Evaluation of RT-PCR data using

 ***SUMO*** 

Christian Schwager, 2013, Christian.Schwager@Med.Uni-Heidelberg.de

Evaluation of RT-PCR using

A (extremely) short introduction to RT-PCR

CT-value

Import ct-values => Expression matrix

Edit the header / data

Analyze / view your data

dCT-Calculation

ddCT – Calculation

Christian Schwager, 2013, Christian.Schwager@Med.Uni-Heidelberg.de

PCR

- run certain number of cycles (e.g. 30)
- analyse amplified product e.g.on agarose gel

Problem: some gene is already amplified, some other gene not vsibile yet
Solution: take aliquots after certain number of cycles (e.g. 10,15,20,25) and analyze

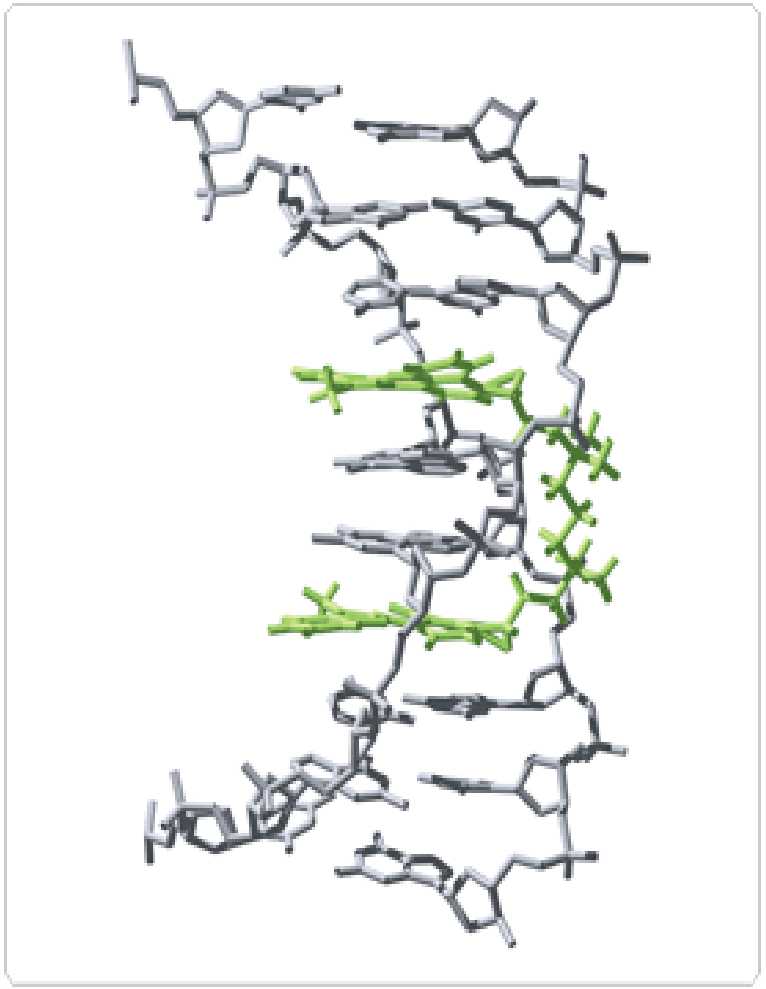
RT-PCR

Continuously monitor amount of synthesized dsDNA
- find when „exactly“a certain „amount“ of dsDNA is synthesized for each sample
- monitor dynamics
- direct access to quantitative data

Online detection - fluorescence measurment

Sybr green:

- doesn't fluoresce by itsself
- intercalates into dsDNA => now it emits



Toto-1 dye, ABI

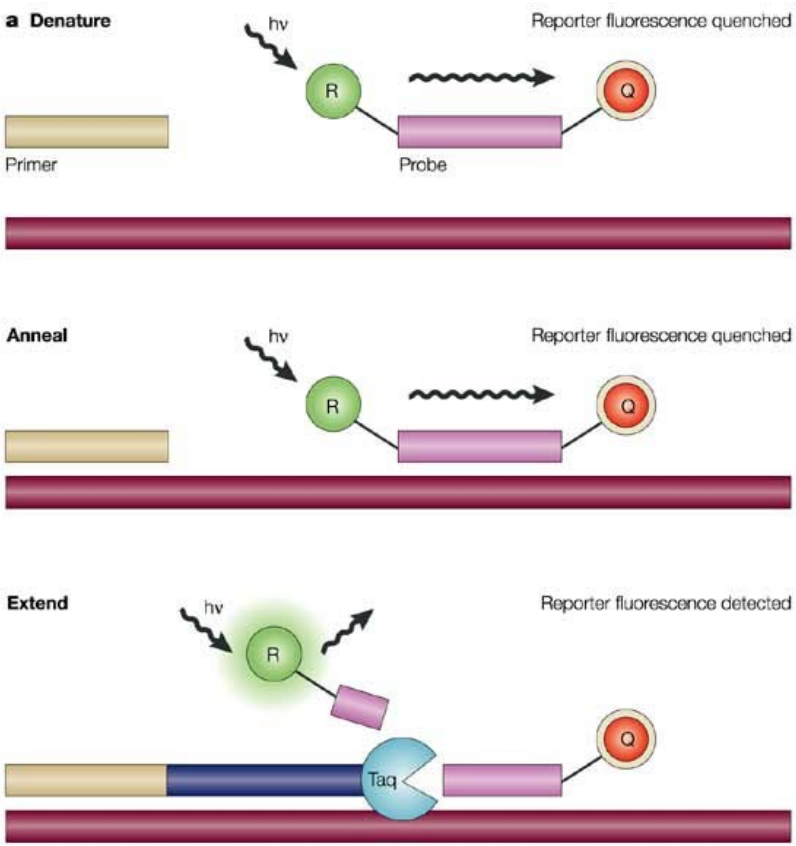
Advantage:
- simple
- cheap

Problem:
- stain any kind of dsDNA
- length dependant – 2xPCR product length mimiques 2x higher expression
- sequence dependant ??

Online detection - fluorescence measurement

TaqMan

- 3rd reporter primer with Fluorophor and Quencher in close proximity, binds in between PCR primers
- reporter primer doesn't fluororesce – quencher internally „digests“ light absorbed by fluorophor
- during second strand sysnthesis, polymerase will digest reporter primer sitting in its way
- quencher gets divided from fluorophor
- fluorophor can emit flurescent light



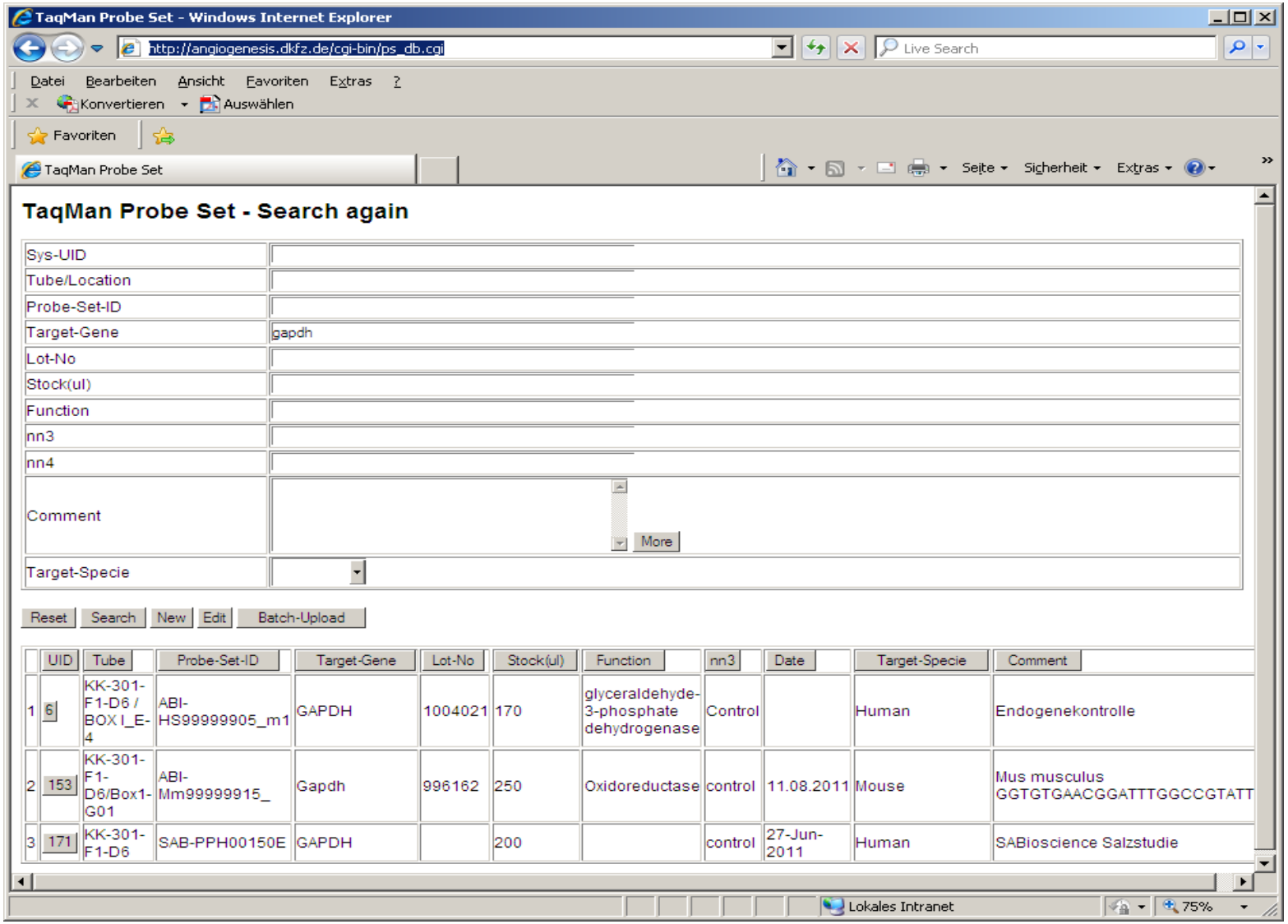
Koch, Nature Reviews Drug Discovery (2004)

Advantage:
- signal direct correponding to synthesized copy number
- higher specificity: 3 primer have to bind in the correct order
- optimized probe sets for nearly all known genes

Problem:
- expensive

How to find TaqMan probesets:

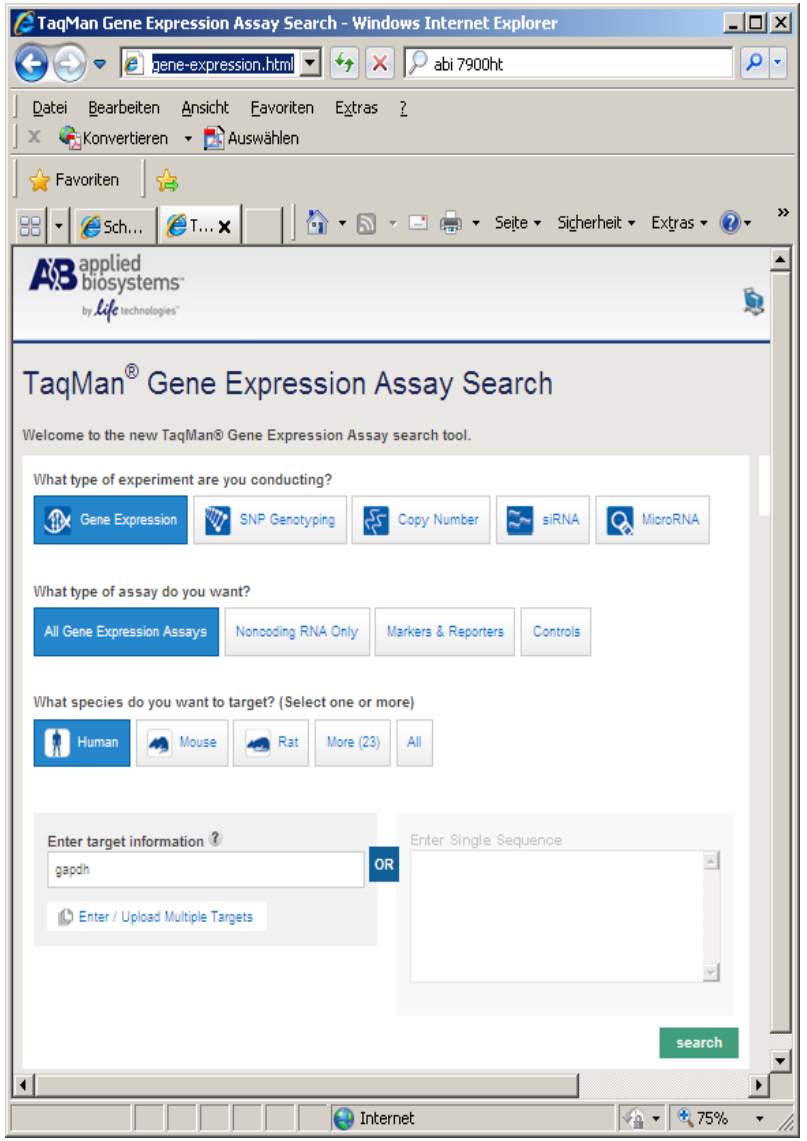
Search our local database: http://...../cgi-bin/ps_db.cgi

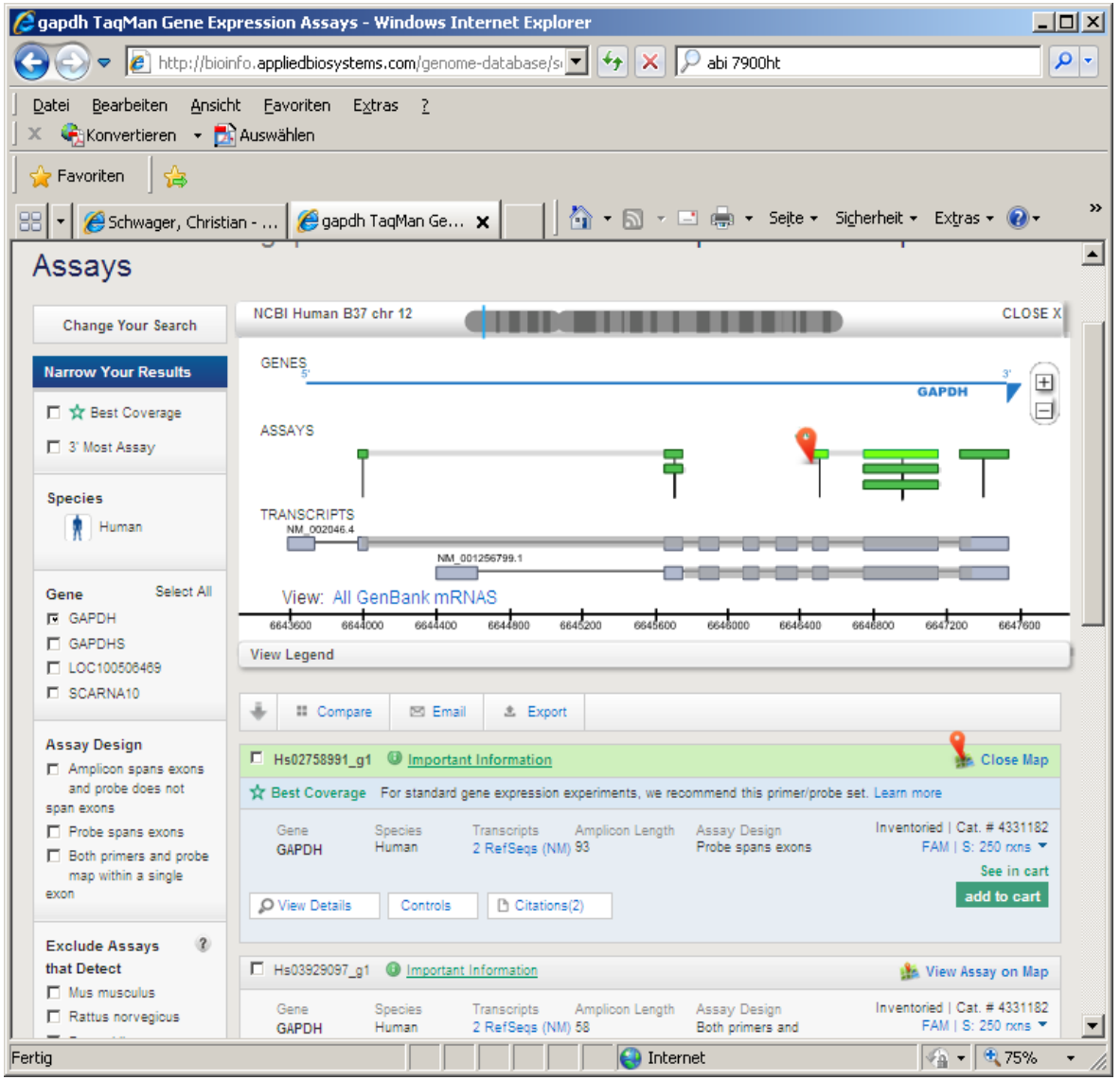


Christian Schwager, 2013, Christian.Schwager@Med.Uni-Heidelberg.de

Search ABI site and order new probesets:

http://bioinfo.appliedbiosystems.com/genome-database/gene-expression.html





Search ==> select best Match, order

Christian Schwager, 2013, Christian.Schwager@Med.Uni-Heidelberg.de

RT-PCR intstruments

Light-Cycler - Roche

- rotor design
- fast heating / cooling with fan

RT-PCR intstruments

ABI 7900HT

- standard MTP design (96/384-well)
- heating with thermostated metal plate

- extend with auto loader for high throughput operation

Christian Schwager, 2013, Christian.Schwager@Med.Uni-Heidelberg.de

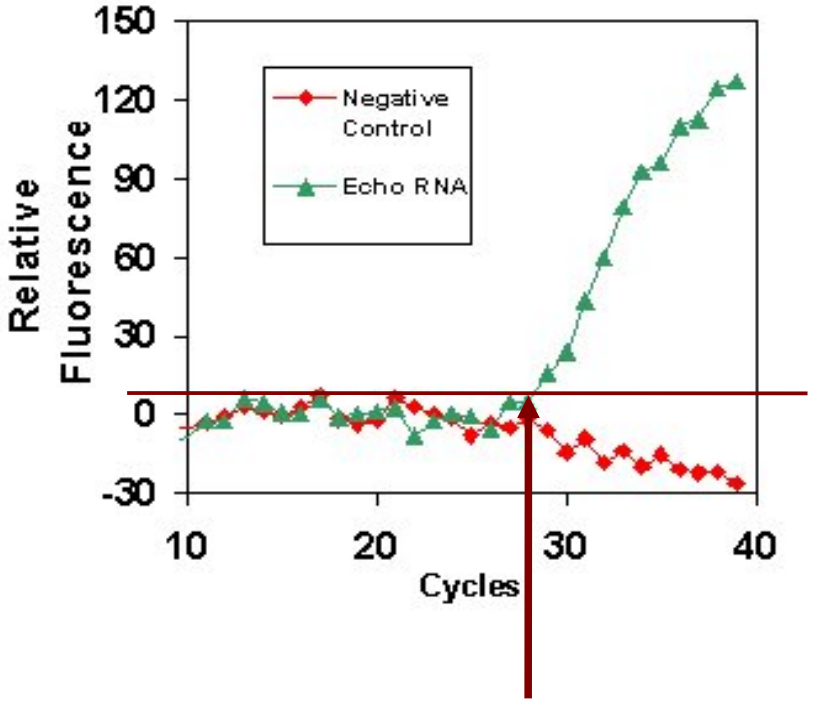
Evaluation - CT value

- measure fluorescence

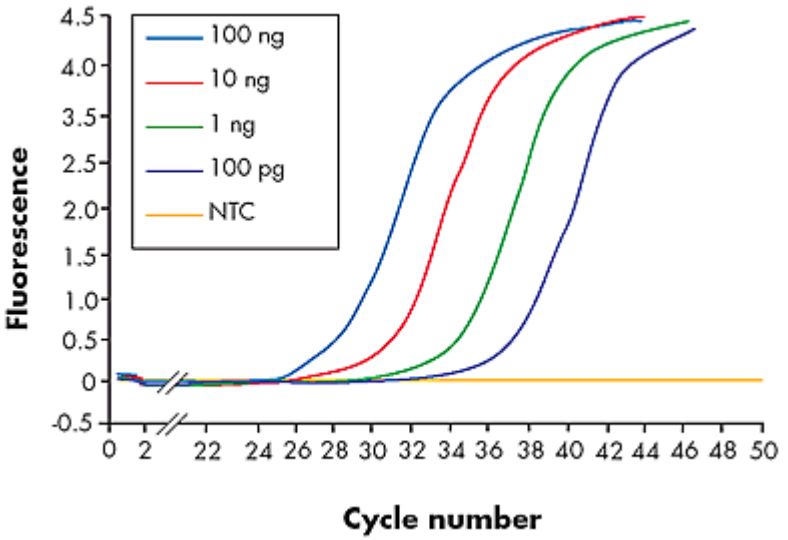
- define background level and noise at first few cylces => define threshold

- as soon as flourescence larger threshold => CT (Cycle at which Threshold crossed)

- CT gives the number of PCR cycles required to generate the same amount of PCR product



Important:
- as higher the CT
- as more cycles required to synthesize a given Amount of DNA
- as less starting DNA
- CT is ~log2:
  10 cycles difference
  => 2^10 => ~1000 times less starting material



Christian Schwager, 2013, Christian.Schwager@Med.Uni-Heidelberg.de

CT-values

As more cycles as higher the probaility you are amplifying also artifacts
- contaminating RNAs / DNAs
- unspecific hybridisations
- primer / primer bindings
…

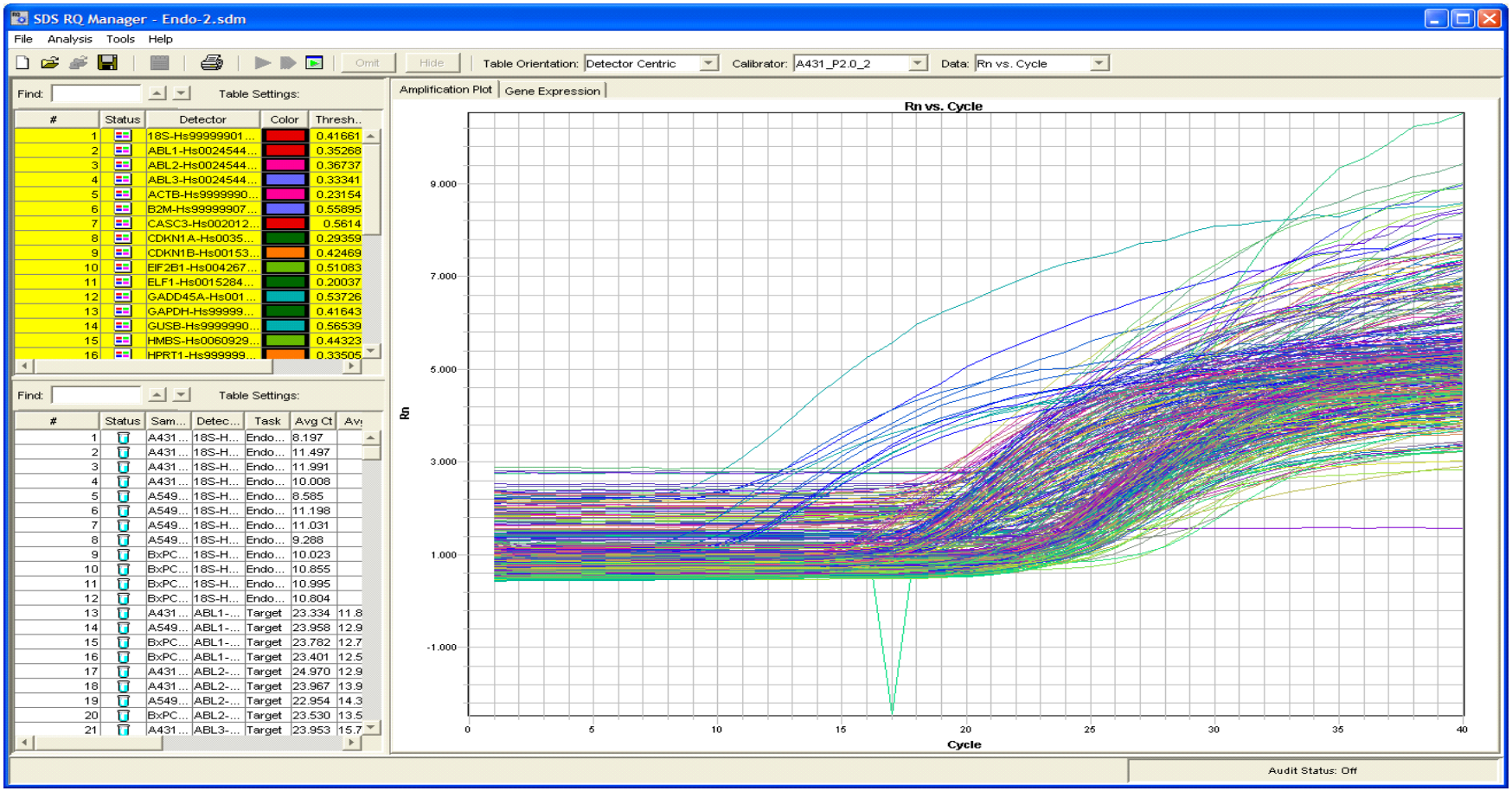
cDNAs with CT>36 are probably meaningless
Don't use them for quantification, only information „very low signal“)

CT of 36 means you need to generate 2^36 ~ 60 000 000 000 copies of your original whatever !!

miRNAs with CT>33 are probably meaningless

Compute CT-values

ABI SDS software for a single plate
ABI RQ-Manager for multiple plates (up to 10)
- export **Amplification data** as text files
- convert with ***SUMO*** into expression matrix, which you can evaluate with ***SUMO*** or any other software.



Christian Schwager, 2013, Christian.Schwager@Med.Uni-Heidelberg.de

Import ct-values => Expression matrix

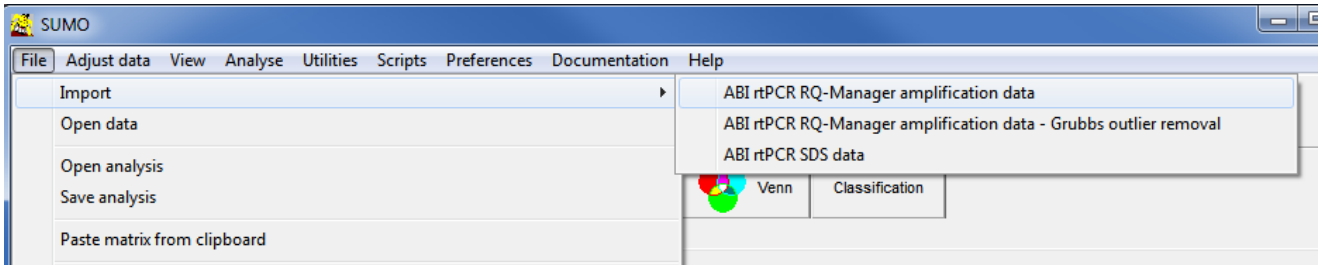
Import raw CT-values from RQ-manager exported „amplification data“

SUMO

File

Import

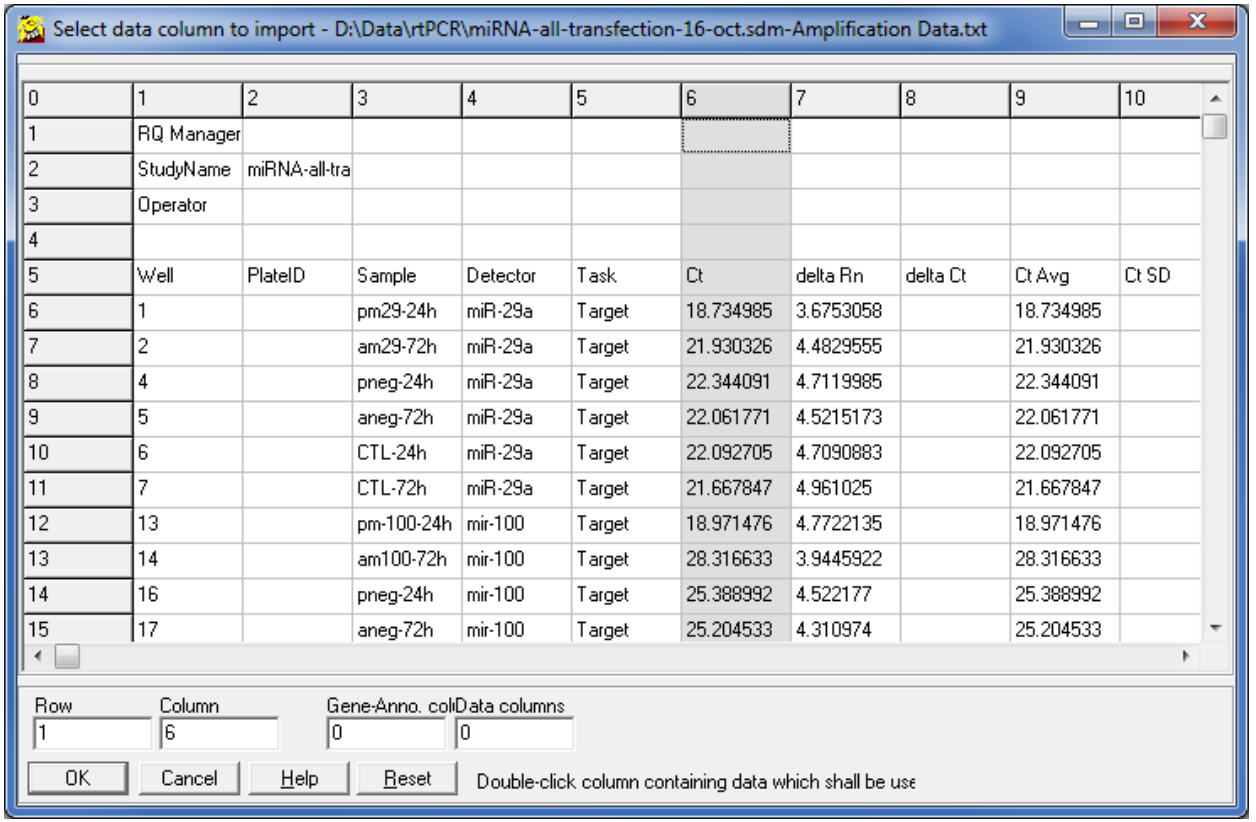
ABI rt-Amplification data



Import ct-values

From the file dialog select one (or multiple) exported amplification data files.

A preview shows a part of the first selectd data file:



0	1	2	3	4	5	6	7	8	9	10
1	RQ Manager									
2	StudyName	miRNA-all-tra								
3	Operator									
4										
5	Well	PlateID	Sample	Detector	Task	Ct	delta Rn	delta Ct	Ct Avg	Ct SD
6	1		pm29-24h	miR-29a	Target	18.734985	3.6753058		18.734985	
7	2		am29-72h	miR-29a	Target	21.930326	4.4829555		21.930326	
8	4		pneg-24h	miR-29a	Target	22.344091	4.7119985		22.344091	
9	5		aneg-72h	miR-29a	Target	22.061771	4.5215173		22.061771	
10	6		CTL-24h	miR-29a	Target	22.092705	4.7090883		22.092705	
11	7		CTL-72h	miR-29a	Target	21.667847	4.961025		21.667847	
12	13		pm-100-24h	mir-100	Target	18.971476	4.7722135		18.971476	
13	14		am100-72h	mir-100	Target	28.316633	3.9445922		28.316633	
14	16		pneg-24h	mir-100	Target	25.388992	4.522177		25.388992	
15	17		aneg-72h	mir-100	Target	25.204533	4.310974		25.204533	

Verify:

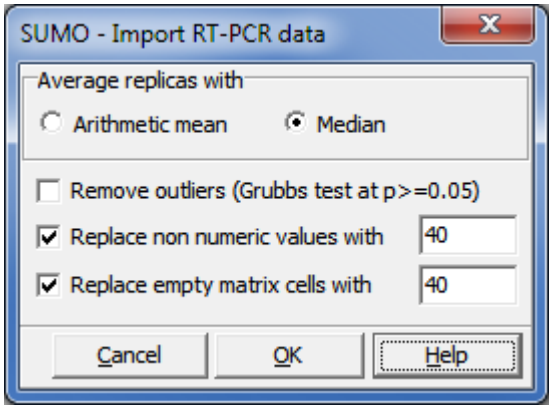
- The selected data file(s) have the correct structure
- The CT column is grayed

Click OK-button to import the data into ***SUMO***

Christian Schwager, 2013, Christian.Schwager@Med.Uni-Heidelberg.de

Import ct-values

A parameter dialog opens up:



Average replicas: *SUMO* detects replicates (i.e. IDENTICAL Gene AND sample name).
Define how to average replicas:

- Arithmetic mean
- Median mean (less sensitive to outliers, recommended)

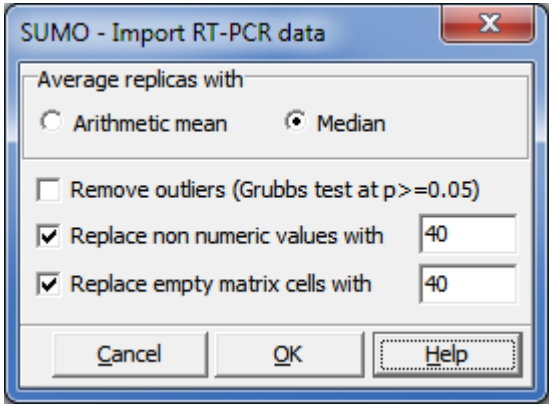
Remove outliers:
Try to detect obvious outliers, applying the standard statistical Grubbs test.
Only use ful for larger replica numbers (>=5)

Replace non numeric values:
RQ-manager exports „not detected“ for each PCR where threshold value was never crossed.
But with „not detected“ you can‘t calculate nor generate graphs – it is not a number.
For most subsequent tasks, ***SUMO*** will ignore „non numerical values“.
Another solution could be to set the „not detected“ to the maximum number of PCR cycles (as defined on the cycler, in our case most often 40).

Christian Schwager, 2013, Christian.Schwager@Med.Uni-Heidelberg.de

Import ct-values

A parameter dialog open up:



Replace empty matrix cells:
In case you have mixed samples with different probesets (e.g.samples S1..S4 with probesets P1..P3, and samples S5..S8 with probesets P4..P6).
The way SUMO will build the expression matrix, it will leave the complementary matrix cells Empty.

	S1	**S2**	**S3**	**S4**	**S5**	**S6**	**S7**	**S8**
P1	21	22	21	22	NAN	NAN	NAN	NAN
P2	22	23	22	23	NAN	NAN	NAN	NAN
P3	30	31	32	31	NAN	NAN	NAN	NAN
P4	NAN	NAN	NAN	NAN	24	25	24	25
P5	NAN	NAN	NAN	NAN	28	29	28	29
P6	NAN	NAN	NAN	NAN	27	26	25	26

It may be useful to replace such values with a fixed value (e.g. 40).

Christian Schwager, 2013, Christian.Schwager@Med.Uni-Heidelberg.de

Import ct-values

SUMO analyzes the data files and builds an „Expression matrix“:

- Find all measurements where RQ-manager didn‘t export a number
  (all samples where threshold never reached or crossed the threshold value)
  Optionally replace the non-numeric values by e.g. 40, the theortically highest ct value
  in an RT-PCR run with 40 cycles)

- Seach all **unique** sample names:
  „Sample1“ is the same as „sample1“
  „sample1“ is not „sample-1“ nor „sample_1“ nor „sample 1“

- Search all **unique** gene names

- Build a matrix with all **unique samples in columns** x all unique genes in rows

- Sort all ct-values into the corresponding cells (unique sample / gene)
- Sort all rn-values into the corresponding cells (unique sample / gene)

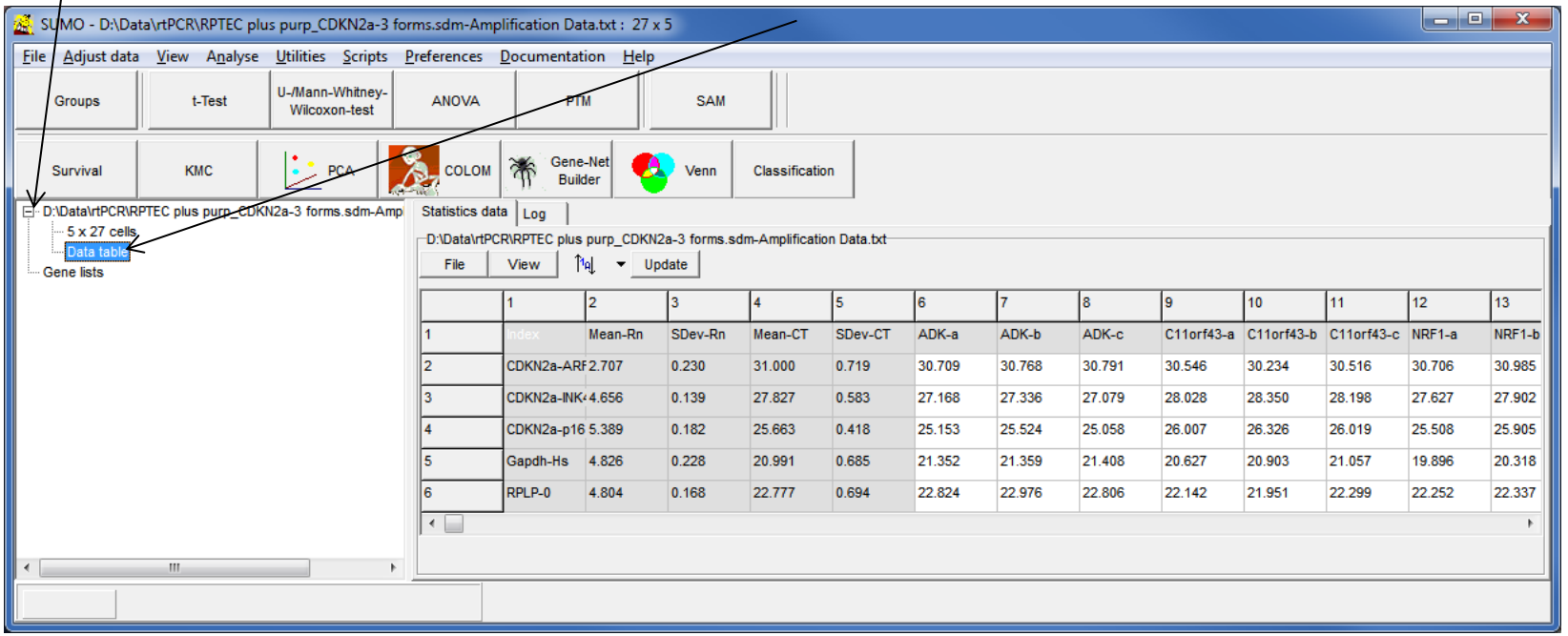
- Average all ct values/rn-values in each cell (e.g. technical replicas with always the same name)

- Compute average and sdev from ct / rn values accross all samples for each gene and add 4 columns to the matrix
  - average ct may be used lateron to filter overall lowly expressed genes
  - average rn may be used lateron to identify genes with overall low fluorescent signal
    which sometimes may generate erraneous ct-values

Christian Schwager, 2013, Christian.Schwager@Med.Uni-Heidelberg.de

Preview the generated matrix (optional):

Open the **experiment tree** and click **Data table** node



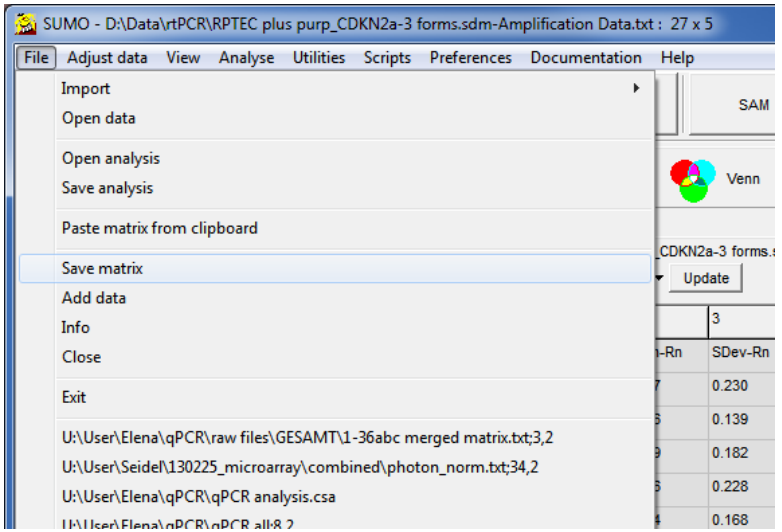
	1	2	3	4	5	6	7	8	9	10	11	12	13
1	Index	Mean-Rn	SDev-Rn	Mean-CT	SDev-CT	ADK-a	ADK-b	ADK-c	C11orf43-a	C11orf43-b	C11orf43-c	NRF1-a	NRF1-b
2	CDKN2a-ARF	2.707	0.230	31.000	0.719	30.709	30.768	30.791	30.546	30.234	30.516	30.706	30.985
3	CDKN2a-INK4	4.656	0.139	27.827	0.583	27.168	27.336	27.079	28.028	28.350	28.198	27.627	27.902
4	CDKN2a-p16	5.389	0.182	25.663	0.418	25.153	25.524	25.058	26.007	26.326	26.019	25.508	25.905
5	Gapdh-Hs	4.826	0.228	20.991	0.685	21.352	21.359	21.408	20.627	20.903	21.057	19.896	20.318
6	RPLP-0	4.804	0.168	22.777	0.694	22.824	22.976	22.806	22.142	21.951	22.299	22.252	22.337

Export the just generated matrix:

SUMO

File

Save matrix



Christian Schwager, 2013, Christian.Schwager@Med.Uni-Heidelberg.de

Edit the header – Sample annotations (optional):

It may be useful to have additional header rows containing treatment details:
- Cell type (fibroblasts, endothelial, a549, …)
- Phenotype (healthy, primary cancer, recurrent, metastasis, …)
- Kind of treatment (drug1, drug2, ….)
- Dose (10mg, 20mg, …)
- …

This may help lateron to (auto-) group the samples for statistic tests / graphs

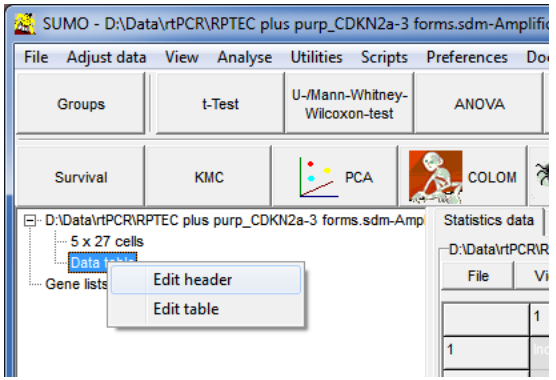
SUMO

Experiment tree

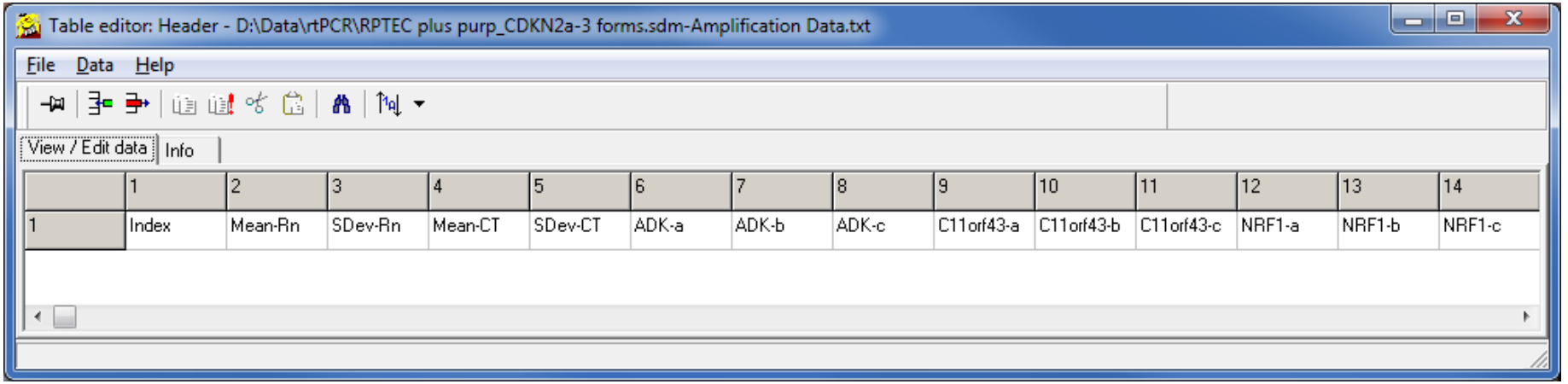
Data table

Context menu

Edit header



A basic table editor opens-up:



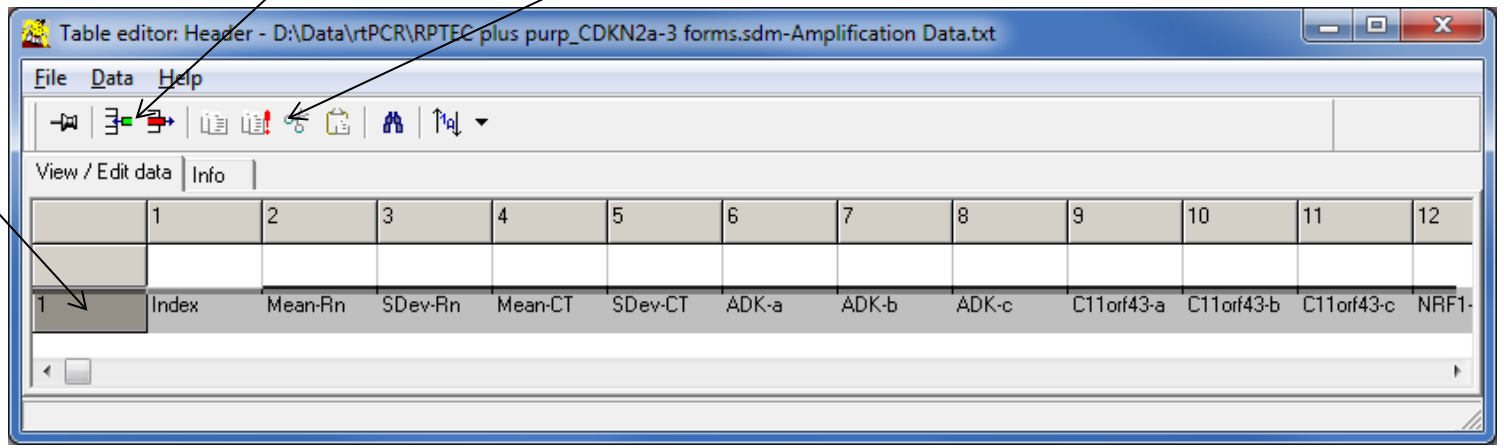
Christian Schwager, 2013, Christian.Schwager@Med.Uni-Heidelberg.de

Edit the header – Sample annotations (optional):

Select a line by clicking the row header:

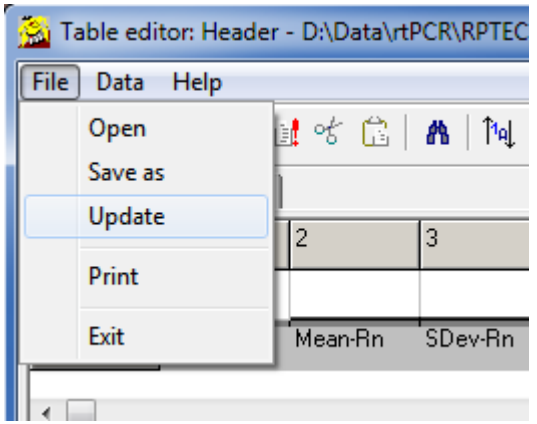
Insert / delete rows

Copy / Paste rows



Edit the contents of any header cell, any way you like.

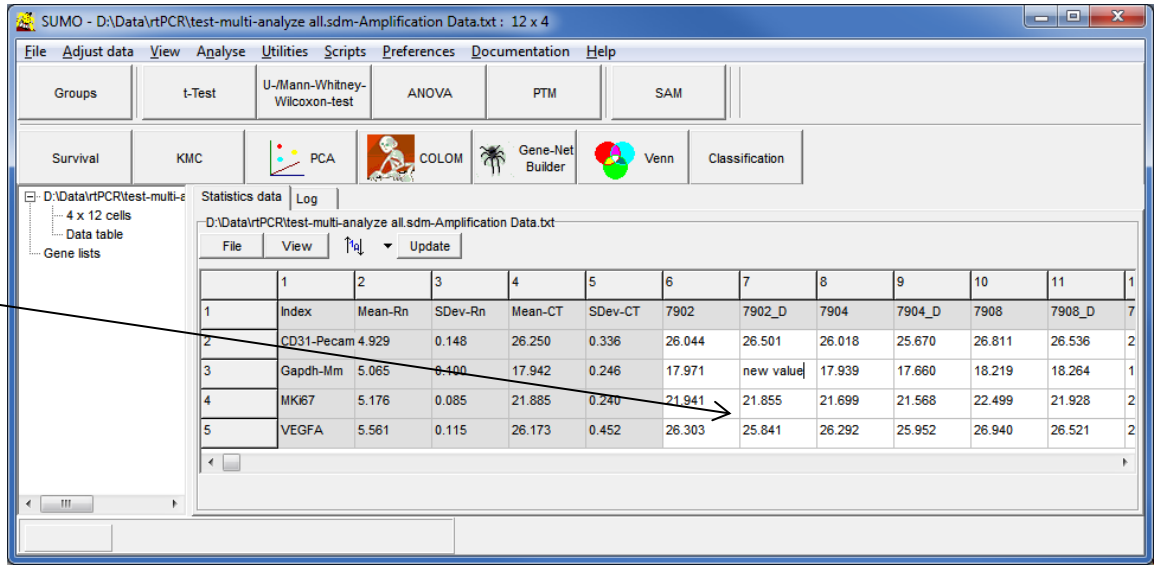
When done **update** the data matrix into ***SUMO***:



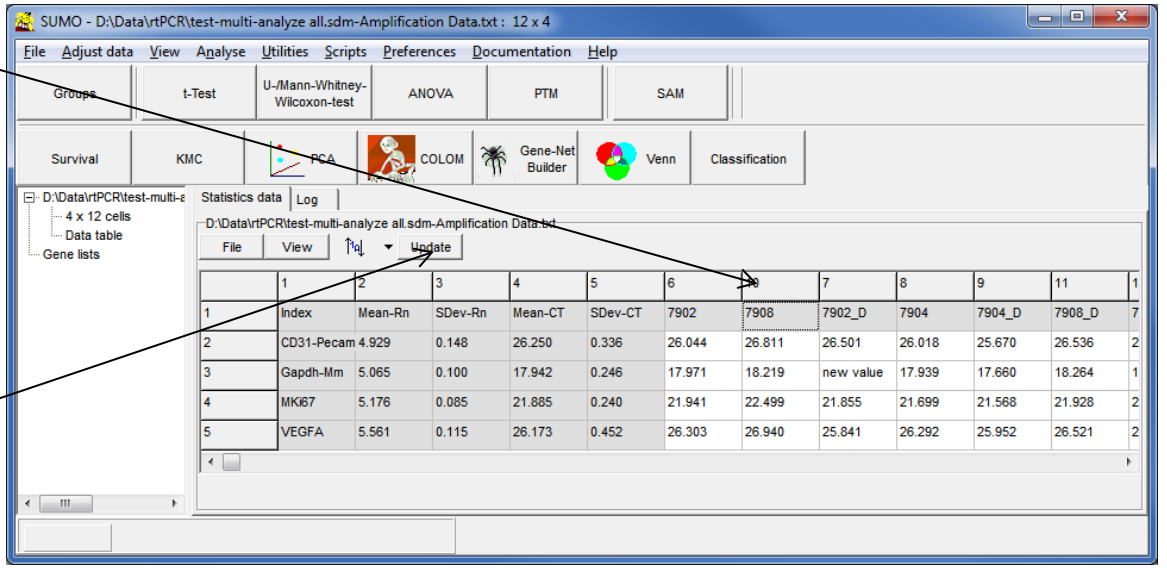
Christian Schwager, 2013, Christian.Schwager@Med.Uni-Heidelberg.de

Edit the data (optional):

- Change any
  data annotation / matrix cell



- Rearrange data colums to sort samples in any convenient order:
  Click into a **column header cell**, and drag the seöected column wherever you like it



- Click **Update** button to save modifications to ***SUMO*** data matrix.

Christian Schwager, 2013, Christian.Schwager@Med.Uni-Heidelberg.de

Analyze / view your data

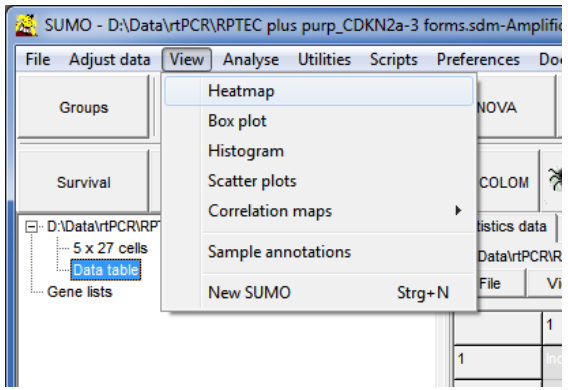
One picture is better then 1250 numbers – View the data as heatmap

- Black = low ct-value => higz abundance
- Bright red = high ct-value => low abundance

SUMO

View

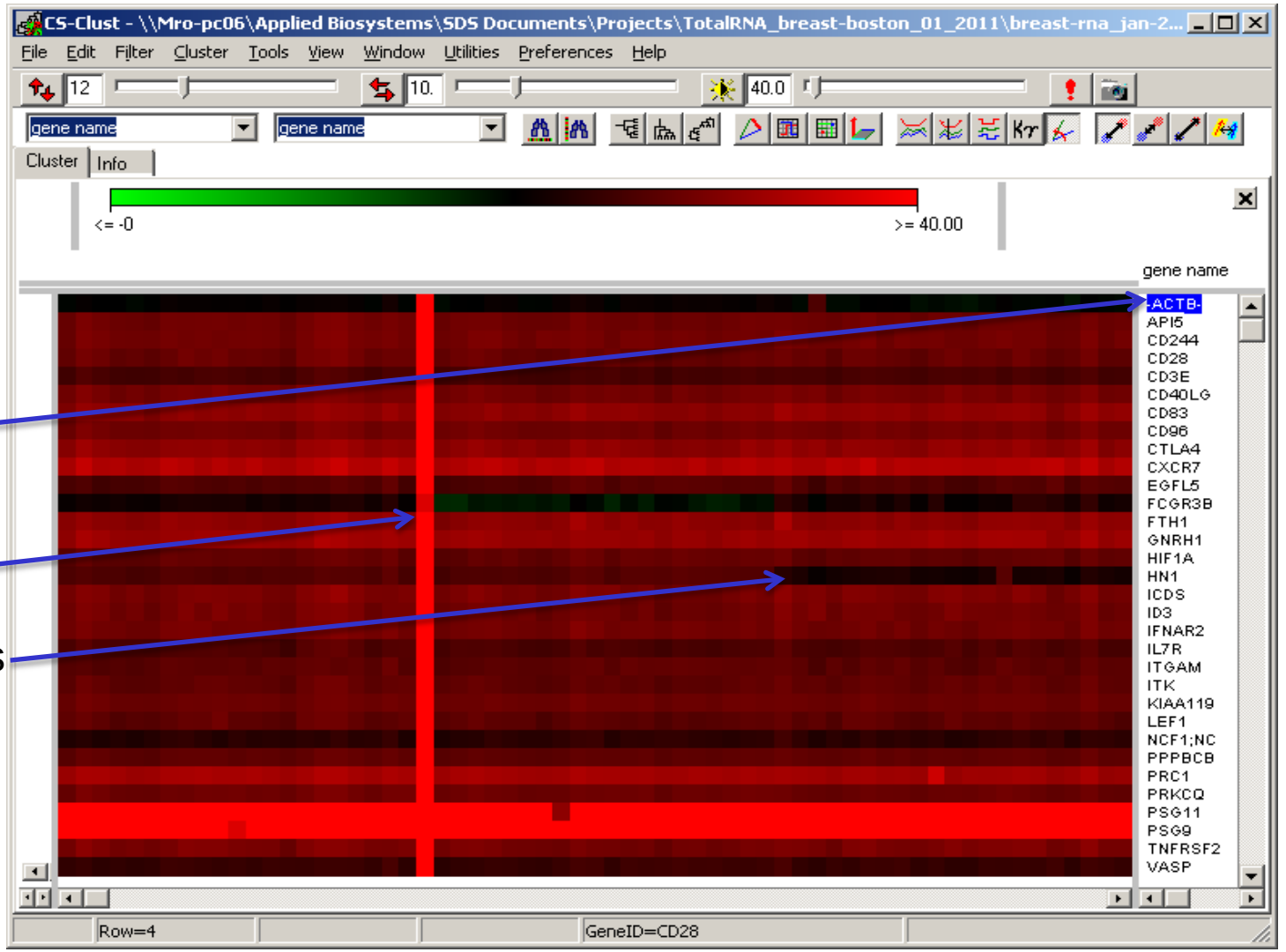
Heatmap



Look at **control genes**

Identify obvious outliers (genes or samples)

Identify interesting genes



Christian Schwager, 2013, Christian.Schwager@Med.Uni-Heidelberg.de

Endogenous controls

CT value is influenced by
- total number of grown cells
- efficiency of cell harvesting
- efficiency of RNA extraction
- RNA quality
  what if RNA is degraded in such a way, that one of the PCR primer sites is affected in one of the genes ?
- RNA concentration measurement
- pipetting accuray
- RT-PCR instrument

Use controls to estimate all effects and compensate them by normalisation
=> Control genes

Best would be a reference which is included in your sample
=> Endogenous controls

- Genes which are always expressed at high level, whatever the cell is doing
- Genes sitting deeply inside vital metabolic pathways

What if the pathway of your endogenous control gene is affected by your treatment?
You would normalize your regulation effect out.

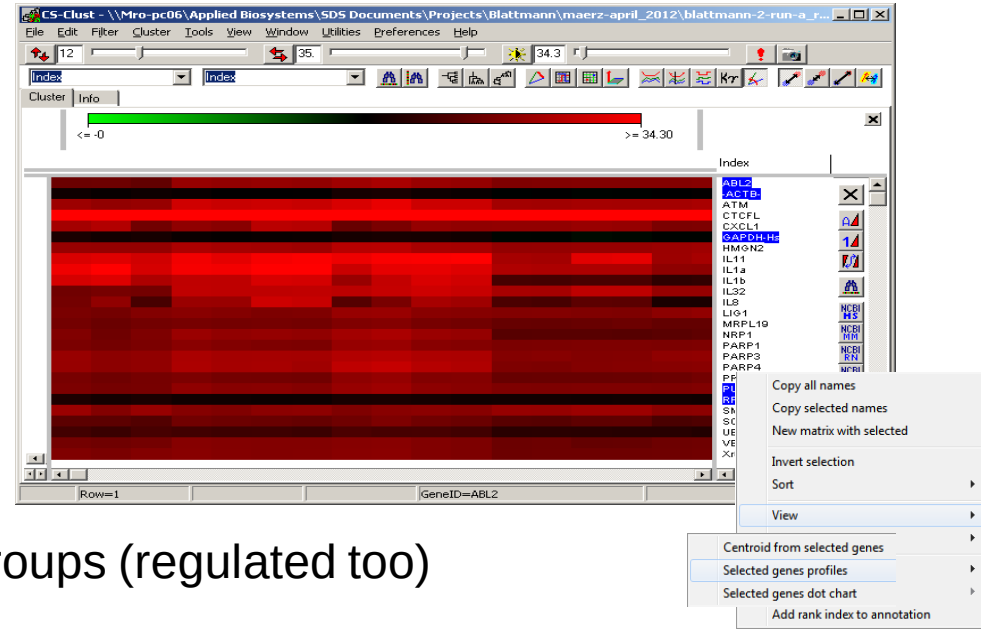
=> Multiple control genes, from different vital metabolic pathways

Christian Schwager, 2013, Christian.Schwager@Med.Uni-Heidelberg.de

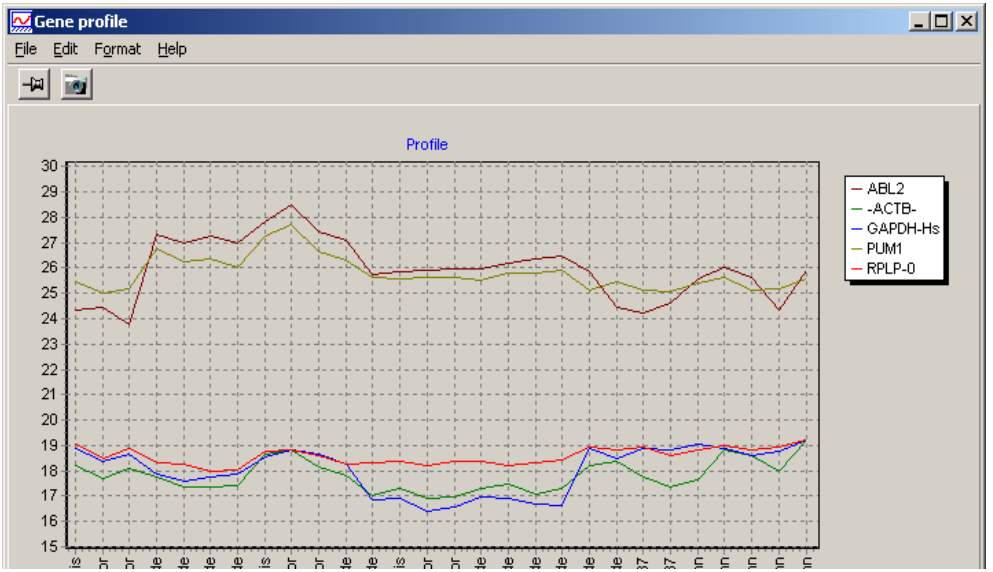
View endogenous controls

Use multiple endogenous control genes from different pathways

Gapdh - energy metabolism
Rpsxx / prlxx - protein synthese pathway
Hprt1 - salvage pathway (purines)
Actb
UBC
....



Review the controls and
- DON'T use obvious outliers
- Don‘t use controls which follow the treatment groups (regulated too)



Use average (mean or median) from controls to normalize your samples

Christian Schwager, 2013, Christian.Schwager@Med.Uni-Heidelberg.de

dCT-values – Normalization by endogenous controls:

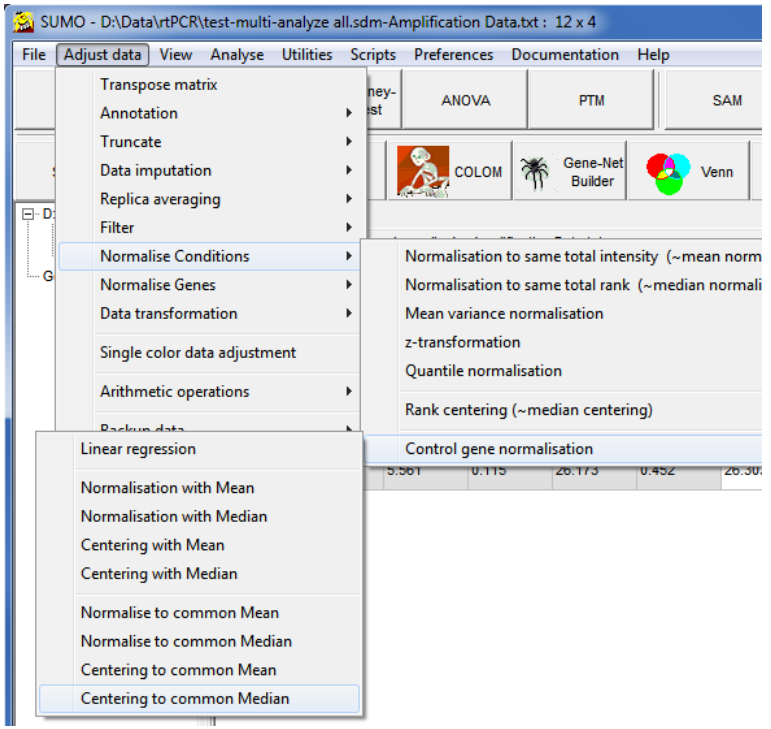
Select

SUMO

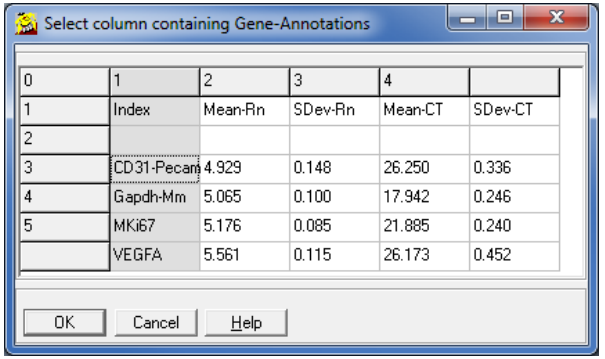
Adjust

Normalize conditons

Centering with / to common Mean /Median



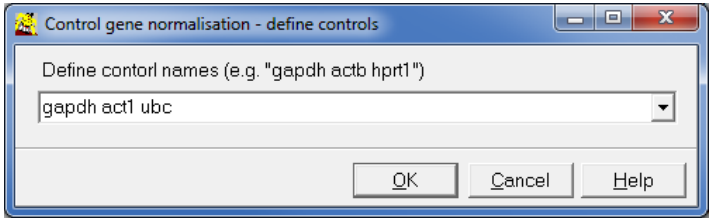
A table opens up, showing all „gene“ annotation columns.



0	1	2	3	4	
1	Index	Mean-Rn	SDev-Rn	Mean-CT	SDev-CT
2					
3	CD31-Pecam	4.929	0.148	26.250	0.336
4	Gapdh-Mm	5.065	0.100	17.942	0.246
5	MKi67	5.176	0.085	21.885	0.240
	VEGFA	5.561	0.115	26.173	0.452

Double click the column, which contains „gene names“

An input dialog opens up:



Define the names of control genes you want to use (partial but unique significant names)

Normalization – Centering ??

Your CT-values are ~log-2
(doubling of DNA per PCR-cycle)

Thus, normalization has to be done by SUBTRACTING (=centering) the respective reference value
(With linear intensities you would divide.)

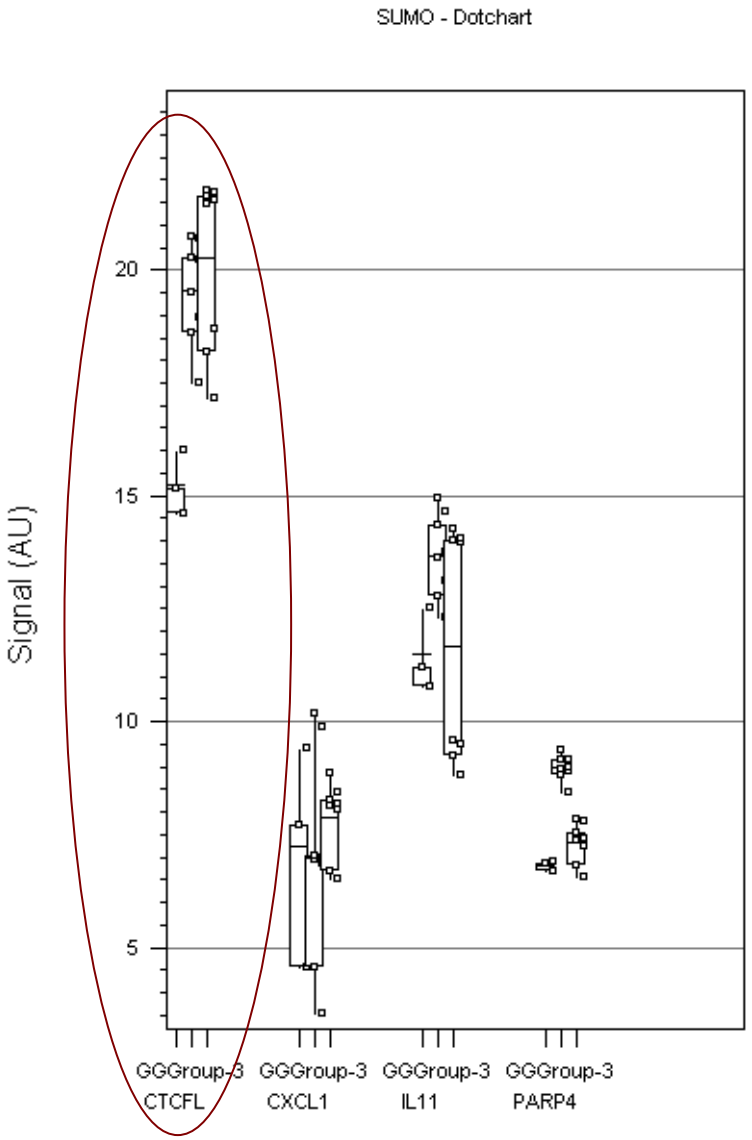
dCT-values – Normalization with endogenous controls:

For each individual sample,
- compute average ct from the respective control(s)
- Subtract this average from the genes to analyze
- => dCT

Now the, the dCT show relative CT-changes against the sample‘s control(s).
Information about averall ct level is lost.

Look at the example:

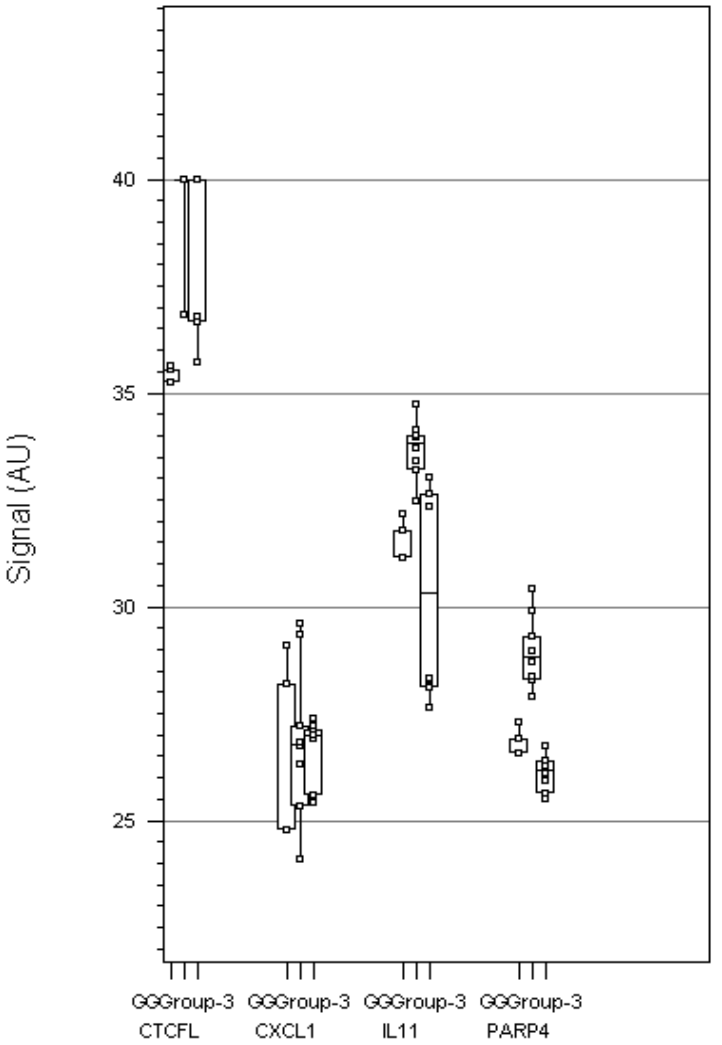
After centering **with** endo-controls,
most interesting gene seems to be CTCFL:
Largest ct-difference between groups !!



Instead of centering **with**, you could also center **to** the endo controls.

- Compute a grand average from **all** endo controls in **all** samples
- Shift all CT-values up/down in each sample for all tested genes in such a way, that the average of endo-controls in this sample will get the grand average
  (i.e. virtually dilute/concentrate your starting material to same amount, defined by controls)

- sample individual effects are removed
- overall information about expression is kept



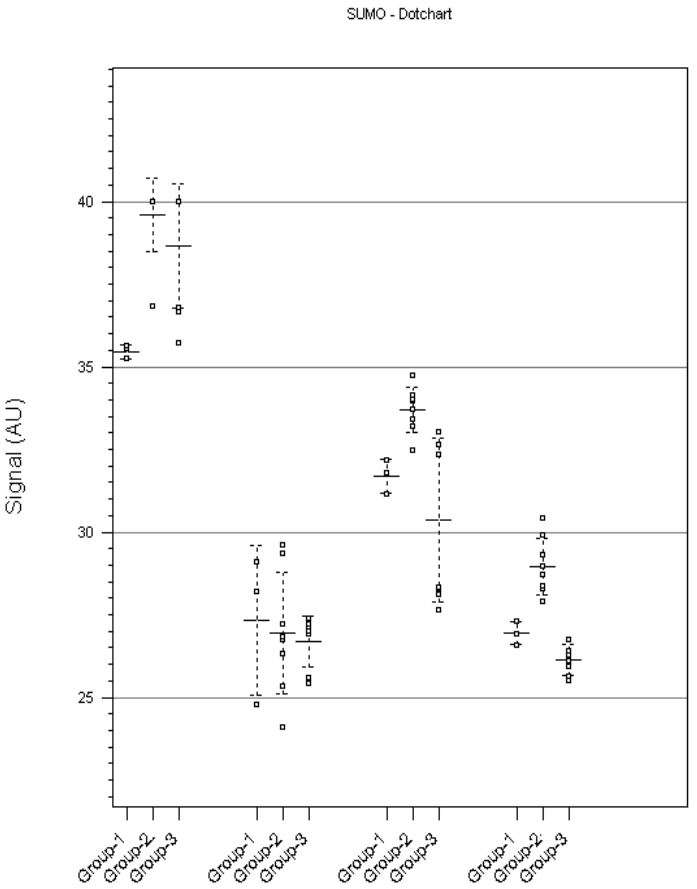
After centering **to** control, it is abvious: CTCFL is useless:

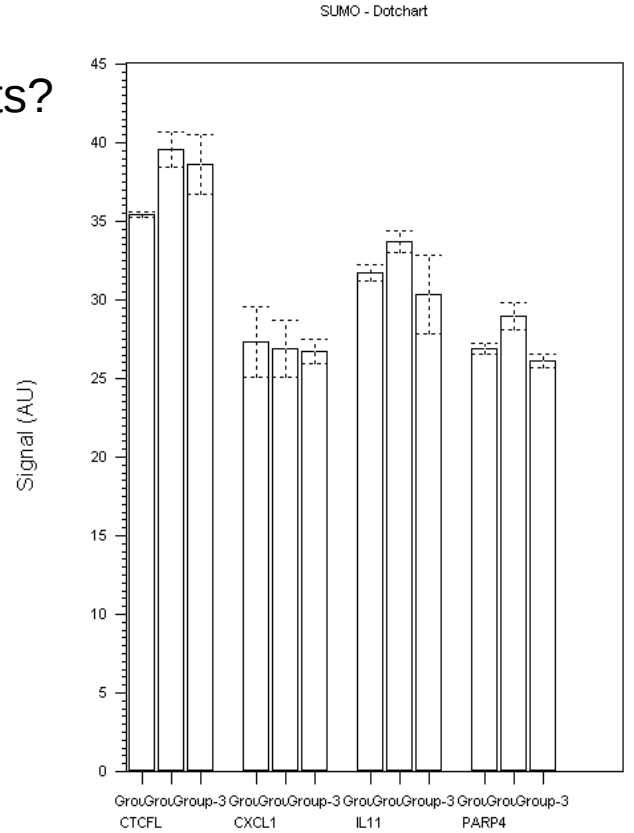
- signal levels above 36 !!
- In two treatment groups even around 40

Most interesting: PARP4

Christian Schwager, 2013, Christian.Schwager@Med.Uni-Heidelberg.de

By the way: Do you prefer bar charts?





Or dot charts?

See ***SUMO*** Help pages for more details how to customize dot chart

Christian Schwager, 2013, Christian.Schwager@Med.Uni-Heidelberg.de

ddCT - Specific regulation of genes in your samples

- Compute regulation against control / reference samples
  (a single or better the mean/median of a set of ref. samples)
- subtract reference-mean from your samples

SUMO main menu
Adjust data
Normalise genes
Hybridisation normalisation/centering
Mean/Median centering

Assign the Reference/Control samples to group 2:
- Select all Controls in **Condition list**
- Double click Group-2 in **Groups list**

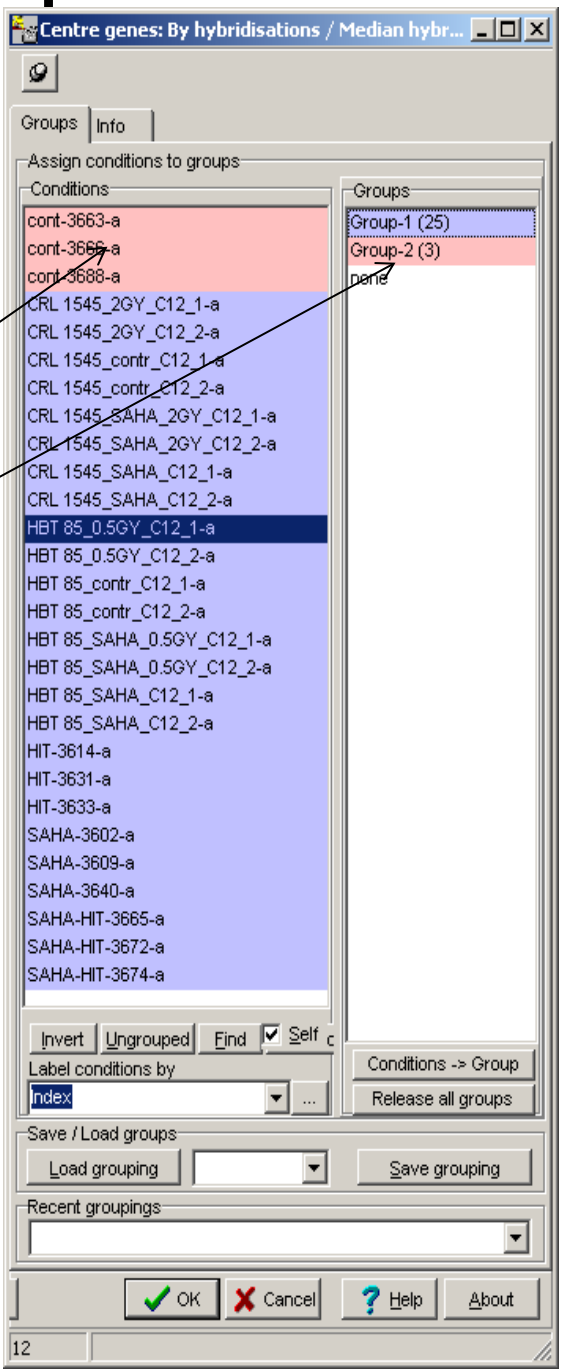
Assign the „treated“ samples to group 1
- Select all Treatments in Condition list
- Double click Group-1 in Groups list

Click **OK-button**

You may perform this for different sample groups independently
(e.g. Normalize all treated fibroblasts fo control fibroblasts,
All endothelial cells to endothelial controls,…)

Don‘t forget to save **new** matrix.

See ***SUMO*** Help pages for more details how to use the grouping tools



Christian Schwager, 2013, Christian.Schwager@Med.Uni-Heidelberg.de

ddCT to Log-2 regulation

Still, our data are **CT-values.**

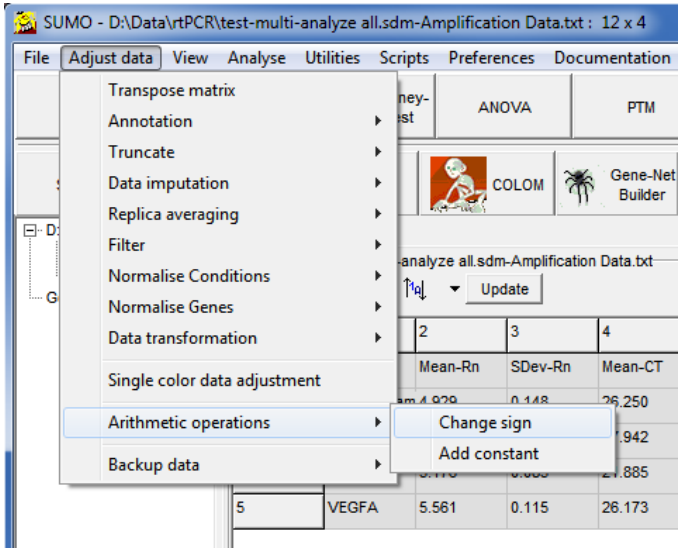
ddCT > 0: more ct-cycles are required to synthesie the same amout of DANN in your sample compared to the control sample
=> Sample is down-regulated compared to control.

ddCT < 0: less ct-cycles are required to synthesie the same amout in your sample compared to the control sample
=> Sample is up-regulated compared to control.

To fix this, invert the sign: -(ddCT-values) => ~ log2 regulation

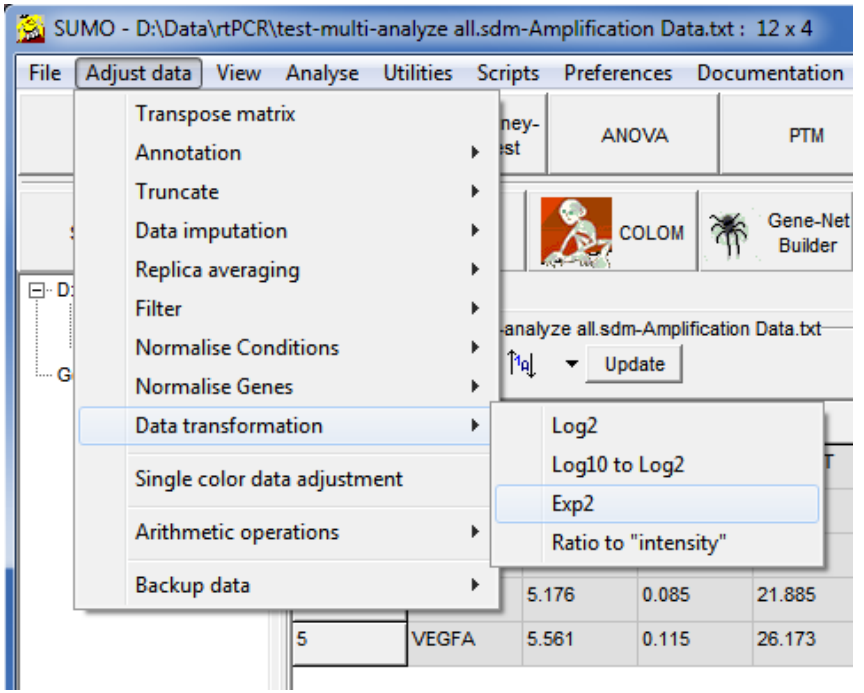
SUMO main menu
Adjust data
Arithmetic operations
Invert sign

Assign **all** samples to **Group1** and press **RUN** button.



Log-2 regulation => relative regulation

SUMO main menu
Adjust data
Data transformation
Exp2



Christian Schwager, 2013, Christian.Schwager@Med.Uni-Heidelberg.de

What to do now ?

Use ***SUMO*** to
- Run statistic test
- generate graphs for your test groups
- save transformed data as tab delimited text (File | Save matrix)

How to get ***SUMO*** – www.oncoexpress.de/software/sumo - Download

What else can I do with ***SUMO*** – Look in ***SUMO*** web pages.

Statistical tests

Question: Which genes are statistically significant differentially expressed between Samples from Treatment1 ⇔ Treatment2 ⇔ Treatment3 ⇔ …

SUMO offers to perform a variaty of standard class tests to find statistically significant regulated genes between your treatment groups:

- Gaussian distrubtion based parametric tests
- Gaussian distrubtion based permutaion tests
- Non-parametric Rank tests

- Single class
- Two class – unpaired
- Paired samples
- Multiclass

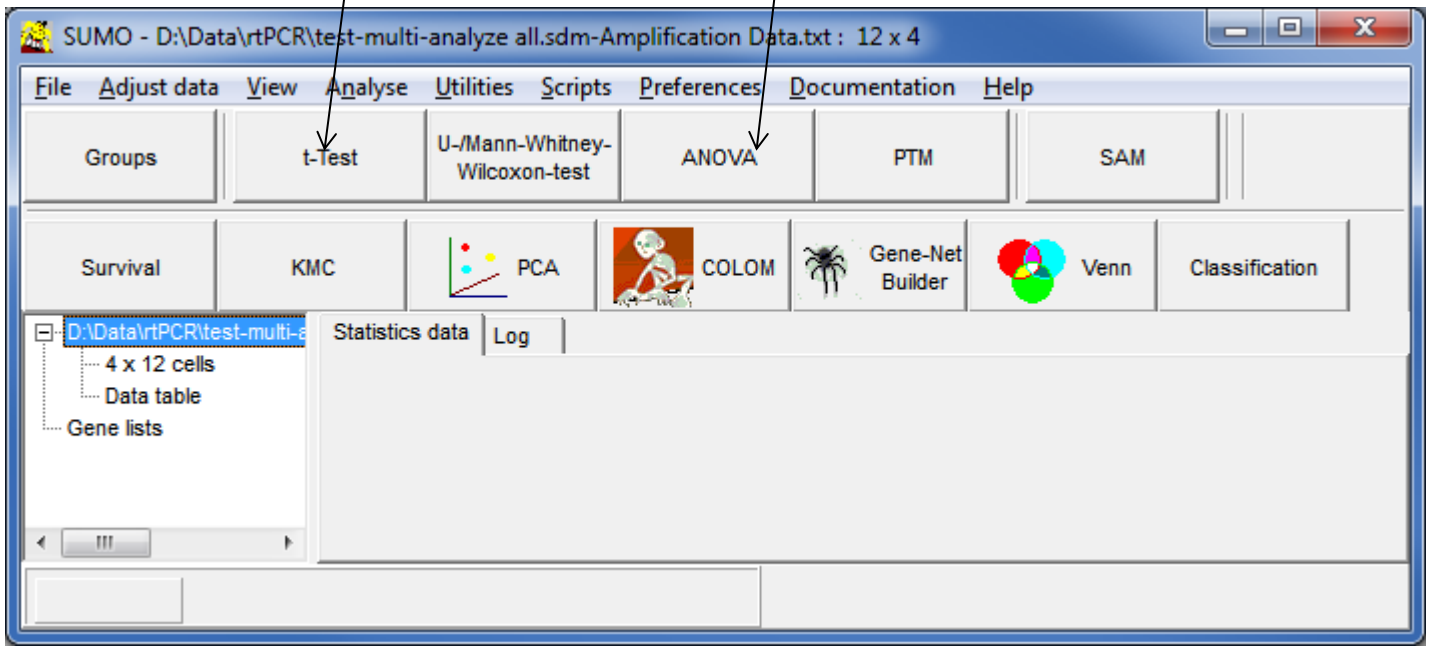
See ***SUMO*** Help pages for more details about hypthesis testing / class tests

Christian Schwager, 2013, Christian.Schwager@Med.Uni-Heidelberg.de

View grouped data

Set up a „fake“ statitical test to arrange samples in groups.

This simplifies to create graphs (heatmaps / dot-,bar, bow-whisker charts
- 2-class tests – for two groups - ANOVA – for 3 or more groups



View grouped data

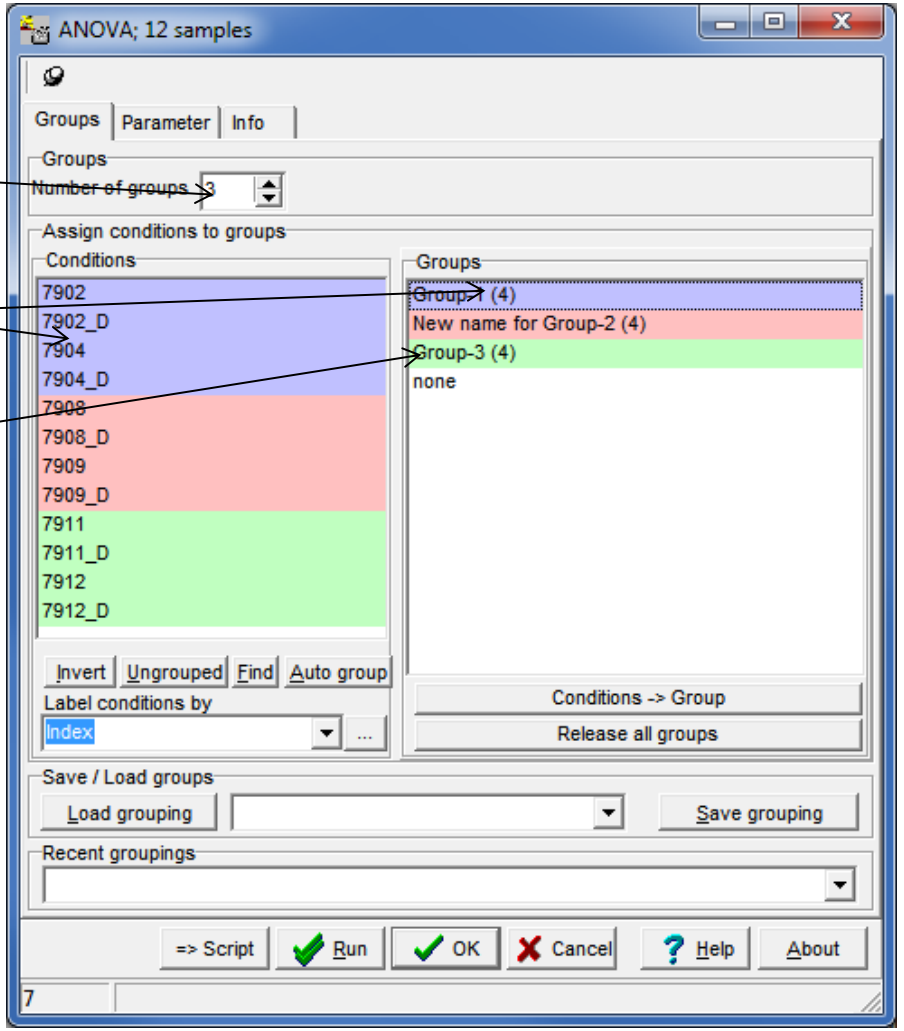
The grouping tool opens up:

Set number of groups (ANOVA)

Select individual samples and
Double click respective group
Repeat this for all relevant groups

Change groups names:
Right click a group name,
select **Rename** from context menu

Reorder Groups:
Select a group,
Drag it to another position in the group list
Repeat this for all relevant groups



See ***SUMO*** Help pages for more details about the grouping tool

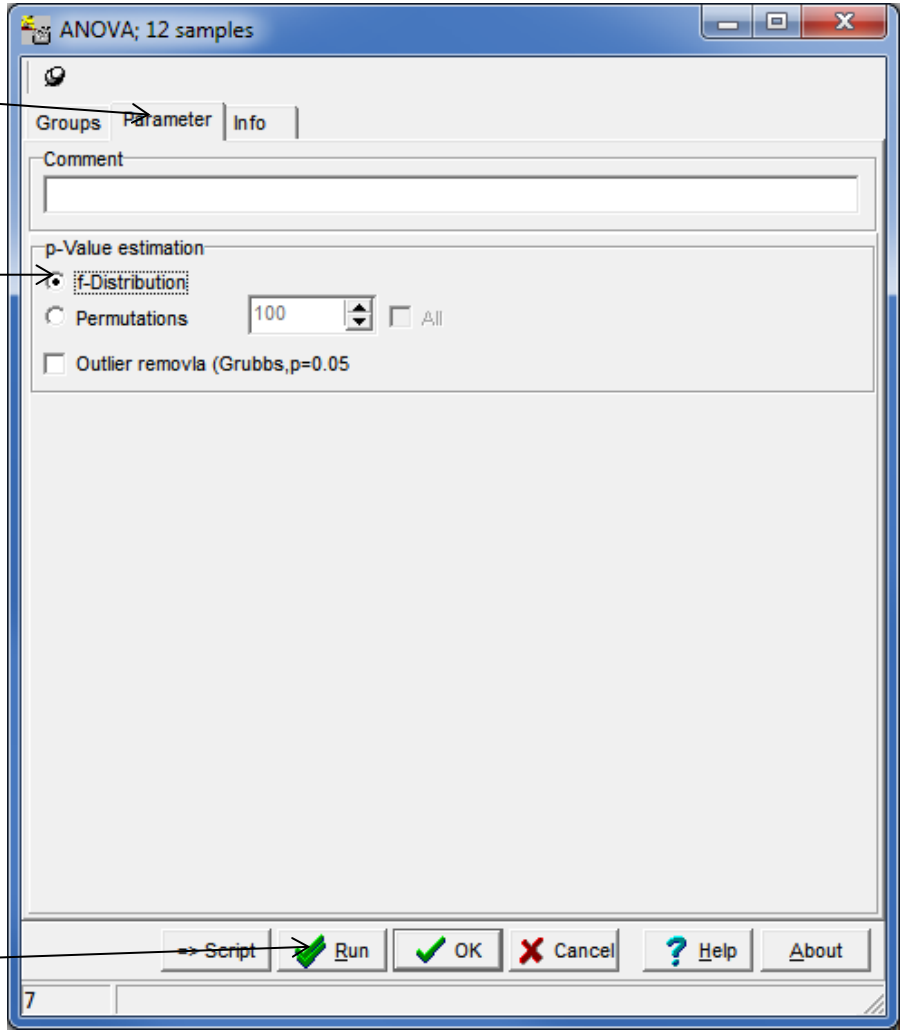
Christian Schwager, 2013, Christian.Schwager@Med.Uni-Heidelberg.de

View group data

Go to **Paramter** page:

Select **t/f distribution**

Click **RUN**-button
to execute the „fake“ test



Christian Schwager, 2013, Christian.Schwager@Med.Uni-Heidelberg.de

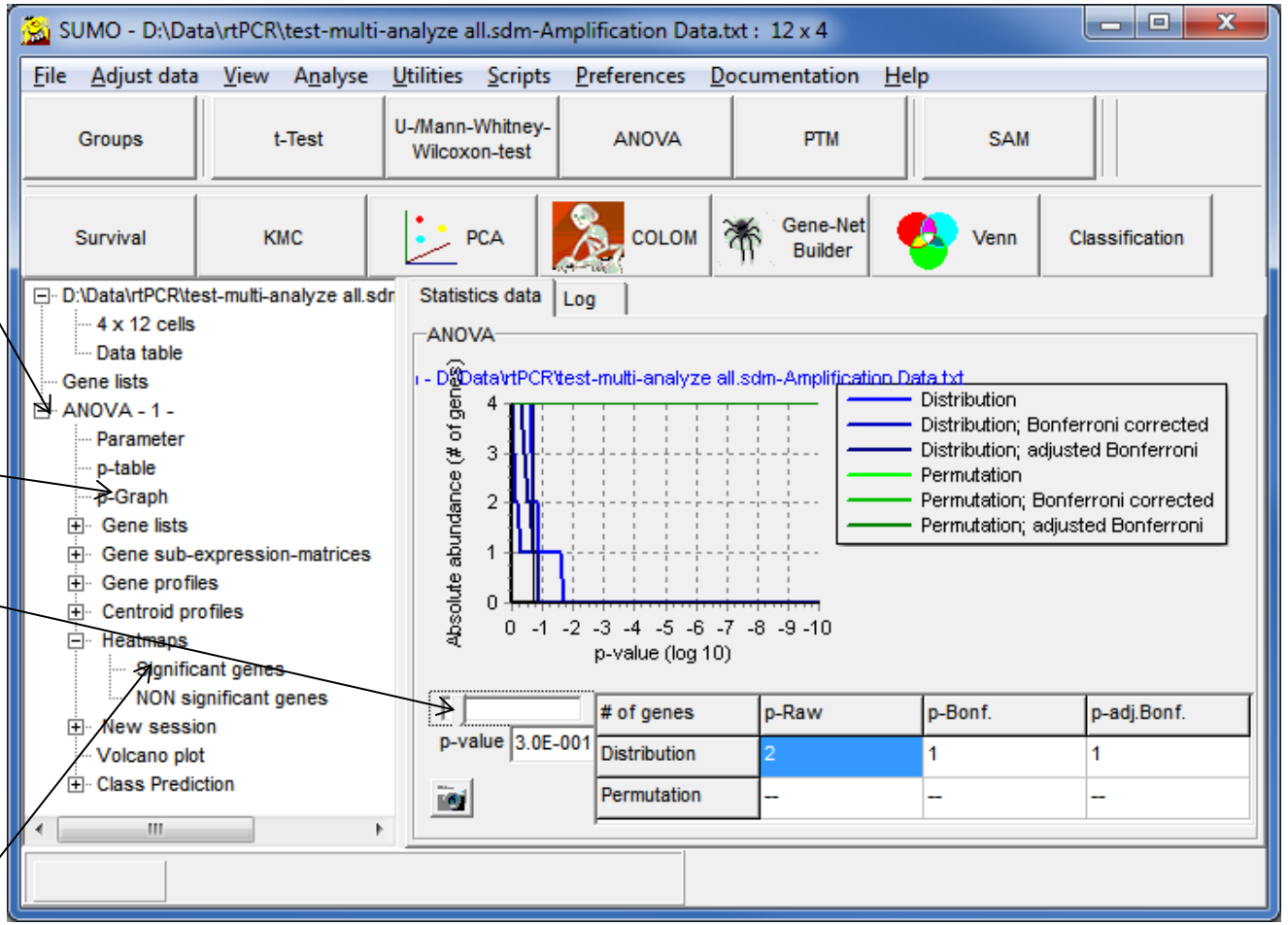
View group data

Open experiment in tree:

Goto p-graph

Adjust p-value with slider,
To set p-value threshold.
Move slider complete left (p=1)
to see all genes

Click
Heatmaps | Significant genes
To view the heatmap of all selected genes



Christian Schwager, 2013, Christian.Schwager@Med.Uni-Heidelberg.de

Heatmap of grouped data

Use slider to adjust height, width of genes, color contrast.

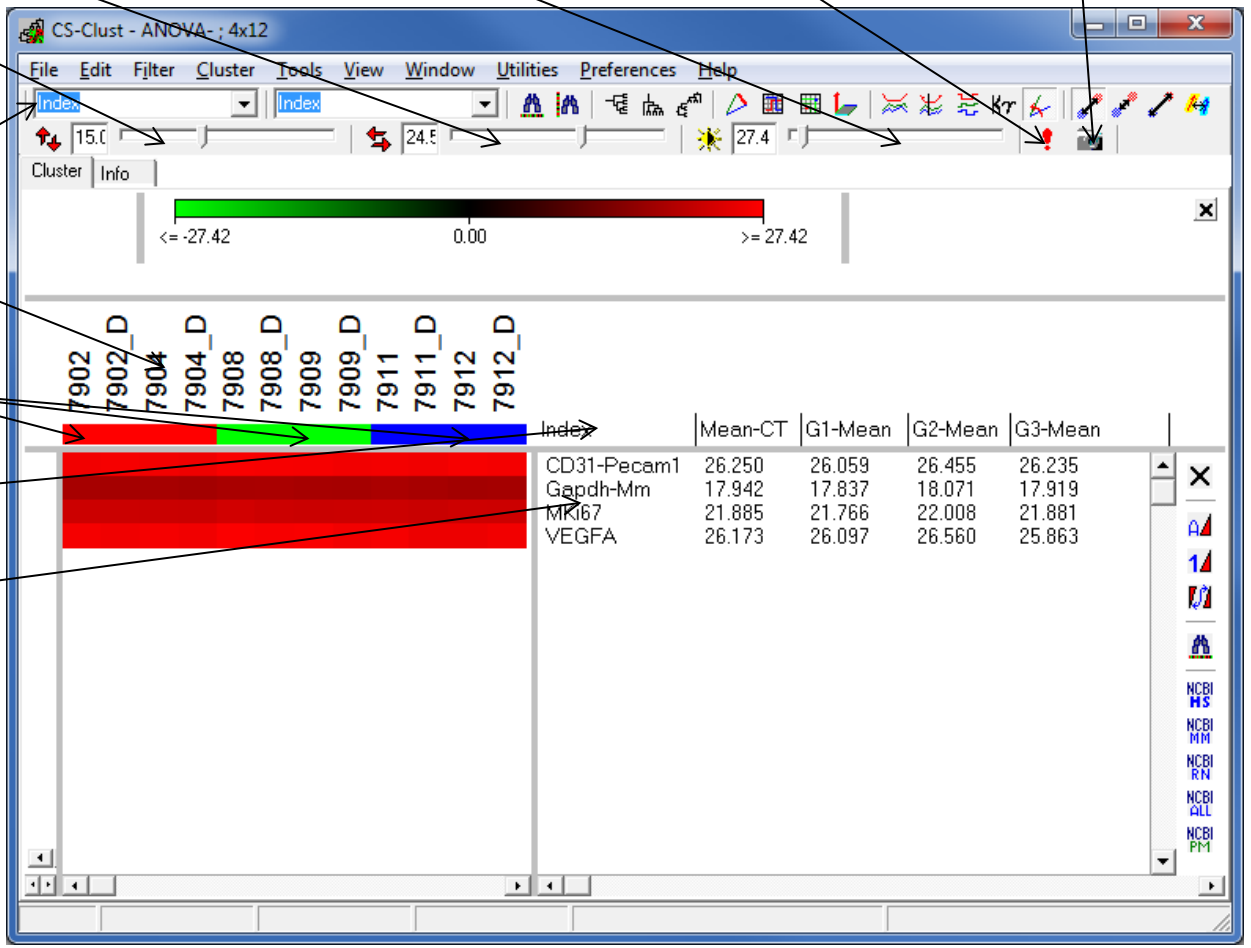
Autoscale all

Snapshot

Select sample annotation to be
Shown on top of heatmap

Color bars indicating groups

Right click into colum header,
to select gene annotation to be
Shown in respective column



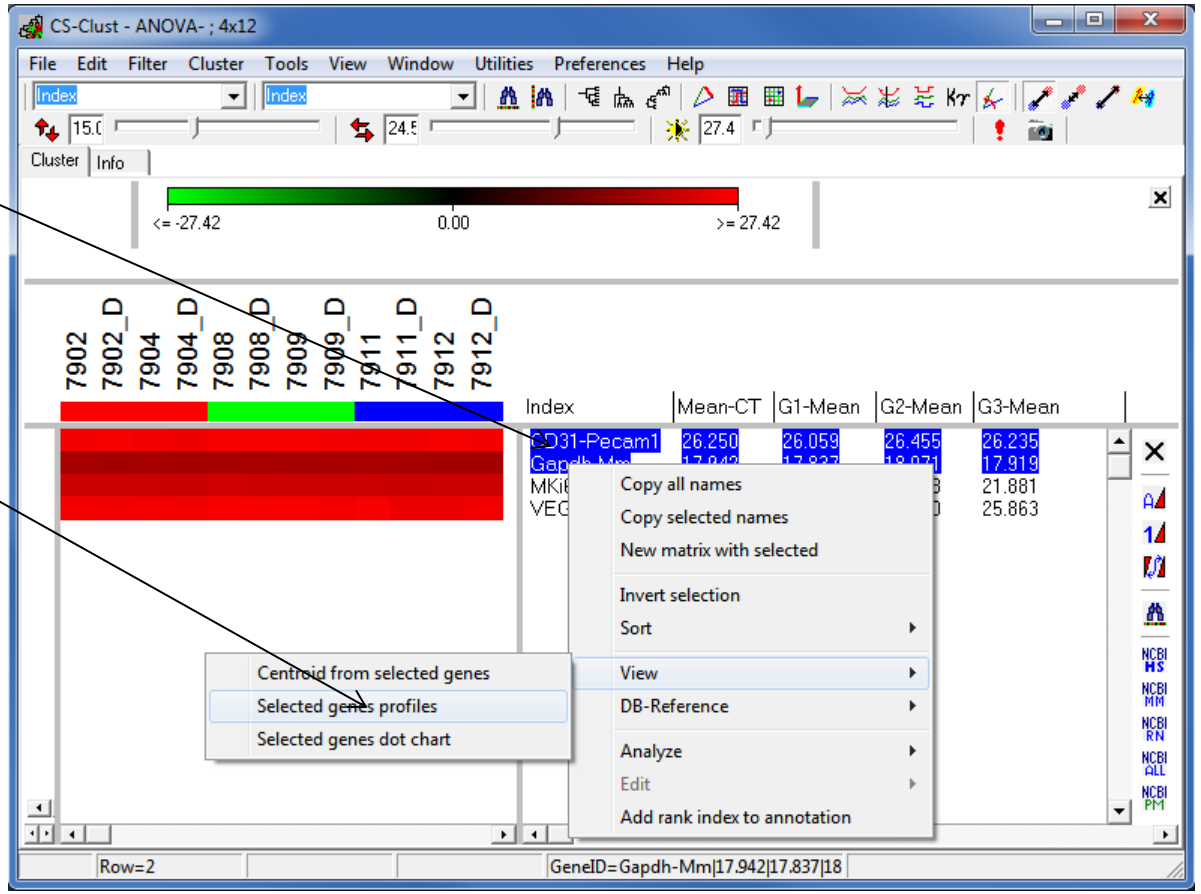
See ***SUMO*** Help pages for more details how to customize the heatmap

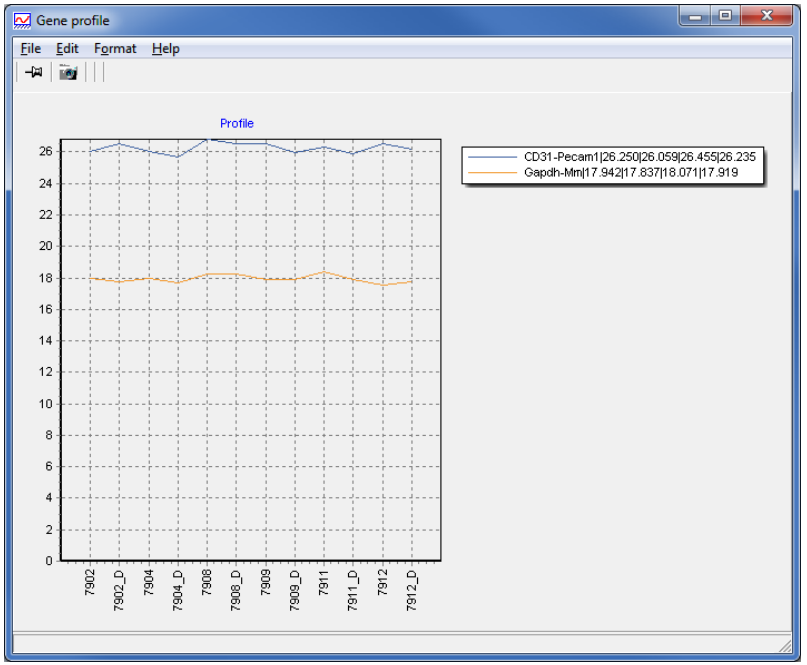
Christian Schwager, 2013, Christian.Schwager@Med.Uni-Heidelberg.de

View group data‘s profiles

Select multiple genes (CTRL+Click)

Click right mouse button,
From context menu select
View | Selected genes profiles



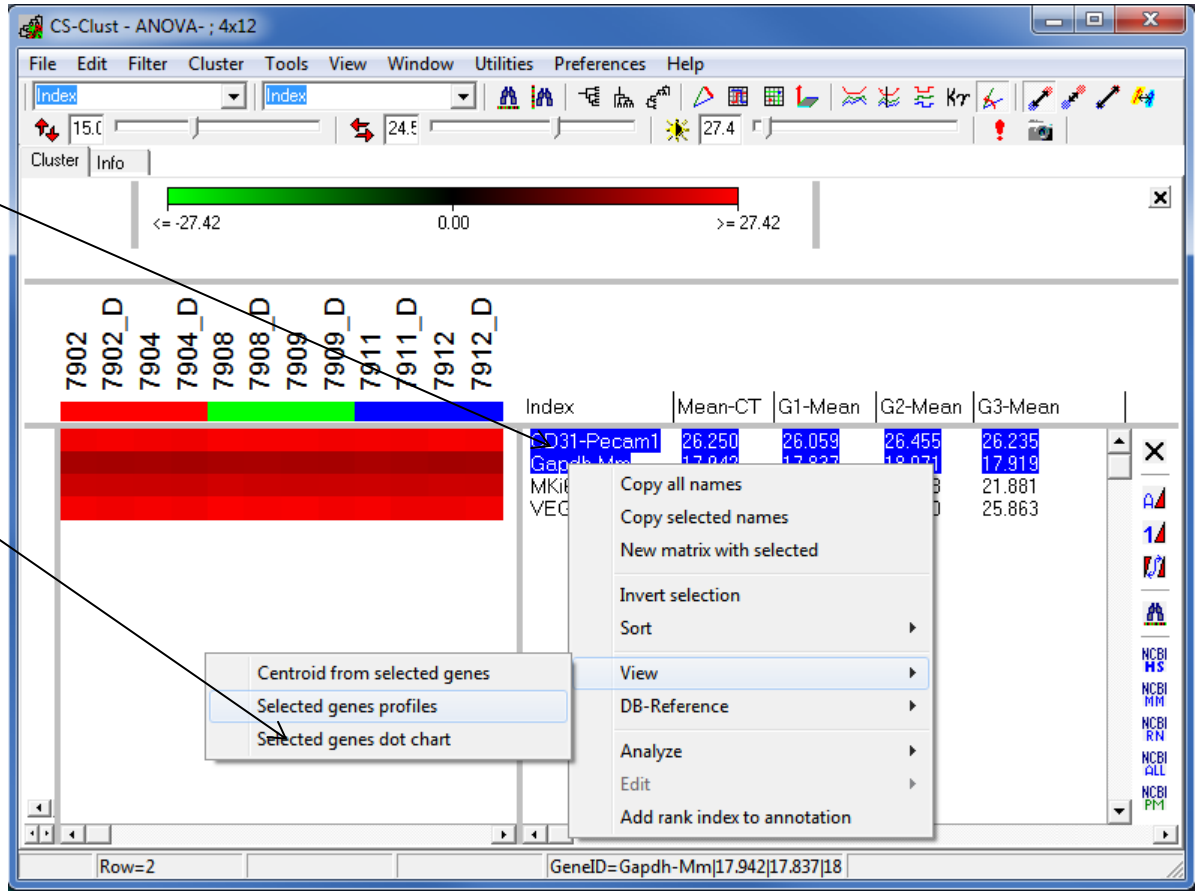


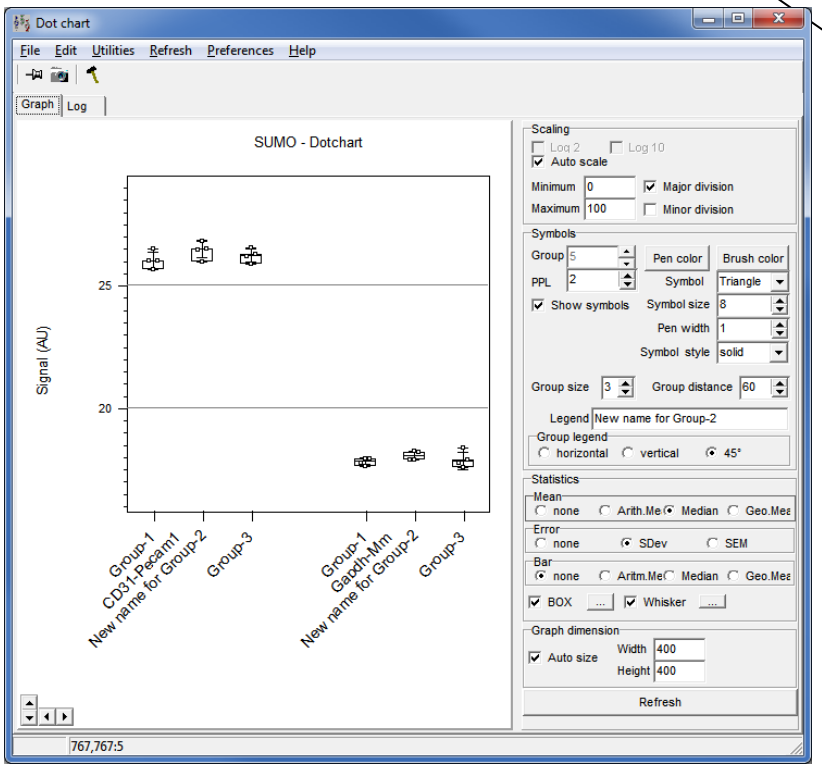
Christian Schwager, 2013, Christian.Schwager@Med.Uni-Heidelberg.de

View group data‘s dot chart (bow whisker plot / bar chart)

Select genes (CTRL+Click)

Click right mouse button,
From context menu select
View | Selected genes dot chart





See ***SUMO*** Help pages for more details how to customize the dot chart.

Christian Schwager, 2013, Christian.Schwager@Med.Uni-Heidelberg.de

How to get ***SUMO ?***

=> http://www.oncoexpress.de/software/sumo

What else can I do with ***SUMO*** – Look in ***SUMO*** web pages.

If I don't want to use ***SUMO*** – what else could I use ?

Any other program where you can work with data matrices.