this reason, the user should keep in mind that successive runs with the same parameters do not necessarily produce the same results [17].

We use a parallel implementation of the t-SNE algorithm developed by Ulyanov [18] and redistributed under the license of TU Delft [a]. For performance reasons, principal component analysis (PCA) is used to reduce the dimensionality of the input to the t-SNE implementation. PCA is performed through singular value decomposition using the Intel® MKL implementation of the LAPack linear algebra libraries [b].

2.4 The Sample Composition tool

The Sample Composition tool allows you to analyze the composition and absolute abundance of distinct cell types in a single sample or groups of samples, and a correlation thereof with different sample traits such as diseases, treatments, developmental stages, etc. The tool is especially valuable when combined with single-cell RNA-Seq compendia.

2.4.1 Getting started

1. Select an experiment of interest

Go to the “Data Selection” dialogue box and select the species, platform and experiment you are interested in (see Chapter 1.4.2). If you have an active “Data Selection” containing more than one experiment, the first experiment in the list will be selected by default.

Both single experiment and compendium-wide analyses are possible. We recommend starting with a single experiment analysis and advancing to compendium-wide analyses after mastering the concept of the tool.

2. Start the Sample Composition tool

Click the icon of the Sample Composition tool on the home screen.

3. Select biological context of X- and Y-axes

Choose the desired metadata category from the drop-down menu (Figure 2.6). By default, the first category will be selected for the Y-axis and the second category for the X-axis.
Figure 2.6: The drop-down menus in the toolbar of the **Sample Composition** tool are used to select the biological context of the X- and Y-axes.

4. Select a visualization

Choose the desired visualization from the drop-down menu in the toolbar of the **Sample Composition** tool (Figure 2.7). The default visualization “Proportion” will display the cell proportion. The other option for visualization, “Counts”, will display cell counts.

Figure 2.7: The drop-down menu in the toolbar of the **Sample Composition** tool is used to select the desired visualization.

2.4.2 Features

- The **Sample Composition** tool provides a powerful overview of sample composition in a study. The results are displayed in a horizontal bar chart (Figure 2.6). Detailed information is available in a tooltip upon resting the mouse on a part of the bar.

- Various combinations of visualizations are possible. The category used for the y-axis can be chosen under “List categories from”. The order can be sorted by clicking on the title of the category. The category used for the x-axis can be chosen under “Color categories from”. The annotation is displayed next to the plot.

- A limit of 30 colors can be displayed on the x-axis. The tool will automatically select and display categories with the biggest number of cells/aggregates/bulk-tissue samples. If there are more annotated categories, the remaining cells/aggregates/bulk-tissue samples belonging to these annotations will be combined and displayed in black. This is typically observed if the tool is used in combination with an entire platform.

- It is possible to select a group of samples by clicking on the horizontal bar chart or the color categories displayed on the right of the chart and save new or add to existing sample selections (e.g., Chapter 1.4.2).

- As in many other tools, it is also possible in the **Sample Composition** tool to export features based on what is displayed in the active selection (Proportion or Counts).
Chapter 3
COMPENDIUM WIDE ANALYSIS: CONDITION SEARCH TOOLS

3.1 Overview of CONDITION SEARCH TOOLS

The CONDITION SEARCH TOOLS allow you to find out which conditions regulate the expression of your genes of interest. All the tools (Figure 3.1) display meta-profiles as described above (see Chapter 1.1) and allow a rapid identification of the conditions affecting the expression of selected genes, e.g., to identify mutations leading to an up- or down-regulation of these genes. The CONDITION SEARCH TOOLS are instrumental in discovery research, validation of existing hypotheses or in generating new hypotheses that can be tested in the laboratory.

![Figure 3.1](Image)

Figure 3.1: Screenshot of the CONDITION SEARCH toolset. The Cell Types, Cell Lines and Cancers tools are specific for the biopharma community.

3.2 General features available for the CONDITION SEARCH TOOLS

3.2.1 Detailed view and experimental / clinical parameters

Each category (or comparison for the Perturbations tool) consists of one or several samples. You can get a detailed view of all the samples aggregated/comprised in a leaf node category/comparison by clicking on a category/comparison of interest (or on several categories by using the control button on the keyboard). A new panel will open at the bottom showing the expression values of your selected genes across all individual samples from this category/comparison (Figure 3.2). On the right of this additional panel, you will find the various experimental parameters attributed to each sample.

In the detailed view, individual samples or samples with specific experimental parameters can be removed from the samples list by clicking on the cross next to a sample or an experimental parameter (Figure 3.2). GENEVESTIGATOR® will recalculate on-the-fly the new results based on this new composition of samples. The reset button on the toolbar allows you to return to the original composition of samples. Note that the filtering used at this level, e.g., removal of all patients 60+ years within a chosen cancer type, will only remove samples within this category. All other categories will still contain these patients. To remove all samples with a particular parameter, see Chapter 1.4.2.2 filter # 3. The samples listed in the detailed view can be used to either create a new “Data Selection” (“New” button) or to be added to an existing one (“Add” button). As for the Samples tool, it is possible to create and export gene signatures from the detailed view (see Chapter 2.1.2).
3.2.2 HELP button

Supplementary information for each tool (features, statistics, etc.) is available under the “HELP” button of the toolbar.

3.3 The Anatomy tool

The Anatomy tool displays how strongly genes of interest are expressed in different anatomical categories, including organs, tissues, cell cultures from primary cells but does not contain data from cancer nor cell lines samples - cancer data are found in the Cancers tool (see Chapter 3.5) and cell lines data in the Cell Lines tool (see Chapter 3.4). In the Anatomy tool, data are plotted against a tree of anatomical categories.

3.3.1 Getting started

1. Create a “Data Selection” (see Chapter 1.4.2)
2. Create a “Gene Selection” (see Chapter 1.4.3)

The plot will automatically be generated (see Figure 3.3). Several options to display the results are available and described in the next section. By default, the “Boxplot-List” view will be displayed (see Chapter 1.5).

3.3.2. Features

- Three types of graphs are available: boxplot (1 gene), scatterplot (up to 10 genes) and heatmap (up to 400 genes)
- The categories can be visualized either as a tree or as a list with the categories ordered according to the (average) expression levels of the gene(s) in focus
- For all types of graphs, results can be displayed in linear or in log₂ scale

Figure 3.2: Detailed view of the “Diaphragm” meta-profile in the Anatomy tool for the human CA3 gene on the Affymetrix Human 133 Plus2 platform. All or only distinct parameters can be displayed. The red crosses highlight the possibility of removing, in the detailed view, individual samples or all the samples sharing a common parameter. The new results will be recalculated on the fly. The reset button restores all samples at once.
A ladder indicating “LOW”, “MEDIUM” and “HIGH” is displayed above the boxplot and the scatterplot, providing a general indication of expression level. MEDIUM is the interquartile range of all expression values for a platform (see Chapter 1.5.4).

By resting the mouse on a “result” or on a category, additional information will appear in a tooltip. This tooltip can be frozen / closed by pressing ‘F2’

A panel with a detailed view of all samples comprised within a category is obtained by clicking on it. The expression value as well as experimental parameters for each sample will be individually displayed (see Chapter 3.2.1). Note that a multiple selection is possible (CTRL + click).

As for the Samples tool, it is possible to create and export gene signatures from this tool (see Chapter 2.1.2).

Figure 3.3: Screenshots of the results obtained with the Anatomy tool for the human CA3, AMPD1 and TPM2 genes on the Affymetrix Human 133 Plus2 platform. The number of samples for each category is listed on the right. **Left image:** the Boxplot-list view displays results for a single gene (probe) listing the tissues showing the highest expression on top. **Right image:** the Heatmap-tree view displays results for up to 400 genes (probes). The categories are displayed as nodes of a tree. Each node can be expanded or collapsed.

### 3.3.3. Statistics

The expression values displayed in the Anatomy tool represent the average expression for a given gene in a tissue across all selected samples. When the anatomical parts are shown as a tree, parent nodes represent the average expression of all samples within this branch. The number of samples aggregated in each category to calculate this average is indicated on the right of the graph. In the boxplot view, the whiskers represent the lowest datum still within 1.5 IQR from the lower quartile, and the highest datum still within 1.5 IQR from the upper quartile. Outliers, represented as stars, are values outside this range. In the scatterplot view, the whiskers indicate the standard error of the mean. Additional statistical parameters such as mean, standard error, 95% confidence interval are available by resting the mouse on a result (see Chapter 1.5.7).
3.4 The Cell Types tool

The Cell Types tool displays how strongly genes of interest are expressed in different cell types for selected tissues or tumors.

3.4.1 Getting started

1. Create a “Data Selection” (see Chapter 1.4.2)
2. Create a “Gene Selection” (see Chapter 1.4.3)

The plot will automatically be generated (see Figure 3.4). Several options to display the results are available and described in the next section. By default, the “Boxplot-List” view will be displayed (see Chapter 1.5).

3.4.2 Features

- Three types of graphs are available: boxplot (1 gene), scatterplot (up to 10 genes) and heatmap (up to 400 genes)
- The categories can be visualized either as a tree or as a list with the categories ordered by decreasing (average) expression levels of the gene(s) in focus
- For all types of graphs, results can be displayed in linear or in $\log_2$ scale
- A ladder indicating “LOW”, “MEDIUM” and “HIGH” is displayed above the boxplot and the scatterplot, providing a general indication of expression level. MEDIUM is the interquartile range of all expression values for a platform (see Chapter 1.5.4)
- By resting the mouse on a “result” or on a category, additional information will appear in a tooltip. This tooltip can be frozen / closed by pressing ‘F2’
- A panel with a detailed view of all samples comprised within a category is obtained by clicking on it. The expression value as well as experimental parameters for each sample will be individually displayed (see Chapter 3.2.1). Note that a multiple selection is possible (CTRL + click)
- As for the Samples tool, it is possible to create and export gene signatures from this tool (see Chapter 2.1.2)

3.4.3. Statistics

The expression values displayed in the Cell Types tool represent the average expression for a given gene in a particular cell type across all selected samples. When the cell types are shown as a tree, parent nodes represent the average expression of all samples within this branch. The number of samples aggregated in each category to calculate this average is indicated on the right of the graph. In the boxplot view, the whiskers represent the lowest datum still within 1.5 IQR from the lower quartile, and the highest datum still within 1.5 IQR from the upper quartile. Outliers, represented as stars, are values outside this range. In the scatterplot view, the whiskers indicate the standard error of the mean. Additional statistical parameters such as mean, standard error, 95% confidence interval are available by resting the mouse on a result (see Chapter 1.5.7).
3.5 The **Cell Lines** tool

The **Cell Lines** tool displays the expression level of genes across various cell lines.

3.5.1 Getting started

3. Create a “Data Selection” (see Chapter 1.4.2)
4. Create a “Gene Selection” (see Chapter 1.4.3)

The plot will automatically be generated (see Figure 3.5). Several options to display the results are available and described in the next section. By default, the “Boxplot-List” view will be displayed (see Chapter 1.5).
3.5.2 Features

- Three types of graphs are available: boxplot (1 gene), scatterplot (up to 10 genes) and heatmap (up to 400 genes)
- The categories can be visualized either as a tree or as a list with the categories ordered according to the (average) expression levels of the gene(s) in focus
- For all types of graphs, results can be displayed in linear or in log₂ scale
- A ladder indicating “LOW”, “MEDIUM” and “HIGH” is displayed above the boxplot and the scatterplot, providing a general indication of expression level. MEDIUM is the interquartile range of all expression values for a platform (see Chapter 1.5.4)
- By resting the mouse on a “result” or on a category, additional information will appear in a tooltip. This tooltip can be frozen / closed by pressing ‘F2’
- A panel with a detailed view of all samples comprised within a category is obtained by clicking on it. The expression value as well as experimental parameters for each sample will be individually displayed (see Chapter 3.2.1). Note that a multiple selection is possible (CTRL + click)
- Categories representing normal tissues can be added to the plot by ticking the “show anatomy” box (Figure 3.5), to compare expression between cell lines and normal tissues
- Hierarchical clustering of a set of genes across Cell Lines or Cell Lines + Anatomy + Cancers meta-profiles can be performed with the Hierarchical Clustering tool (see Chapter 5.1)
- As for the Samples tool, it is possible to create and export gene signatures from this tool (see Chapter 2.1.2)

3.5.3. Statistics

The expression values displayed in the Cell Lines tool represent the average expression for a given gene in a particular cell line across all selected samples. When the cell lines are shown as a tree, parent nodes represent the average expression of all samples within this branch. The number of samples aggregated in each category to calculate this average is indicated on the right of the graph. In the boxplot view, the whiskers represent the lowest datum still within 1.5 IQR from the lower quartile, and the highest datum still within 1.5 IQR from the upper quartile. Outliers, represented as stars, are values outside this range. In the scatterplot view, the whiskers indicate the standard error of the mean. Additional statistical parameters such as mean, standard error, 95% confidence interval are available by resting the mouse on a result (see Chapter 1.5.7).
Figure 3.5: Screenshot of the Cell Lines tool showing in a Boxplot-tree the expression of the human LINGO1 gene on the Affymetrix Human Genome U133 Plus2 platform across the normal tissues and the cell lines (neoplastic and non-neoplastic cell lines).

3.6 The Cancers tool

The Cancers tool displays how strongly genes of interest are expressed in different cancer types. The cancer data classification is compliant with international standards (ICD-10 and ICD-O3). By default, only data from cancer samples are displayed, but non-cancer data (normal tissues and cell lines) can be added by ticking the "show anatomy" and/or "show cell lines" boxes, respectively, in the toolbar (Figure 3.6, lower image). Results can be displayed as a sorted list of cancer types or as classification tree of cancer types.

3.6.1 Getting started

1. Create a “Data Selection” (see Chapter 1.4.2)
2. Create a “Gene Selection” (see Chapter 1.4.3)

The plot will automatically be generated (see Figure 3.6). Several options to display the results are available and described in the next section. By default, the “Boxplot-List” view will be displayed (see Chapter 1.5).
3.6.2 Features

- Three types of graphs are available: boxplot (1 gene), scatterplot (up to 10 genes) and heatmap (up to 400 genes)
- The categories can be visualized either as a tree or as a list with the categories ordered according to the (average) expression levels of the gene(s) in focus
- For all types of graphs, results can be displayed in linear or in log2 scale
- A ladder indicating “LOW”, “MEDIUM” and “HIGH” is displayed above the boxplot and the scatterplot, providing a general indication of expression level. MEDIUM is the interquartile range of all expression values for a platform (see Chapter 1.5.4)
- By resting the mouse on a “result” or on a category, additional information will appear in a tooltip. This tooltip can be frozen / closed by pressing ‘F2’
- A panel with a detailed view of all samples comprised within a category is obtained by clicking on it. The expression value as well as experimental parameters for each sample will be individually displayed (see Chapter 3.2.1). Note that a multiple selection is possible (CTRL + click)
- Categories representing normal tissues and cell lines can be added to the plot by ticking the “show anatomy” and/or “show cell lines” boxes (Figure 3.6, lower image), to compare expression between cell lines and normal tissues
- Hierarchical clustering of a set of genes across Cell Lines or Cell Lines + Anatomy + Cancers meta-profiles can be performed with the Hierarchical Clustering tool (see Chapter 5.1)
- As for the Samples tool, it is possible to create and export gene signatures from this tool (see Chapter 2.1.2)

3.6.3. Statistics

The expression values displayed in the Cancers tool represent the average expression for a given gene in a particular cancer across all selected samples. When cancers categories are shown as a tree, parent nodes represent the average expression of all samples within this branch. The number of samples aggregated in each category to calculate this average is indicated on the right of the graph. In the boxplot view, the whiskers represent the lowest datum still within 1.5 IQR from the lower quartile, and the highest datum still within 1.5 IQR from the upper quartile. Outliers, represented as stars, are values outside this range. In the scatterplot view, the whiskers indicate the standard error of the mean. Additional statistical parameters such as mean, standard error, 95% confidence interval are available by resting the mouse on a result (see Chapter 1.5.7).
Figure 3.6: Screenshot of the results obtained with the **Cancers** tool for human CA3 gene on the Human 133 Plus2 platform with the boxplot-tree view. The categories are displayed as nodes of a tree. The number of samples for each category is listed on the right. **Upper image:** only the cancer data are displayed. All the categories are collapsed beside cancers of bone/articular cartilage. **Lower image:** the anatomy data and cell lines have been added to the graph ("show anatomy" and "show cell lines" checkboxes, respectively).
3.7 The Perturbations tool

The Perturbations tool provides a summary of gene expression responses to a wide variety of perturbations, such as chemicals, diseases, hormones, stresses, mutations, etc. Its purpose is to easily identify experimental conditions causing an up- or down-regulation of genes of interest.

In contrast to all other tools, in which the level of gene expression is shown, the Perturbations tool represents relative values from a comparison of experimental versus control samples (see Chapter 1.5). Each item in the list of Perturbations is a comparison between samples belonging to the same experiment. If the same biological condition is tested in several independent experiments, this condition will appear multiple times in the list as separate comparisons. The values reflect up- or down-regulation of genes and are given as ratios (linear scale) or log₂-ratios (log₂ scale). These ratios indicate how big the difference is between the average of the expression in the experimental samples and the average in the control samples.

3.7.1 Getting started

1. Create a “Data Selection” (see Chapter 1.4.2)
2. Create a “Gene Selection” (see Chapter 1.4.3)

The plot will automatically be generated (see Figures 3.7A/B). Several options to display the results are available and described in the next section. By default, the “Scatterplot-List” view will be displayed (see Chapter 1.5).

3.7.2 Features

- Two types of graphs are available: scatterplot (up to 10 genes) and heatmap (up to 400 genes)
- The categories can be visualized either as a tree or as a list with the categories ordered according to the (average) expression levels of the gene(s) in focus. In the “-Tree” views, the perturbations are classified according to their types (chemical, drug, disease, etc...)
- For all types of graphs, results can be displayed in linear or in log₂ scale (default)
- For the scatterplots, a ladder indicates the fold-change (“down-regulated” on the left, “up-regulated” on the right)
- For the heatmaps, the ratios are displayed by default using a green-red color-coding but can be changed to blue-yellow (see Chapter 1.5.1) (Figure 1.19, C)
- By resting the mouse on a “result” or on a comparison, additional information will appear in a tooltip. This tooltip can be frozen / closed by pressing ‘F2’
- A panel with a detailed view of all samples comprised within a comparison is obtained by clicking on it. The expression value as well as experimental parameters for each sample will be individually displayed (see Chapter 3.2.1). Note that a multiple selection is possible (CTRL + click)
- Comparisons can be filtered by p-value (significant changes in expression between experimental and control samples via nominal t-tests) and/or by fold-change
The relevant Perturbations can be used to create a new "Data Selection" or added to an existing one (Figure 3.7B) for further analysis with the SIMILARITY SEARCH TOOLS, e.g., using the Co-Expression tool to find genes responding to the same Perturbations and expressed in the same tissues (see Chapter 5.2).

As for the Samples tool, it is possible to create and export gene signatures from this tool (see Chapter 2.1.2).

Figure 3.7A: Screenshot of the results obtained with the Perturbations tool for the human CA3, AMPD1 and TPM2 genes on the Affymetrix Human 133 Plus2 platform. The “Heatmap-List” view was chosen here. The Perturbations are ordered based on the expression fold-change of the gene in focus, here AMPD1, with the highest fold-change at the top. The Perturbations have been filtered to keep only the comparisons leading to a 3-fold-change at least and a p-value of maximum 0.05 as indicated in the red rectangle.
Figure 3.7B: A new “Data Selection” containing only the Perturbations affecting a gene of interest can easily be created or added to an existing one for further analysis.

3.7.3 Statistics

Each category (= comparison) is composed of experimental samples versus control samples coming from a single experiment.

For each gene/probe ID, the value shown while called the log-ratio is the difference between the mean log expression for experimental samples and the mean log expression for control samples. The log-ratio and the fold-change indicate how big the difference is between the average expression in the experimental samples and the average expression in the control samples.

The p-value takes into account not only the difference in the averages but also the variance and size of the two sample groups. A low p-value indicates that the averages are different and that this is probably not a coincidence, the average for a high number of replicates is likely to be clearly different.

For microarray data, the p-values are computed using the t-test.

For RNA sequencing data, the t-test is slightly modified to take into account the discrete nature of the underlying read mapping data.

3.8 The Development tool

The Development tool summarizes the expression of genes across distinct stages of development of an organism’s life cycle. Each organism has its own developmental stage ontology: from the fertilized egg cell via embryo, fetus and newborn up to the adult organism for mammalian organisms, or from the germinated seed up to the senescent plant for higher plants. This tool is not available for human data as for ethical reasons, no data on pre-natal stages of development are obtainable. Although the samples could theoretically be partitioned into more fine-grained development categories, generally between 10 and 15 stages are defined for each organism. This is to diminish the dominance of local experimental conditions that affect the expression of genes but are not related to the developmental stage itself. As a result, each stage comprises up to several hundred samples from which the average values are calculated. Nevertheless, the Development tool must be interpreted with caution. Large patterns reveal a general trend, but local peaks may be the result of specific conditions occurring at that particular stage of development.

3.8.1 Getting started

1. Create a “Data Selection” (see Chapter 1.4.2)
2. Create a “Gene Selection” (see Chapter 1.4.3)

The plot will automatically be generated (see Figure 3.8). Several options to display the results are available and described in the next section. By default, the scatterplot view will be displayed (see Chapter 1.5). For a given probe/gene, the expression value indicated for a given stage of development is the simple average of expression of all samples annotated as such.

3.8.2 Features

- Two types of graphs are available: scatterplot (up to 10 genes) and heatmap (up to 400 genes)
- For all types of graphs, results can be displayed in linear or in log2 scale
- A ladder indicating “LOW”, “MEDIUM” and “HIGH” is displayed above the boxplot and the scatterplot, providing a general indication of expression level. MEDIUM is the interquartile range of all expression values for a particular platform (see Chapter 1.5.4)
- By resting the mouse on a “result” or on a category additional information will appear in a tooltip. This tooltip can be frozen / closed by pressing ‘F2’

![Figure 3.8: Screenshot of the results obtained for 3 genes of Oryza sativa (rice) with the Development tool on the Affymetrix OS_51K platform. Expression of one of them (LOC_Os10g13800) is unchanged during the course of development while expression of the 2 others (LOC_Os04g26550, LOC_Os07g48620) peaks at the flowering stage (asterisk).](image-url)
Chapter 4

COMPENDIUM WIDE ANALYSIS: GENE SEARCH TOOLS

4.1 Overview of GENE SEARCH TOOLS

While the CONDITION SEARCH TOOLS (see Chapter 3 and Figure 3.1) allow you to investigate the expression of selected genes across various conditions, the GENE SEARCH TOOLS (Figure 4.1) do the opposite: they help you identify genes that are specifically expressed in a chosen set of conditions (“target categories”) compared to a larger set of “base” categories. A typical example is the search for biomarker genes, e.g., specifically up-regulated in response to a perturbation but minimally regulated in all other perturbations. Such queries are available for all types of meta-profiles, i.e., for Anatomy, Cell Types, Cell Lines, Cancers, Perturbations and Development. For the Anatomy, Cell Types, Cell Lines, Cancers and Development tools, results show absolute expression values in selected categories, whereas for the Perturbations tool, results show relative values (log₂-ratios) (see Chapter 1.5).

Additionally, the GENE SEARCH TOOLS comprise the RefGenes tool (see Chapter 4.4) to quickly find genes having the highest stability of expression across a chosen set of conditions and the Ortholog Search tool (see Chapter 4.5) to find most likely functional orthologous gene in other species.

![GENE SEARCH TOOLS](image)

*Figure 4.1: Screenshot of the GENE SEARCH toolset. The Cell Lines and Cancers tools are specific for the biopharma community.*

4.2 GENE SEARCH across Anatomy, Cell Types, Cell Lines, Cancers and Development

4.2.1 Getting started

To search for genes specifically expressed in chosen anatomical parts, cell types, cell lines, cancers, or developmental stages, proceed as follows:

1. Create a “Data Selection” (see Chapter 1.4.2)
   - E.g., “Homo sapiens” and “Human133-2: Human Genome 47k array”. For most analyses, a selection containing the largest possible number of samples is recommended

2. Click on the icon of the tool you want to use, e.g., Anatomy (Figure 4.1)

3. Choose one or several target categories, for which you want to find specifically expressed genes
4. Select the categories against which you want to run the analysis

- By default, the search will be made against all other categories, but it is possible to compare your "Target" category (-ies) against only a subset of "Base" categories. To use this feature, mark the checkbox "show bases" in the toolbar (Figure 4.2, red rectangle). Deselect all bases by clicking on the top checkbox, just below the column called "Base". Select your "Target" category (-ies), and then the "Base" categories against which you would like to specifically run the comparison, e.g., anatomical category "Umbilical cord" against all the other gestational structures.

- Note that, for the tool to work well, the set of "Base" categories should be significantly larger than the set of "Target" categories.

![Figure 4.2: Target and Base selection. By limiting the search of gene specific to umbilical cord against only the categories ("Base") "gestational structure" as shown in A, the algorithm will only consider the categories selected as "Base" in the search. The expression of the resulting genes will be displayed in the other categories and can be relatively high as shown in B.](image)

5. Choose the number of genes to be displayed in the “Limit” dropdown list (toolbar) (Figure 4.3)

- By default, 10 genes will be displayed

6. Click on "Run"
Figure 4.3: The toolbar for the Anatomy, Cell Types, Cell Lines, Cancers, Perturbations and Development tools. From left to right: the “Run” button to start the analysis; the “Reset” button to reset all “target” and “base” selections; the “show bases” checkbox to display the bases categories against which the analysis is performed; the “show anatomy” checkbox (specific to the Cell Lines and Cancers tools) to add normal tissues to the plot; the “show cell lines” checkbox (specific to the Cancers tool) to add cell lines to the plot; the “Limit” dropdown menu to define the number of genes being displayed; the “Copy”, “New”, “Add” buttons to copy a list of genes (e.g., into MS Excel), to create a new “Gene Selection” with it or add it to an existing selection (genes are actually copied as probe IDs); the “HELP” button to obtain more information.

4.2.2 Features

- The results represent absolute expression values and show genes which are highly expressed in the “Target” categories and lowly expressed in the “Base” categories.

- A heatmap plot will be created showing a list of genes having the most specific expression in the chosen “Target” category relative to all other categories. The genes are sorted by descending score, i.e., the genes with the highest specificity (highly expressed in the target categories and minimally expressed in the base categories) are at the top of the list (Figure 4.4).

- By default, the search will be made against all available categories (“Base”) for the sample selection in focus, i.e., expressed in the selected “Target” categories and minimally expressed in the “Base” categories. The “show bases” feature allows you to restrict the comparison to a smaller set of “base” categories defined by yourself.

- In the Cell Types tool, you can optionally add the tissues of origin (“show anatomy”) and/or the cell states (“show cell states”) to screen for genes specific for a chosen cell type but not expressed in normal tissues or in other cell states (Figure 4.5).

- In the Cell Lines tool, you can optionally add the normal tissues (“show anatomy”) or the cell types (“show cell types”) to screen for genes specific for a chosen cell line but not expressed in other cell lines or in normal tissues or cell types.

- In the Cancers tool, you can optionally add the normal tissues (“show anatomy”) or the cell types (“show cell types”) and/or the cell lines (“show cell lines”) to screen for genes specific for a chosen cancer but not expressed in other cancers or in normal tissues or cells and/or cell lines.
Figure 4.4: Screenshot of the search for genes specific for “squamous cell carcinoma, NOS, micro-dissected” (“Target”) and not or minimally expressed in all other categories of “neoplasms” of lip/oral cavity/pharynx (“Base”). Additional information about the gene LRRC25 is displayed in the mouse-over tooltip. Since the Cancer tree is hierarchical, signals for child nodes or branches (B) are also counted in parent nodes (A) where they appear in “diluted” form (less intense color). The Anatomy and the Cell Lines tools have identical functionalities.

- The “Copy” button in the toolbar allows the export of the list of genes resulting from the search. By clicking on the button, the gene list is copied to the computer's clipboard and can be inserted into any other application or into the GENEVESTIGATOR® “Gene Selection” dialogue by pressing “CRTL + V”
  - By clicking on the “New” button, the list of genes resulting from the search will be copied directly into a new folder in the “Gene Selection” panel and can be used as list of genes for further analysis with other tools. The list of identified genes can also be added to an existing gene selection by clicking on the “Add” button
  - As for the Samples tool, it is possible to create and export gene signatures from these tools (except from the Development tool) (see Chapter 2.1.2)

- Under the “HELP” button you will find additional information about the tool you are currently using
  - For microarray data, the search for genes is done with probe IDs, since they represent the physical units measured on the arrays. Nevertheless, the plot legends will display corresponding gene model identifiers as selected in “Gene Label” (see Chapter 1.5.8) and the corresponding probe will be indicated in parentheses
4.2.3 Statistics

The results represent absolute expression values and show genes which are highly expressed in the “Target” categories and lowly expressed in the “Base” categories. More specifically, the score for each gene is defined as the sum of average signal values of all “Target” categories divided by the sum of average signal values of all “Base” categories. A score of 0.9 therefore means that 90% of the sum of all average signal values is within the “Target” categories. More precisely, the score $K$ of gene $g$ ($K_g$) is calculated as:

$$K_g = \frac{\sum_{i \in T} s_{i,g}}{\sum_{i \in B} s_{i,g}}$$

where $s_{i,g}$ is the average expression for category $i$ and gene $g$, $T$ is the set of all “Target” categories and $B$ is the set of all “Base” categories. You can find the score for each gene by pointing the mouse to any cell of the heatmap (Figure 4.4). The score $K_g$ is calculated for all genes and the highest scoring genes are displayed in a heatmap.

Expression-level filter

Some genes, particularly in the single-cell platforms, may have very low or zero expression for most base categories. A slightly higher expression in the target categories, essentially at the level of measurement noise, could result in high score values for these genes, but these high scores are often not statistically significant.

The expression-level filter allows to exclude from the search all genes whose average expression in the target categories (denominator of $K_g$ divided by number of elements in $T$) is below a chosen threshold. Based on our experience, the default threshold was chosen as 50 TPM to give reasonably good results in most cases, but the actual value can be adjusted by the user if needed.

4.3 GENE SEARCH across Perturbations

4.3.1 Getting started

The Perturbations tool provides a summary of gene expression responses to a wide variety of Perturbations, such as chemicals, diseases, hormones, stresses, mutations, etc. The basic workflow is similar as described above (see Chapter 4.2.1), except that you must specify if they are looking for genes that are up- or down-regulated in the “Target” categories:

1. Create a “Data Selection” (see Chapter 1.4.2)
   - E.g., “Homo sapiens” and “Human133-2: Human Genome 47k array”. For most analyses, a selection containing all samples is recommended
2. Click on the icon of the Perturbations tool (Figure 4.1)
3. Choose one or several “Target” categories, for which you want to find specifically up- or down-regulated genes. It is highly recommended to start the search with a single “Target” category and to add further categories of interest only if they show similar expression profiles than the initial “Target” category. This can be done iteratively until a distinct profile appears grouping Perturbations causing common responses (Figure 4.5)
4. Choose the number of genes to be displayed in the “Limit” dropdown list (toolbar) (Figure 4.3). By default, 10 genes will be displayed.

5. Click on “Run”

4.3.2 Features

- In the **Perturbations** tool, results are represented as expression $\log_2$-ratios.

- A heatmap plot will be created showing a list of genes having the most specific expression in the chosen “Target” category relative to all other categories. The genes are sorted by descending score, i.e. the genes with the highest specificity (highly expressed in the “Target” categories and minimally expressed in the “Base” categories) are at the top of the list (Figure 4.5).

- By default, all “Base” categories are selected but can be modified by clicking on “show bases” and then selecting only a subset of “Base” categories. The “Tolerance %” button allows to tolerate a certain percentage of base categories that are co-regulated with the target category (default is 0%).

- The “Copy” button in the toolbar allows the export of the list of genes identified in a gene search. By clicking on the button, the list of genes (more exactly: probe IDs) is copied to the computer’s clipboard and can be inserted into any other application or into the GENEVESTIGATOR® “Gene Selection” dialogue by pressing “CRTL + V.”
  
  - By clicking on the “New” button, the list of genes resulting from the search will be copied directly into a new folder in the “Gene Selection” panel and can be used as list of genes for further analysis with other tools. The list of identified genes can also be added to an existing gene selection by clicking on the “Add” button.

  - As for the **Samples** tool, it is possible to create and export gene signatures from this tool (see Chapter 2.1.2).

- Under the “HELP” button you will find additional information about the tool you are currently using.

  - For microarray data, the search for genes is done with probe IDs, since they represent the physical units measured on the arrays. Nevertheless, the plot legends will display corresponding gene model identifiers as selected in “Gene Label” (see Chapter 1.5.8) and the corresponding probe will be indicated in parentheses.
Figure 4.5: Screenshot of a search across Perturbations for Human. Genes down-regulated in acute coronary syndrome were searched. The search was carried out against all Perturbations (all “Base” categories, default setting). The identified genes are specifically down-regulated in this condition but show minimal change of expression in other categories.

4.3.3 Statistics

The score $K_g$ measures how specific the up- and down-regulation of a gene is in the “Target” categories compared to the “Base” categories. Additionally, the ratio threshold option (in the toolbar; by default set to 0.5) allows you to discard all genes having an expression log$_2$-ratio below the given threshold in at least one of the chosen “Target” categories.
The score $K_g$ is calculated as:

$$K_g = \frac{\sum_{i \in T_u} s_{i,g}}{\sum_{i \in T_d} s_{i,g}} + \frac{\sum_{i \in B^+} s_{i,g}}{\sum_{i \in B^-} s_{i,g}}$$

where $s_{i,g}$ is the meta-profile log$_2$-ratio for category $i$ and gene $g$, $T_u$ is the set of all “Target” categories in the up-regulated group, $T_d$ is the set of all “Target” categories in the down-regulated group, $B^+$ is the set of all “Base” categories with positive log-ratios ($s_{i,g} > 0$) and $B^-$ is the set of all “Base” categories with negative log-ratios ($s_{i,g} < 0$). You can find the score for each gene by pointing the mouse to any cell of the heatmap. The score $K_g$ is calculated for all genes and the highest scoring genes are displayed in a heatmap.

### 4.4 The RefGenes tool

Reference genes (frequently also called “housekeeping genes”) are often used as internal controls in transcript quantification assays such as qRT-PCR. In many laboratories, reference genes from commercial panels such as GAPDH or ACTB are routinely used to normalize qRT-PCR raw data. Unfortunately, under many conditions these commonly used genes are inappropriate for normalization because their expression is not universally stable as reported in several independent studies.

**RefGenes** is a new type of tool that allows the identification of the genes having the highest stability of expression across a chosen set of conditions. Rather than testing a handful of “housekeeping” genes, **RefGenes** selects candidate genes from the entire genome (Hruz et al., [6]). The primary objective is to provide scientists performing qRT-PCR experiments an objective choice of reference genes specific for their experimental context. As GENEVESTIGATOR® contains data covering hundreds of different experimental conditions, it is often possible to choose a group of conditions very similar to that of one’s own qRT-PCR experiment and to find the most stable genes within this group, e.g., same tissue type.

Ideally, reference genes have two characteristics:

- They must have a stable level of expression across the conditions being compared
- Their overall expression level is preferably similar to that of the target gene(s) being amplified by PCR (there are several practical and theoretical reasons for this, although this is not an absolute requirement)

The RefGenes tool can fulfill both conditions by allowing you to a) choose conditions closely related to your experiment, and b) choose the optimal range of transcript abundance of candidate reference genes by comparing with the expression range of your target gene(s). It then computes the variance of expression for each gene (probe) across the chosen conditions and selects the 20 best scoring genes (i.e. those with lowest variance).

Figure 4.6 shows an example for mouse small intestine tissue represented by 176 samples on the Affymetrix Mouse Genome 430 2.0 Array. Five commonly used reference genes (first 5 genes shown in various colors in the upper part of the plot) were given as target and a search for gene very stably expressed in this tissue was made using **RefGenes**. The expression range is automatically defined by the expression range of the given target genes but can be changed. The top-scoring reference genes found by **RefGenes** are shown “Reference genes” section of the plot. As shown here, all 5 commonly used reference genes have a large variance in expression across these samples, whereas the reference gene candidates proposed by **RefGenes** show lower variance.
4.4.1 Getting started

1. Create a “Data Selection” (see Chapter 1.4.2) and select samples having similar experimental conditions as in your qRT-PCR experiment using the filter “Refine selection by conditions” (see Chapter 1.4.2.1)
   - To get reasonable results, we recommend using a minimum of 50 samples from at least 3 independent experiments. Regarding the conditions to choose, we recommend choosing the same tissue type (or related tissues), and then verify the stability of expression of the candidate genes obtained against other dimensions such as perturbations.

2. Enter the target gene(s) that you want to amplify by qRT-PCR to focus the RefGenes search on a suitable expression range (see Chapter 1.4.3)
   - RefGenes will show signal intensity and variance for your target genes and propose an expression range near to that of your target genes. The range can also be manually adjusted by changing the “Range” values in the toolbar (Figure 4.6).

3. Click on “Run”
   - The 20 best scoring reference genes will be displayed with their expression intensity and variance. If you want to compare with other reference genes that you may have considered using (e.g., commonly used ones such as ACTB), add them to your list in the “Gene Selection” panel.

Figure 4.6: “Housekeeping” genes in mouse small intestine. The 5 traditional reference genes, B2M, SDHA, GAPDH, YWHAZ and ACTB show a much larger variance of expression across samples for this tissue than the reference genes proposed by RefGenes. A video tutorial opening in the browser is available under the camera icon in the toolbar.
4.4.2 Features

- The results are displayed as boxplots.
- The expression range in which the most stable genes are searched is automatically defined by the entered target genes but can be changed manually.
- The “Copy”, “New” and “Add” features described for the other GENE SEARCH TOOLS are also present in RefGenes (see Chapter 4.2.1).
- A video tutorial is available directly in the tool by clicking on the camera icon or on the GENEVESTIGATOR® website: https://genevestigator.com/support/

4.4.3 Statistics

The expression values displayed in the RefGenes tool represent the average expression for a given gene across all selected samples. The expression values are “normalized” as described in Chapter 7 for microarray data and Chapter 8 for RNA-Seq data.

In the boxplots, the whiskers represent the lowest datum still within 1.5 IQR from the lower quartile, and the highest datum still within 1.5 IQR from the upper quartile. Outliers, represented as stars, are values outside this range. Median value and standard deviation are indicated on the right of the plot. Additional statistical parameters are available by resting the mouse on a result (see Chapter 1.5).

4.5 The Ortholog Search tool

The Ortholog Search tool allows you to find the most probable functional orthologs to a gene of interest in other species. This tool is unique in the way that it uses both sequence and expression data for the prediction of functional orthologs.

4.5.1 Getting started

1. Create a “Gene Selection” (see Chapter 1.4.3)
2. Click on “Run”

4.5.2 Features

- The results will be displayed in a table grouped by organism (Figure 4.7). For each gene, a sequence-based score (PAM = point accepted mutation) and an expression-based score will be shown. The shade of the color and the displayed numerical value indicate the distance of the orthologous gene to the query gene. The PAM is provided by the OMA database (https://omabrowser.org) [19]. The expression-based score is calculated by GENEVESTIGATOR®. This score is an indicator of functional orthology between the query gene and the displayed orthologs based on expression values only. The color ranges from red (positive scores) for orthologs to blue (negative scores) for less related genes. Additionally, for the query gene and the identified orthologous genes, the probe ID, the platform and the number of tissues used for computing the expression-based score are displayed.
The organisms and platforms used to search for orthologs can be modified by clicking on the “Organisms and Platforms” button. A dialogue will open, allowing the user to define platform and organism (Figure 4.8). To optimize the search, we recommend including the default platform for each species as it contains the highest number of samples.

The “Copy”, “New” and “Add” features described for the other GENE SEARCH TOOLS are also present in Ortholog Search (see Chapter 4.2.1).

A new query gene can be entered by clicking on the “Change...” button.

Figure 4.7: Ortholog search for the FOS human gene. Orthologs have been found for dog, mouse, rhesus monkey and pig. All orthologs have a high sequence similarity (low PAM value, dark gray) and a high expression similarity (high value and dark red). The probe ID, the platform and the number of tissues used for computing the expression-based score are displayed for both the query gene and the identified orthologs.
Creation of a list of orthologs from an existing gene selection

It is possible to translate a gene selection from one species to another, using a mapping provided by OMA (see statistics below). To create a new selection with orthologs from another species, simply right-click on the gene selection of interest, go to “Create Orthologs” and select the desired organism (Figure 1.17). A new gene selection containing the corresponding orthologs will appear as a new folder in the “Gene Selection” panel.

4.5.3 Statistics

4.5.3.1 Distance-based score

The Ortholog Search tool uses Point Accepted Mutations (PAM) as a distance measure for the sequence score which is provided by the OMA database (https://omabrowser.org) [19].

4.5.3.2 Expression-based score

The Ortholog Search tool uses the expression profiles of genes over anatomical categories to obtain information about their relation of orthology. It bases its evaluation on modeling two probability distributions:

- The distribution of the expression over the anatomical categories for pairs of orthologous genes
- The distribution of the expression over the anatomical categories for pairs of unrelated genes

Both models are calculated by fitting multivariate normal distributions, once for pairs of orthologous genes of the two organisms and two more times for the genes of the two organisms separately. This results in two normal probability distribution functions, one describing the expression of pairs of orthologs, the other describing the expression of pairs of arbitrary genes. This last distribution is computed as the product of the normal models for the separate organisms. For each pair of genes from the two organisms, the tool then displays the logarithm of the likelihood ratio of the two following probabilities:

- The probability that the observed expression of the two genes results from orthologous genes
- The probability that the observed expression of the two genes results from non-orthologous genes

The relevance of the likelihood ratio can be seen as a consequence of the Neyman–Pearson Lemma. (See Simple-vs-simple_hypotheses on Wikipedia.) According to Bayes Theorem, the likelihood ratio, (also called Bayes factor) is exactly what is needed to compute a posterior estimate of the probability that two given genes are orthologs from prior probabilities. Typically, the prior probability will come from sequence information. Alternatively, the likelihood ratio can simply be used as an indicator of orthology.
Chapter 5

COMPRENDIUM WIDE ANALYSIS: SIMILARITY SEARCH TOOLS

The SIMILARITY SEARCH TOOLS allow the grouping of your genes of interest according to their similarity across chosen expression profiles or to find conditions generating an expression pattern similar to a given signature. This toolset currently consists of six different tools (Figure 5.1):

![SIMILARITY SEARCH TOOLS](image)

**Figure 5.1:** SIMILARITY SEARCH toolset comprising six individual tools.

- The Hierarchical Clustering tool based on the classical approach that was initially proposed by Eisen et al., [7], for clustering gene expression data
- The Co-Expression tool to identify genes being the most correlated to a target gene across a dataset you defined
- The Signature tool to identify experimental conditions causing similar biological responses as in your own experiment based on expression values that you enter
- The Biclustering tool based on a more recent approach that looks at similarity of gene expression under subsets of conditions rather than across all conditions (for an assessment of different methods, see Prelić et al., [8]). What makes the GENEVESTIGATOR® cluster analysis different than other clustering software is its integration with a large curated database. Thus, it is possible to cluster genes based on meta-profiles, i.e., within their biological context
- The Gene Set Enrichment tool to search for gene sets similar to a gene selection
- The 2-Gene Plot tool to visualize the expression of two genes simultaneously across a data selection

5.1 The Hierarchical Clustering tool

The Hierarchical Clustering tool helps you group genes and/or conditions having similar profiles across all conditions or genes selected, respectively. It works on the expression matrix defined by the current sample and gene selections. Different options influencing the clustering results are available via drop-down lists in the toolbar (Figure 5.2).

5.1.1 Getting started

1. Create a “Data Selection” (see Chapter 1.4.2)
2. Create a “Gene Selection” (see Chapter 1.4.3) 

3. Select a biological context (Samples, Anatomy, Development, Perturbations, Cell Lines Cancers, Anat./Cancer/Cell.) from the drop-down menu in the toolbar (Figure 5.2) 

4. Click on “Run” 
   - A heatmap will automatically be generated for the chosen profile with the entered genes and the samples selected

![Hierarchical Clustering tool](image)

*Figure 5.2: The toolbar of the Hierarchical Clustering tool split into two parts. Diverse options are available to adapt the clustering to your requirements. Drop-down lists allow you to choose the biological context (or profile), the type of distance measure and the leaf-ordering that will be used.*

5.1.2 Features and Statistics

- The Hierarchical Clustering algorithm allows you to cluster the matrix in one or in both directions, i.e., group genes with similar expression patterns across the selected conditions, or group conditions with similar expression patterns for the selected genes. Select the checkboxes “Genes” or “Conditions” in the toolbar accordingly (Figure 5.2)
  
  - The “distance measure” option: similarities between expression profiles can be determined using different measures. Possible settings are: Pearson correlation, Euclidian distance, Manhattan distance. In GENEVESTIGATOR®, Euclidian distance is the default setting. Additionally, GENEVESTIGATOR® uses average linkage with the chosen distance measure to calculate the distance between two clusters. Different “distance measures” are available in a drop-down list in the toolbar (Figure 5.2)

  - The “leaf-ordering” option: in a clustering tree, branches can be turned either way at each node. Leaf ordering consists of turning the branches around so that similar vectors (i.e., clusters with similar patterns) are positioned close to each other (from left to right) but the clustering tree remains the same. There are situations, such as a time-course or a dose response series, where leaf-ordering can be very useful to obtain a better visualization. The ordering of the leaves is computationally more intensive than the clustering itself. Despite the use of the fastest known algorithm for leaf ordering (Bar-Joseph et al., [9]) it can take several dozens of seconds for large data matrices. Therefore, the tool was specified to apply leaf-ordering by default for small matrices but to be optional for larger matrices (default is then set to "no leaf-ordering"). Figure 5.3 demonstrates the benefits of a well-chosen leaf-ordering
Individual branches of the gene cluster can be selected and the list of genes can be copied to the clipboard ("Copy"), to a new gene selection ("New") or added to an existing gene selection ("Add") (Figure 5.2)

Individual branches of the conditions cluster can be selected and the list of samples included in these conditions can be copied to the clipboard ("Copy"), to a new “Data Selection” ("New") or added to an existing “Data Selection” ("Add") (Figure 5.2)

As for the Samples tool, it is possible to create and export gene signatures from this tool (see Chapter 2.1.2)

**Figure 5.3:** Hierarchical clustering of genes against the Development meta-profile in Arabidopsis. **A:** Hierarchical clustering without leaf-ordering. **B:** Hierarchical clustering with leaf-ordering. Leaf-ordering allows a better visualization of sequential processes, such as developmental stages (shown here) or time-course experiments. The trees are identical, only the branches and leaves are displayed in a different order.

### 5.2 The Co-Expression tool

The Co-Expression tool helps you identify genes having the most similar profile to a target gene across a dataset selected or created from the database. As the GENEVESTIGATOR® engine processes the correlations on the fly, any dataset (i.e. a selection of samples) can be defined, representing either individual experiments or experimental conditions relevant for the target gene.
5.2.1 Getting started

1. Create a “Data Selection” (see Chapter 1.4.2)
   - The choice of dataset underlying a correlation analysis is crucial. We strongly recommend to first identify the conditions that are relevant for a target gene using the Perturbations tool from the CONDITION SEARCH TOOLS and to create a “Data Selection” with these conditions (see Chapter 3.6.2). Then, to run a correlation using the Perturbations profile
2. Create a “Gene Selection” (see Chapter 1.4.3)
   - If you have a list of genes, the target gene is the first marked gene in the list. You can define a new target gene by creating a new gene selection (“New” button in the “Gene Selection” panel) or by clicking on the “Change...” button in the results panel (Figure 5.4)
3. Select a biological context (Samples, Anatomy, Development, Perturbations, Cell Lines, Cancers, Anat./Cancer/Cell) from the drop-down menu in the toolbar (Figure 5.4)
4. Click on “Run”

![Figure 5.4: Toolbar of the Co-Expression tool. Several parameters must be selected from drop-down menus: the biological context, the number of genes to be displayed and the type of correlation. The target gene can be changed directly in the result panel by clicking on the “Change...” button. A video tutorial opening in the browser is available upon clicking the camera icon.](image)

5.2.2 Features

- By default, the target gene is the first marked gene in the gene selection in focus
- Since GENEVESTIGATOR® processes the correlations on the fly, any dataset composed of individual samples (Samples) or of summarized data into meta-profiles Anatomy, Development, Cancers, Perturbations, Anat./Cancer/Cell can be selected
- The number of genes to be displayed can be selected in the “Limit” drop-down menu in the toolbar (Figure 5.4)
- Two types of correlations (“Positive” or “Negative”) are available
- The tool will correlate all genes (more precisely: all probes) against the target gene (probe) across the profiles generated from the selected dataset. The resulting genes are displayed in the circular plot and described in the adjacent table (Figure 5.5)
- The “Copy”, “New” and “Add” features described for the other GENE SEARCH TOOLS are also present in the Co-Expression tool (see Chapter 4.2.2) and the co-expressed genes can easily be further analyzed using other tools like the Hierarchical Clustering tool (see Chapter 5.1)
Figure 5.5: Co-expression analysis for the human PSCK9 gene. A: the correlation was done with the conditions in which PCSK9 is significantly regulated identified with the Perturbations tool from the CONDITION SEARCH TOOLS and using the Perturbations profile. The top 50 co-expressed genes are displayed on a circular plot. A cluster of mutually correlated gene is apparent. B: both correlation scores can be changed by sliding the cursor along the correlation scales. The correlation score can be used to filter the number of correlated genes to be displayed. The mutual correlation score will allow visualizing connections between mutually correlated genes. C: the 50 resulting genes are listed and described in an adjacent table and can be copied to the clipboard or used to create a new gene selection using the Copy, New and Add buttons on top of the window. The highlighted gene corresponds to the red squared dot in the plot. Genes can also be copied to the clipboard, together with the displayed scores and descriptions, using the usual keyboard shortcuts, e.g. CTRL+C.
In the circular plot, the target gene is in the center and the correlated genes are displayed around it at a distance corresponding to their correlation with the target (see Chapter 5.2.3). The closer they are to the center, the higher their correlation with the target gene across the selected dataset (see axis indicating Pearson's correlation coefficients). The positions around the center are determined by hierarchical clustering with optimal leaf-ordering, i.e., genes that more strongly correlate with each other than with the remaining genes will group together. This allows you not only to identify genes having a strong correlation with a given target gene, but to get an idea whether there are groups of differently regulated genes that correlate well with the target gene. To visualize this grouping more easily, it is possible to add an edge between each pair of genes that have a correlation coefficient above a chosen threshold. The threshold can be defined manually using the slide bar below the plot (Figure 5.5).

- A video tutorial is available for this tool directly from the tool (camera icon) or under the following link: https://genevestigator.com/support/

### 5.2.3 Statistics

In the current version of this tool, we have chosen the Pearson correlation coefficient as measure of similarity between genes, both for identifying co-expressed genes as well as to define the pairwise correlation between genes in the plot. This score is calculated on log₂-scaled expression data that is processed from the GENEVESTIGATOR® database. To learn more about how data are normalized in GENEVESTIGATOR®, please see [Chapter 7](microarray data) and [Chapter 8](RNA-Seq data). The positioning of the dots in the plot is obtained by a circularized hierarchical clustering with average linkage and optimal leaf-ordering.

### 5.3 The Signature tool

The Signature tool allows you to put your own results into the context of thousands of other experiments. It allows the user to enter any gene expression signature obtained, e.g., by RT-qPCR, RNA-Seq or microarray and compare it with the GENEVESTIGATOR® content. A signature consists of a list of genes and the corresponding values of expression level or log₂-ratio. The Signature tool will then correlate the entered signature with expression data from all the curated experiments of the “Data Selection” and output those conditions having the most similar/different profiles.

#### 5.3.1 Getting started

1. Create a “Data Selection” (see Chapter 1.4.2)
   - Ideally you should select the default platform for an organism to search against the maximum number of conditions

2. Click on the “Enter Signature” button present in the toolbar (Figure 5.6)

3. In the “Enter Signature” dialogue (Figure 5.7) enter a signature of at least 8 genes or probe IDs and the corresponding expression values (either by typing them or pasting them) and specify:
   - The scaling of data: linear or log-scale
   - The type of expression data: absolute or relative values
Figure 5.6: The toolbar of the Signature tool split into two parts. The “Run” and “Enter Signature” buttons are highlighted by red rectangles. Analysis parameters such as the type of profile, the type of similarity and the type of distance are available in drop-down lists. A video tutorial opening in the browser is available under the camera icon.

Figure 5.7: The “Enter Signature” dialogue. The organism is defined based on the “Data Selection” in focus. A minimum of 8 genes or probe IDs are required. The genes and expression values can be manually entered or directly pasted from another file. Both the “Scaling of data” and the “Type of data” must be defined.
4. Choose from the drop-down menu in the toolbar the type of profile GENEVESTIGATOR® should search (Figure 5.6)

   - **Absolute** expression values will allow you to search across the **Samples, Anatomy, Cell Lines, Cancers** or Development profiles

   - **Relative** expression values will allow you to search across the **Perturbations** profile

5. Choose from the drop-down menu in the toolbar the type of similarity (“Most similar” or “Most different”) (Figure 5.6)

6. Choose from the drop-down menu in the toolbar the type of distance (“Euclidean Distance” “Manhattan Distance” “Pearson Correlation”) (Figure 5.6)

7. Click on “Run”

   - GENEVESTIGATOR® will return a list of conditions (according to the profile type chosen) in which gene expression is most similar/different to your signature (Figure 5.8)

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**Figure 5.8:** The **Signature** tool retrieved the 50 Perturbations having the most similar expression pattern to the entered signature. The Perturbations are ranked based on their relative similarity (indicated on the right).

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5.3.2 Features

   - A video tutorial is available for this tool directly from the tool (camera icon) or under the following link: [https://genevestigator.com/support/](https://genevestigator.com/support/)

   - Two types of similarity are available: “Most similar” and “Most different”

   - Three types of distance measurements are available: “Euclidean Distance”, “Manhattan Distance” and “Pearson Correlation”
The “New” and “Add” features described for the other GENE SEARCH TOOLS are also present in the Signature tool (see Chapter 4.2.1) and the samples comprised in the “Most similar” or “Most different” conditions can easily be further be analyzed using other tools.

As for the Samples tool, it is possible to create and export gene signatures from this tool (see Chapter 2.1.2).

Creation of gene signatures for use in other tools

Previously, it has been possible from certain tools in GENEVESTIGATOR® to copy a list of genes either to the clipboard or to a gene selection. This function has now been enhanced to allow exporting the list of genes together with the corresponding expression values (“signatures”) from a given condition. To create such a “signature”, select the condition of interest (Anatomy or Perturbations), and click in the toolbar on the Signature: “Copy”, “New” or “Add” buttons.

The “signature” will appear in the “Gene Selection” panel with a “S” tag.

A “signature” can be opened directly in the Signature tool and allows you to compare it with data from other studies. Such a comparison is useful to find other studies giving similar or opposite results, or to verify the nature of a given sample.

5.3.3 Statistics

The results show conditions which are similar or different to the entered genes and expression values. The level of similarity is given as “relative similarity” and indicates the degree of their resemblance: The higher the value, the higher the similarity relative to the average similarity.

More precisely if the similarity si is defined as 1/di with di the distance of category i to the signature, then the relative similarity R of a category c is calculated as

$$R_{sc} = \frac{s_c}{\frac{1}{N} \sum_{i \in I} s_i}$$

where si is the similarity of category i and I the set of all conditions.

For statistical reasons, expression similarity measures are calculated on log-scaled data. Therefore, if the data entered is in linear scale, it will be log-transformed by the tool for the calculation of similarity.

5.4 The Biclustering tool

In contrast to the Hierarchical Clustering tool, which groups genes by measuring their similarity of expression across all selected conditions, the Biclustering tool identifies groups of genes that exhibit similarity only in a subset of conditions, irrespective of their expression profiles in other conditions. The rationale for using biclustering is that each gene has its own individual set of regulators and that, under certain conditions, it shares common regulation with other genes. Therefore, the Biclustering tool searches for modules of co-regulated genes, in subsets of conditions under which they are regulated in the same way, irrespective of how they are regulated in other conditions.
Several biclustering algorithms have recently been developed for the analysis of expression data. For an empirical comparison see Prelić et al., [8].

5.4.1 Getting started

1. Create a “Data Selection” (see Chapter 1.4.2)
2. Create a “Gene Selection” (see Chapter 1.4.3)
3. Choose from the drop-down menu in the toolbar the type of profile (Samples, Anatomy, Development, Perturbations, Cell Lines, Cancers or Anat./Cancer/Cell.) (Figure 5.9)
   - For the Perturbations dimension, specify if you are looking for modules of genes that are commonly “up-” or commonly “down-” regulated or both up- and down-regulated (Figure 5.10)
4. Click on “Run”

![Figure 5.9: Toolbar of the Biclustering tool split into two. The drop-down menu to select a profile is open and the binary matrix box is unchecked. A video tutorial opening in the browser is available under the camera icon.](image)

Identifying a list of interesting biclusters requires in many cases several runs. A critical issue is the running time as the number of biclusters can get huge depending on the settings and size of the matrix and it is easily possible to specify settings for which the algorithm would require hours to complete. Thus, it is best to start with a high discretization threshold, e.g., one leading to approximately 5-10% black cells in the discrete matrix and high numbers for the minimal factors and minimal genes settings, e.g., 5x5 or more. If no biclusters or only a small number of biclusters are found, these numbers can then be reduced step by step for further runs (Figure 5.10).

The list of biclusters identified in a run is displayed on the right. Selecting one of the biclusters re-orders the matrix in such a way that this bicluster appears in the upper left corner of the matrix.
5.4.2 Features

- A video tutorial is available for this tool directly from the tool (camera icon) or under the following link: https://genevestigator.com/support/

- As in the Hierarchical Clustering tool, a list of genes can be displayed against various biological dimensions (Samples, Anatomy, Development, Perturbations, Cell Lines, Cancers, Anat./Cancer/Cell). Discretization “Threshold” is set at 0.8 by default but another value can be entered. To verify your choice, visualize the matrix by clicking the “binary matrix” checkbox – the discretized matrix will be displayed instead of the original expression matrix. Ideally, the number of black cells should only represent a small percentage of the whole matrix (5-10% of black cells) (Figure 5.10)

- The “min factors” and “min probes” options can be used to specify the minimal numbers of factors and genes that a bicluster must contain. By default, both are set to 5 (Figure 5.9)

- As in other tools, the "Copy", "New" and "Add" buttons can be used to export the gene list to the clipboard, to create a new gene selection or to add the list to an existing gene selection (see Chapter 4.2.1)

- As for the Samples tool, it is possible to create and export gene signatures from this tool (see Chapter 2.1.2)

- For the Perturbations profiles, you can specify whether you want to find biclusters of upregulated genes or downregulated genes or biclusters with genes that changed their expression in either direction

5.4.3 Statistics

GENEVESTIGATOR® uses the BiMax algorithm (Prelić et al., [8]) because it is an exact algorithm that identifies all biclusters in a matrix. The algorithm works on a binary matrix (i.e., data having two states for instance, up-regulated versus not up-regulated). The expression data matrix is first discretized to 0 and 1 according to a user-specified threshold, e.g., the expression data are categorized into either “changed” (black) versus “not changed” (white). Based on this binary matrix, BiMax identifies all maximal biclusters. A bicluster is defined here as a set of genes and a set of conditions for which all values are 1 and where "maximal" means that this bicluster is not completely contained in any other bicluster.

Specifying a threshold

In case of the Perturbations profiles, the threshold is specified as a log ratio between treatment and control. A threshold of 1 is equivalent to two-fold up-regulation, a threshold of 2 is equivalent to four-fold up-regulation and a threshold of -1 is equivalent to two-fold down-regulation. For all other profiles which show absolute expression values, the discretization is based on the percentage of the expression potential (in log scale). The expression potential estimates the maximum expression level that a gene can reach. Thus, a threshold of 0.5, for example, marks all genes as highly expressed that reach half of their expression potential.

Limitations

The biclustering tool has several limitations to help prevent crashes of the client. The first is that no matrix greater than 250,000 elements (e.g., 500x500, 200x1250, 10x25000, etc...) can be biclustered. In addition, if the algorithm encounters many biclusters during evaluation, it will abort computation and return with a warning message.
Figure 5.10: Biclustering of genes with the *Perturbations* profile for Human. In the bottom image, the matrix was discretized into 0 (white; below threshold) and 1 (black; above threshold). The selected bicluster is placed at the top left corner.

5.5 The *Gene Set Enrichment* tool

The *Gene Set Enrichment* tool allows you to compare an input gene set with a background collection of gene sets such as Reactome pathways or Gene Ontology categories. The tool helps you to understand to what pathways or biological process the genes of your input list are related.

5.5.1 Getting started

1. Create a “Gene Selection” (see Chapter 1.4.3). By default, the “Gene Selection” in focus will be used as “Input Gene Set”
2. Choose a background collection of gene sets by clicking on the “Change…” button beside the label “Background collection of gene sets” (Figure 5.11) (e.g., Reactome annotations)
3. Click on “Run”
Figure 5.11: Selection of (a) gene set background collection(s). By clicking on the “Change…” button a dialogue displaying the list of existing gene set collections will open. One or several collections can be selected for an analysis (e.g., all collections for Reactome annotations or only some of them).

5.5.2 Features

The upper right table displays the list of the sets of the background collection (Figure 5.12 A). For each set, a p-value measuring its similarity with the input gene set is given, as well as a false discovery rate (FDR) computed from all the p-values. The first column of the table provides a visual representation of the overlap between each gene set of the collection and the input gene set. The colors refer to the Venn diagram on the left (Figure 5.12 B). Additional information about a gene set appears when the mouse is hovering over it.
Figure 5.12: Gene Set Enrichment analysis. The results are displayed in a Venn diagram and two tables. A. The upper right table displays the list of gene sets for the background collection and indicates for each set a p-value and an FDR.

A Venn diagram occupies the left side of the view (Figure 5.12 B). It represents the input gene set and up to three additional sets from the background collection. The input gene set is represented in pink and the additional sets are represented by distinct colors (green, violet and grey). By default, the three gene sets having the highest similarity to the input set are displayed, but other gene sets from the background collection can be chosen using the check boxes of the upper right table. The numbers indicate how many genes belong to each region. By selecting one region (or several, using the CTRL/CMD key), the corresponding genes in the lower right table will automatically be selected.

The lower right table (Figure 5.12 C) contains a row for each gene belonging to a gene set checked in the upper right table and displayed in the Venn diagram. It also lists descriptions for those genes. The first column of the table displays the colors of the sets of the Venn diagrams of which the genes are members.

5.5.3 Statistics

The similarity between the input gene set and the background gene sets is indicated using a p-value. The p-value for a pair of gene sets is the probability that two random sets of the same size have an at least equally large intersection. The p-values are calculated by a method usually known as the hypergeometric test or Fisher’s exact test. Since the test corresponds to the rather unrealistic null hypothesis that the genes of one set were chosen at random independently, its results should usually be used heuristically.
5.5.4 Alternative use case

Besides being used to compare an input gene set to a large predefined collection of sets, the tool can also be used to examine the relationship between a few selected gene sets (either gene selections or predefined gene sets). More precisely, two (or more) gene lists that were either created from a GENEVESTIGATOR® tool or imported from another application can be compared in order to find the genes shared between the lists. In this second case, one set of interest should be used as input set, while the other sets of interest should be taken as "Background collection of gene sets".

5.6 The 2-Gene Plot tool

The 2-Gene Plot tool helps you visualize the expression of two genes simultaneously in one plot. You can display the expression values across the whole sample selection or only in a subset: the different biological categories available in GENEVESTIGATOR® can be selected from a drop-down menu. The existing sub-categories and sample level annotations of each category will be listed in separate tables and can be highlighted by manually assigning different colors.

5.6.1 Getting started

1. Create a “Data Selection” (see Chapter 1.4.2).
2. Create a “Gene Selection” (see Chapter 1.4.3). By default, the first two genes of the active "Gene Selection" are used for the plot.
3. Start the 2-Gene Plot tool.
4. A plot will automatically be created, but you can still select a biological context (Samples, Anatomy, Development, Perturbations, Cell Lines, Cancers, Anat./Cancer/Cell.) from the drop-down menu in the toolbar (Figure 5.14). The default context is Samples.

![Figure 5.14: The drop-down menu in the toolbar of the 2-Gene Plot tool to select the biological context.]

5.6.2 Features and Statistics

The 2-Gene Plot tool is a powerful yet simple visualization tool, which plots the expression values of two genes against each other. The “Perturbations” category displays log_{2} ratios, all other categories (Samples, Anatomy, Development, Cell Lines, Cancers, Anat./Cancer/Cell.) show the log_{2} expression values.

The results are displayed in a scatterplot (Figure 5.15). More information on a sample is available in a tooltip upon resting the mouse on a data point. This information includes the expression values (or ratios) of the two genes and additional information that varies depending on the selected category, e.g., details about the experiment.
When one of the Anatomy, Development, Perturbations, Cell Lines, Cancer or Anat./Cancer/Cell. category is selected, a tree next to the plot is showing the ontologies available for that category in the current sample selection. To visualize gene expression according to your biological question, you can assign individual colors to the ontologies by dragging-and-dropping from the color palette on top of the table.

If the Samples category is selected, the existing sub-categories are listed in a table next to the plot. Selecting one of these sub-categories creates a second table containing the sample level annotations available in the current sample selection and sub-category. To visualize gene expression according to your biological question, you can assign individual colors to these annotations by dragging-and-dropping from the color palette on top of the table.

As in many other tools, it is also possible in the 2-Gene Plot tool to select groups of samples with the lasso tool and/or by CTRL-clicking and save new or add to existing sample selections (e.g., Chapter 1.4.2).

In the Samples category, the log₁₀ expression values of the two selected genes are plotted without any further statistical analysis. In the other categories, the displayed value represents the average log₁₀ expression value or log₂ ratio (Perturbations) of all samples in the selected ontology.

Figure 5.15: The 2-Gene Plot tool used to visualize cell-specific expression of molecular markers. The human default platform was searched for the research area “multiple sclerosis” and filtered for “diseased” samples only. When the “Samples” category in the drop-down menu (top left) is selected, the expression of the first two genes of the active Gene Selection (here: TAF11 and CTSS) is plotted across all samples (A). The sample level categories available for this selection are listed next to the plot (B). Selecting the category “Anatomical Part” results in a list of all sample level annotations of this category in a new table (C). The color of each annotation was adjusted manually.
Chapter 6
Saving results and exporting figures & data

GENEVESTIGATOR® has several functions to save your analysis results and to allow you to continue working with it in another session or to export results for use in other applications. The current state of your analysis can be saved as a workspace and figures or data can be exported.

6.1 Saving workspaces

The “Save workspace” function in the “File” menu saves all sample selections and all gene selections (on the level of probes) of the current analysis to a local file on your computer. Workspaces are not stored on the GENEVESTIGATOR® servers. Via the “Load workspace” menu function such a file can be opened again and the analysis re-established. Workspaces are not associated with a user account, i.e., you can send such a file to a colleague to share your results or to collaborate on an analysis.

6.2 Exporting figures

In the “Results” menu under “Export Image”, GENEVESTIGATOR® provides extended possibilities to export the figures from your analysis results for further use (Figure 6.1). After previewing and scaling the figure, you have the choice between four file formats: PDF, PNG, GIF or JPG. The first is ideally suited for print publications, since it allows highest possible resolution and resizing without loss of information (vector graphics). The latter three are designed for publishing figures on the Web. Their resolution is lower, especially if figures are subsequently increased in size using image processing software (see Figure 6.2 for a comparison of the formats).

Figure 6.1: The figure export dialogue. Figures can be resized to the desired size and shape and exported either in PNG, GIF, JPG or PDF format.
Working with figures in PDF

PDF (Portable Document Format) is a graphics file format, making it easier to embed within another document. A PDF file can contain any combination of graphics, images and text, being therefore the most versatile format currently available. The main advantage of PDF for exporting figures from an application like GENEVESTIGATOR® is that it is vector-based and not pixel-based. For example, a line is stored as a stretch with start and end points, color, and thickness. In an image format such as JPEG, GIF or PNG, a line does not explicitly exist, it simply appears as a line because the pixels along this line possess the same color, but basically, they are “independent” (Figure 6.2).

![Comparison of the PNG and PDF formats for exporting figures. Since browsers cannot interpret PDF format directly, and PNG uses high compression, PNG is best suited for websites or in a presentation. PDF is the preferred choice for print publications.](image)

**Figure 6.2:** Comparison of the PNG and PDF formats for exporting figures. Since browsers cannot interpret PDF format directly, and PNG uses high compression, PNG is best suited for websites or in a presentation. PDF is the preferred choice for print publications.

Working with figures in PNG

PNG (Portable Networks Graphics) is a bitmap image format that uses lossless data compression. PNG performs better and now often replaces the older but more familiar GIF format. PNG supports palette-based, grayscale and RGB images, but not CMYK. Its lossless data compression allows you to open, edit, and re-save the image without loss of quality (in contrast, for example, to JPEG, which deteriorates the image after re-saving). Most browsers support the format and correctly render PNG image files.

6.3 Exporting data from figures

This feature is only available if you have a **Professional** or **Enterprise** license of GENEVESTIGATOR®. It is not available in trial licenses.

In every tool, you can export the expression data underlying a graph using the “Export data” function in the “Results” menu. The results can be exported to three different file types: Microsoft Excel (*.xlsx), comma-separated values (*.csv) or tab-delimited (*.txt). The latter two formats can be directly read in spreadsheet applications like Microsoft Excel, Open Office Calc and in most statistical analysis software. This feature is ideal if you want to use separate software for creating the figures based on the same data. In the export dialogue, you can choose which gene aliases or descriptions should be added to the signal data in the export.

Note: Several spreadsheet software products, such as Microsoft Excel, allow a smaller number of columns (e.g., max. 256 columns in some older Excel versions) than lines (e.g., max. 65,000 lines in older Excel versions). Depending on the data size you want to export, please check how many genes and conditions are being exported and choose the layout accordingly so that you can open it in your preferred spreadsheet application.
6.4 Exporting entire studies or data compendia

This feature is only available if you have an Enterprise license of GENEVESTIGATOR®. It allows you to export all data from one or multiple studies, or all data behind a selection you have created in GENEVESTIGATOR®, such as all blood samples across all studies, together with the metadata of each sample.

In the Datasets menu, the first two items are to export either the metadata ("Annotations") or the expression levels ("Signals") of the data selection that is currently in focus. There is no limit on the number of studies or samples that are combined. The data matrix is exported as flat file.

The “Single Experiment (GSE)…” item is to choose and export a single experiment/study and export both metadata and expression values in the GSE format. This allows you to import it into any other tool that has a GEO importer feature. The difference with importing the same study directly from GEO is that the metadata has been curated and enriched by NEBION, and the signal values have been quality controlled and processed using our pipeline and are comparable to any other data exported from GENEVESTIGATOR®.

The “Current Selection” menu item allows to export the data matrix for your current Data and Gene Selections, either in tab-delimited or *.csv format or in *.gedata format to be imported into the Qlucore Omics Explorer software.
Chapter 7
Expression quantification for microarray data

The decision of which algorithm to choose for expression quantification depends, besides statistical considerations, on several factors such as experimental design, number of arrays to analyze, diversity of tissue types being analyzed, and type of data integration. The GENEVESTIGATOR® database hosts data from a variety of platforms and a wide variety of experimental conditions and designs. The GENEVESTIGATOR® Team has extensively tested a variety of methods and has chosen robust methods that best fulfill the requirements of single- and multi-experiment analysis.

7.1 Meaning of expression values

Expression values from microarrays are unitless values that allow comparison between the expression level of a given gene in one sample to the expression level of this gene in another sample, and to a limited degree between the expression levels of two different genes in the same sample. The absolute value for the expression of a gene does not generally have a meaning. In GENEVESTIGATOR®, the expression values are scaled such that the trimmed mean of all expression values in each experiment equals 1000.

7.2 Data preprocessing and normalization in GENEVESTIGATOR®

GENEVESTIGATOR® does not consist of a mere collection of curated experiments analyzed individually. The essence of GENEVESTIGATOR® is to integrate all experiments and perform meta-analysis across thousands of experiments.

We therefore looked for a method delivering:

1. Robust values for comparisons within individual experiments.
   o precise differential expression analysis between groups of experimental variables
   o compare absolute expression values between samples

2. Approximate values for comparisons between experiments.
   o aggregate data across all experiments to generate meta-profiles for anatomy or cancer
   o roughly compare absolute expression values across different studies to find those with extreme expression values (+/- 5% is acceptable for this purpose)

After intensively testing several methods, we decided to use a two-step approach:

1. STEP 1: a quantile normalization for samples from a given experiment and, subsequently,
2. STEP 2: a global scaling scheme for adjusting between experiments.

STEP 1: raw data is processed using quantile normalization as, e.g., implemented for RMA in Bioconductor:
STEP 2: **global scaling** of the trimmed mean (10% trim of all values of a chosen experiment) to a common target value (=1000):

For Affymetrix data, the Bioconductor (Gentleman et al., [10]) package 'affy' and a customized version of the package 'affyExtensions' with standard parameter settings is used. The RMA preprocessing is applied to the microarrays of each study separately. Regarding the second step, the trimmed mean consists of calculating the mean of all expression values of an experiment (across all samples) after excluding the top 5% and bottom 5% values.

Note: until April 2011, GENEVESTIGATOR® Affymetrix data were normalized using MAS5 with a target value of 1000. Earlier publications from GENEVESTIGATOR® data must be interpreted accordingly.

For Agilent and Illumina BeadChip data, standard quantile normalization methods are used. Specifically, we use the algorithms from the Bioconductor limma package [15].
Chapter 8
Expression quantification for RNA sequencing data

8.1 Meaning of expression values

RNA sequencing is a method for measuring gene expression. For more information please visit: http://rnaseq.uoregon.edu

Gene expression is measured in TPM (transcript per million). The TPM of a gene or an isoform is the expected number of mRNA transcribed from this gene or isoform in a hypothetical sample of 1 million mRNA. The TPM measure is inherently relative to the total number of transcripts in a sample and therefore values between different samples are comparable (to the extent that the assumption holds that the total number of transcripts is the same across samples).

In GENEVESTIGATOR®, while expression is measured in TPM, behind the scene, the number of reads mapping to each transcript (expected counts) is also used to quantify the uncertainty of measurement.

8.2 Trimming

Read libraries are trimmed to remove adapters from the reads using Trimmomatic (Bolger et al., [11]) but are not trimmed according to quality. Our use of sophisticated mappers is usually considered as making quality trimming obsolete (e.g., Williams et al., [12]).

8.3 Computation of expression values

TPM and expected counts are obtained by mapping libraries of reads to transcriptome references. Currently, two different methods are used to compute TPM and expected counts. For some species, alignments are calculated by Bowtie (Langmead et al., [13]) and used by RSEM (Li and Dewey, [14]) to compute both TPM and expected counts. For other species, TPM and expected counts are computed using Salmon in quasi-mapping-based mode. After comparing the performance of RSEM and Salmon, we are currently in the process of switching from using RSEM to using Salmon. While the RSEM-Bowtie pair is slightly more accurate than Salmon, it is much slower and tends to require more ad hoc adjustments.

8.4 Gene level and isoform level quantification

Both the expected count and the TPM are measures of expression for which the expression of a gene is simply the sum of the expression of its isoforms. In GENEVESTIGATOR®, gene expression is computed as a sum of isoform expressions. However, the expression of the isoforms is often not made available to the user. The reason is that it is sometimes much less accurate and stable than the expression of the associated genes. This is due to an instability in the estimation of the provenance of reads that are common subsequences of several isoforms of the same gene.
8.5 Single-cell RNA-Seq data in GENEVESTIGATOR®

Single-cell RNA-sequencing (scRNA-Seq) data can be visualized and analyzed in GENEVESTIGATOR® using the same tools, and basically in the same manner, as described for bulk RNA-Seq data. The data is available in one of two different formats depending on the type of library used: on the level of single cells (Smart-Seq), or the level of cell aggregates (droplet-based, 10x).

To make scRNA-Seq data accessible in GENEVESTIGATOR®, the raw data of each scRNA-Seq experiment needs to go through NEBION’s processing and curation pipeline, which comprises of three major steps: (1) data processing, (2) clustering and cell-type identification, and (3) biocuration of study and sample characteristics. At the end of the pipeline, the scRNA-Seq data is quality-controlled, has accurately allocated cell types, is enriched with metadata, and is principally ready for analysis in GENEVESTIGATOR®. To reduce the complexity of the experiments generated using droplet-based (10x) libraries, individual cells from those experiments will additionally be grouped into aggregates. In other words, cells from the same biosample that have the same cell type and cell state will be grouped into one aggregate. So, while on the single-cell level platforms in GENEVESTIGATOR® one sample represents one cell, on the aggregate-level platforms one sample represents one aggregate of cells having common attributes. Detailed information on the composition of each aggregate is available in the tooltip.

The calculation of the expression values of the aggregates is done in three consecutive steps. (1) The raw sequencing data are processed on a cell level. (2) Starting from the read counts per cell, we are then summing up the counts per aggregate for each gene. (3) Finally, we calculate the TPM from the counts, and we obtain one expression value per measured gene for a group of cells. Here the TPM are actually counts per million, because in 10x libraries there is no length normalization, because transcripts of different lengths like titin and actin will produce reads/ mRNA molecules at similar rates.
Chapter 9
Quality control

9.1 General principles and goal

Since the experiments from GENEVESTIGATOR® were performed in many different labs, NEBION has no control over experimental design, sample preparation and processing. Therefore, we ensure the quality of the samples using quality control (QC) on the raw data and the expertise of the curators.

An extensive QC analysis is performed for every experiment to detect samples of questionable quality. Often, there are no fixed thresholds to qualify samples as problematic, because parameter ranges can vary between organisms and platforms.

Major differences in QC statistics can occur between organisms and sometimes between microarray platforms. Therefore, besides understanding the meaning of each of the QC plots, probably the most critical point is that the results should be consistent between samples from a given experiment, especially between biological and/or technical replicates, and an experiment from a given organism and platform should be consistent with other experiments from the same organism and platform.

For microarray technology, the GENEVESTIGATOR® QC analysis is performed with the R statistical analysis software using a variety of packages and libraries. For Affymetrix data, we use customized versions of the simpleaffy and affyQCReport packages (and depending packages) from Bioconductor. For Agilent and Illumina arrays, we created QC reports using various Bioconductor libraries.

The GENEVESTIGATOR® QC pipeline includes several probe-level analyses which serve to check signal intensity and variance. RNA degradation, border elements, and array-to-array correlation. Samples are excluded from GENEVESTIGATOR® if the QC shows unusually high variance, high RNA degradation levels, overall low signal levels (poor hybridization or labelling), or if a sample shows poor correlation with replicate samples from the same experimental condition.

For RNA sequencing, the GENEVESTIGATOR® QC analysis is performed with FastQC and custom scripts displaying information using the output of RSEM.

The GENEVESTIGATOR® QC pipeline includes graphs obtained from FastQC giving information about reads and their quality. The pipeline also includes a sequence of plots describing how the reads map to the reference transcriptome, the distribution of expression among transcripts, and correlation of expression between samples.


References


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