Real-Time Solutions

At the clinical science interface

PrimerDesign Ltd.

geNorm™ Housekeeping Gene Selection Kit Handbook

For the establishment of the optimal normalising genes
Using SYBR® green detection chemistry

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Kit Contents

1. **Lyophilised primer sets for 6 or 12 reference genes**
   Sufficient reagents are supplied for 200 20µl reactions per gene.

2. **25ml of PCR-Grade water**

3. **CD-ROM containing geNorm VBA applet for Microsoft Excel**
   Also included on the CD-ROM is a detailed geNorm user manual in PDF format and annotated Excel files for data input.

Reagents and Equipment to Be Supplied by User

1. **Real-Time PCR instrumentation**

2. **Mastermix or mastermix components**
   This kit should work well with any commercially available 2xqPCR Mastermix. However the primers have been tested using PrimerDesign 2xqPCR Mastermix which is recommended.

3. **SYBR® green**

4. **Pipettors and Tips**

5. **Vortex and centrifuge**

6. **Sample cDNA**
   The quality of cDNA will directly affect the quality of data generated using this kit.
   10 cDNA samples is the minimum recommended for geNorm analysis.
   When assessing expression stability in different groups (e.g. diseased vs. healthy) make sure that you use the same number of samples from each group in order to avoid bias towards the group with the largest number of samples

7. **PC running windows XP or 2000 and Microsoft Excel version 2000, XP or 2003**

Storage

The PrimerDesign geNorm kit should be stored at -20ºC on arrival. Once reconstituted, repeated freeze/thawing should be avoided.

Installing geNorm

Unzip the *geNorm_3.4.zip* file. After unzipping, a geNorm directory is created, which contains the *geNorm.xls* applet, and an InputData directory and OutputData directory.

The InputData directory contains a demo data file (*fibroblast.xls* described in Vandesompele et al., 2002, Genome Biology), and the OutputData directory contains a detailed user manual as a PDF file).

Licensing Agreement and Limitations of use

The geNorm VBA applet for Microsoft Excel is freely available (on request) from the author (http://medgen.ugent.be/~jvdesomp/genorm/) to all those involved in not-for-profit academic research.
Commercial use of geNorm software is available only via a limited licence when used in conjunction with PrimerDesign kits.

PCR is covered by several patents owned by Hoffman-Roche Inc and Hoffman-LaRoche, Ltd. Purchase of Primer Design kits does not include or provide licence with respect to any patents owned by Hoffman-La Roche or others.

SYBR® green is a registered trade mark of Molecular Probes Inc.

Primer Design Satisfaction Guarantee

PrimerDesign takes pride in the quality of all its products. Should this product fail to perform satisfactorily when used according to the protocols in this manual, Primer Design will replace the item free of charge.

Quality Control

As part of our routine quality assurance programme all Primer Design products are monitored to ensure the highest levels of performance and reliability.

Introduction

For accurate gene expression measurements, it is essential to normalise results from your quantitative real-time PCR experiments to a fixed reference; one that is not affected by your experimental conditions. Although normalising to a constitutively expressed housekeeping gene is the most common method, there is no universal reference gene that is constant in all experimental situations.

For example, reference gene expression levels may vary by:

- The tissue origin of your sample. e.g. brain vs. heart.
- The disease origin of your sample. e.g. carcinoma vs. healthy tissue.
- Your experimental parameters. e.g. stimulated cells vs. non-stimulated.

If your selected reference gene expression is variable, then normalising to this gene will severely limit the accuracy and sensitivity of your assay. Therefore, it is necessary to find the ideal reference genes for YOUR particular experimental system and to establish that these genes are indeed not regulated.

The PrimerDesign geNorm kits each contain a panel of 6 or 12 candidate reference genes. The expression of these genes can be measured by quantitative real-time PCR and the data analysed by the geNorm software.

The analysis will reveal the best reference gene for accurate normalisation in your experimental system by ranking the candidate reference genes according to their expression stability.

Having used geNorm to select the best normalising genes for your experiment, PrimerDesign can provide ready to use high quality normalising gene detection kits for your next experiments. geNorm customers will receive a 25% discount on their first normalising gene detection kit order. Many of our normalising gene detection assays are available as a multiplex. So you can measure two normalising genes in the same reaction to maximise the quality of your normalising whilst minimising cDNA use.

Bench-side Protocol

To minimise the risk of contamination with foreign DNA, we recommend that all pipetting be performed in a PCR clean environment. Ideally this would be a designated PCR cabinet. Filter tips are recommended for all pipetting steps

1. **Pulse-spin each tube in a centrifuge before opening.**
   This will ensure lyophilised primer and probe mix is in the base of the tube and is not spilt upon opening the tube.

2. **Reconstitute all lyophilised primer mixes in 220µl of PCR-grade water (Provided).**
   Briefly vortex each tube to ensure complete reconstitution.

3. **When using PrimerDesign 2XqPCR Mastermix. Make up a Mastermix containing all qPCR reagents according to the protocol below for each reference gene.**

Component	1 Reaction
Reconstituted primer mix	1 µl*
PrimerDesign 2X qPCR Mastermix	10 µl
SYBR® green in DMSO	0.375 µl
PCR-Grade water	3.625 µl
Final volume	**15 µl**

*working concentration of primers = 400nM in a 20µl reaction

4. **Pipette 15µl of the mastermix into each well according to your plate set up.**

ALL SAMPLES FOR EACH REFERENCE MUST BE RUN ON THE SAME PLATE. HOWEVER, DIFFERENT REFERENCE GENES MAY BE RUN ON SEPARATE PLATES. IF USING MULTIPLE PLATES, POUR ALL PLATES ON THE SAME OCCASION.

RUN ALL DATA POINTS IN DUPLICATE WELLS

	Reference Genes											
	ACTB				UBC				GAPDH			
cDNA samples	1	1	9	9	1	1	9	9	1	1	9	9
	2	2	10	10	2	2	10	10	2	2	10	10
	3	3	11	11	3	3	11	11	3	3	11	11
	4	4	12	12	4	4	12	12	4	4	12	12
	5	5	13	13	5	5	13	13	5	5	13	13
	6	6	14	14	6	6	14	14	6	6	14	14
	7	7	15	15	7	7	15	15	7	7	15	15
	8	8	Water	Water	8	8	Water	Water	8	8	Water	Water

Example plate layout for a geNorm analysis using 15 cDNA samples.

5. **Prepare 40µl (6 gene kit) or 80µl (12 gene kit) of cDNA for each sample at a concentration of 5ng/µl in PCR grade water.**

   If the concentration of cDNA is not known, then dilute your RT reactions 1:10 **(10µl of RT and 90µl of water)**. Ensure you have high quality cDNA before proceeding.

6. **Pipette 5µl of diluted cDNA into each well of your 96-well plate according to your plate layout .**
   The final volume in each well is 20µl.

7. **Proceed to the Real-Time PCR Amplification step.**

Amplification Protocol

1. **Amplification conditions using PrimerDesign 2X qPCR Mastermix.**

Highly Recommended

	Step	Time	Temp
	Enzyme activation	10min	95ºC
Cycling x50	Denaturation	15s	95ºC
	DATA COLLECTION*	60s	60ºC

***Fluorogenic data should be collect during this step through the SYBR® green channel.**

2. **If you are using alternative qPCR reagents then follow the manufacturer's instructions for enzyme activation while keeping the cycling conditions as above.**

geNorm Analysis

1. **Transform CT values into relative quantification data using the deltaCT method.**
   Before Ct values from the quantitative real-time PCR are input into geNorm, all CT values from must be transformed into relative quantification data.
   To achieve this **subtract the highest Ct value from all other Ct values for each gene measured**. Hence each Ct value has been transformed in to a "delta CT" value, with the highest deltaCT value as 0. All other values are less than 0.
   Then for each data point **apply the equation $2^{(-\text{delta Ct})}$**. Hence all data is expressed relative to the expression of the least expressed gene.

2. **Prepare input file for geNorm analysis.**
   Data should be laid out in Microsoft Excel with the first column containing the sample names and the first row containing the gene names. The first cell of the first row and column (cell A1) should be empty.
   EMPTY CELLS ARE NOT ALLOWED.

	A	B	C	D	E	F	
1		SDHA	CANX	ACT	GAP	B2M	A2
2	1	0.574349	0.784584	0.267943	0.25	0.554785	0.2
3	2	0.757858	0.217638	0.153893	0.366021	0.196146	
4	3	0.466516	0.267943	0.108819	0.129408	0.353553	0.1
5	4	0.574349	0.392292	0.217638	0.392292	0.277392	0.1
6	5	0.482968	0.392292	0.241484	0.420448	0.707107	0.3
7	6	1	0.267943	0.203063	0.31864	0.183011	0.3
8	7	0.392292	0.392292	0.133972	0.164938	0.233258	0.1
9	8	0.277392	0.594604	0.307786	0.757858	0.68302	0.3
10	9	0.225313	1	1	1	1	
11	10	0.287175	0.406126	0.203063	0.366021	0.258816	0.7
12	11	0.088388	0.353553	0.120742	0.03983	0.112656	0.0
13							
14							

3. **Save the file in the InputData directory.**
   This folder will be found in the geNorm directory created upon geNorm installation.

4. **Close all running instances of Microsoft Excel.**

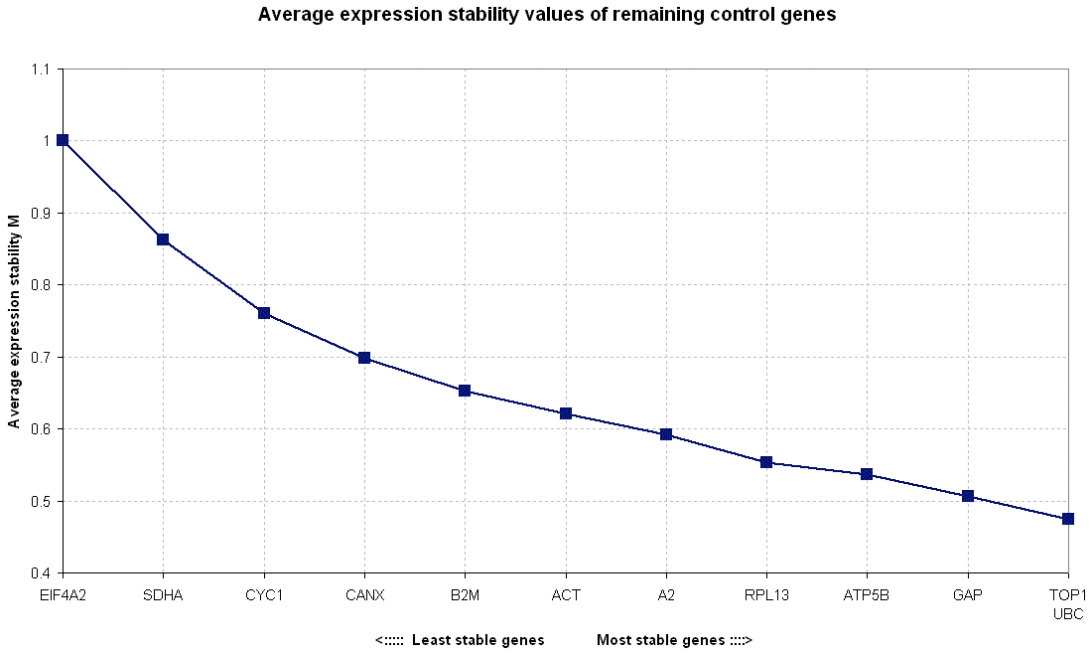
5. **Start up the geNorm applet (in Excel: *Open File*, or double click on the *geNorm.xls* file).**

6. **Enable macros when prompted.**

7. **Load the Input file.**
   This is achieved by clicking Load input data icon in the tool bar. 

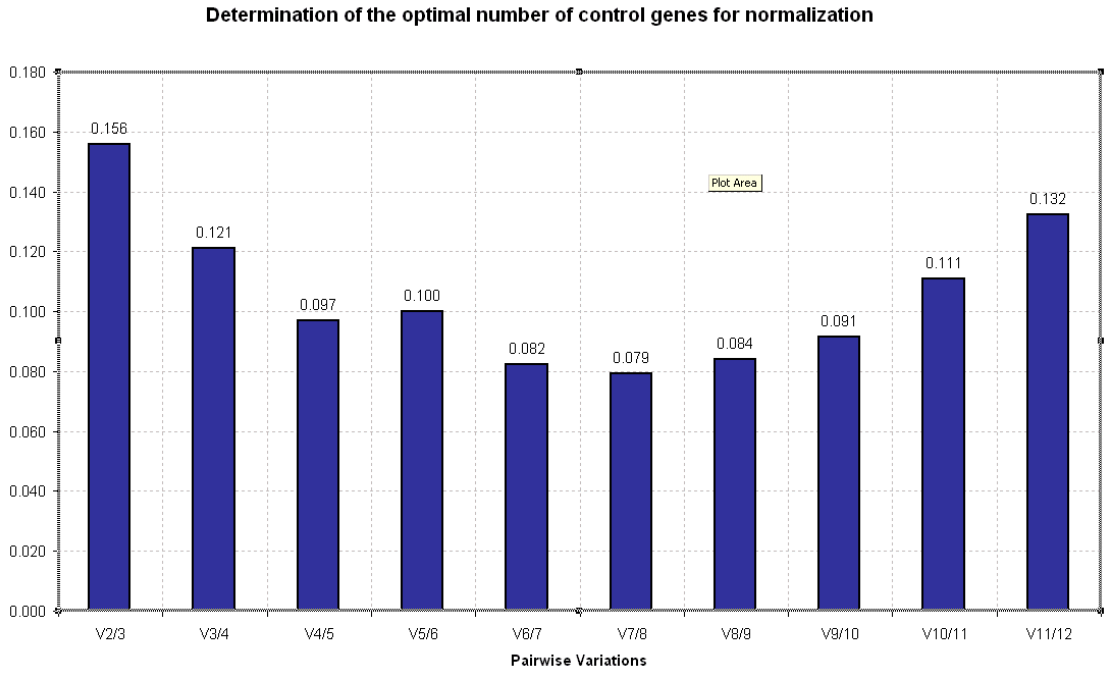
8. **Click the automated analysis icon.**

   Automated analysis icon . The first geNorm chart is generated.
   Automated analysis is recommended although manual analysis can be performed if required (see detailed geNorm manual PDF.)



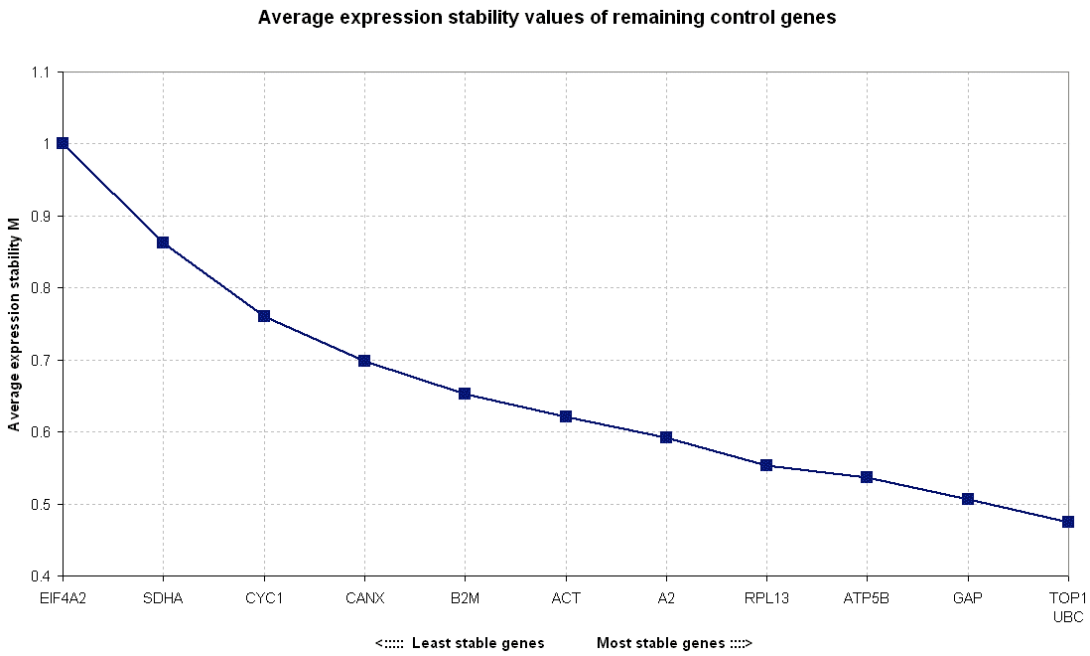
9. **Click the automated analysis icon a second time.**

   Automated analysis icon . A second geNorm chart is generated.



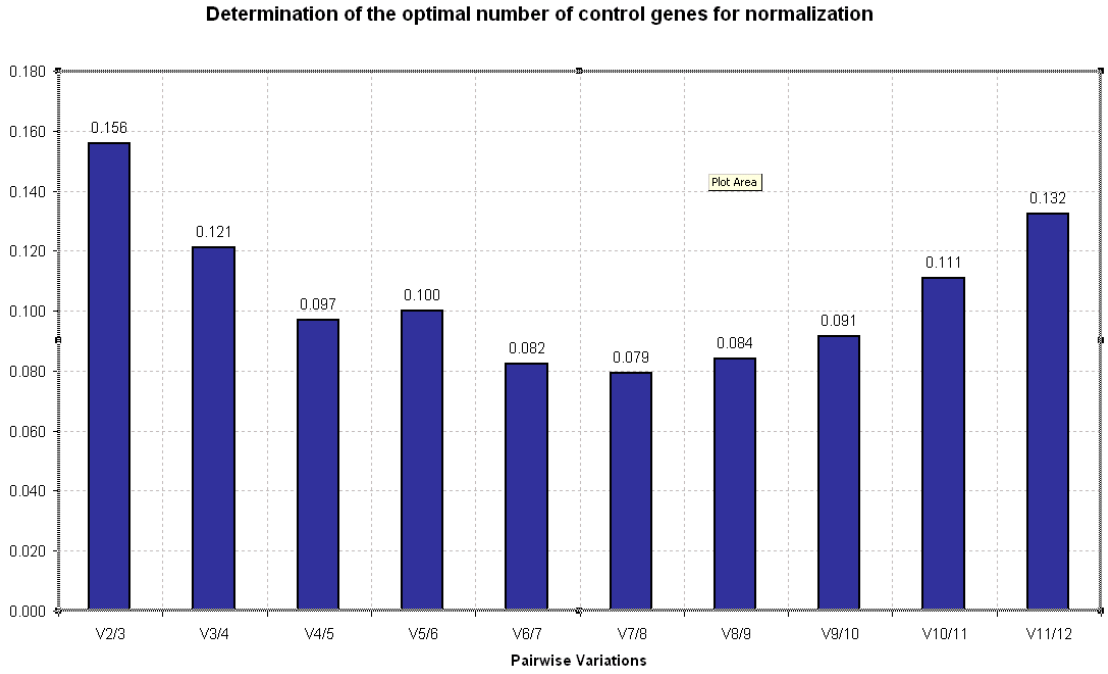
Interpreting geNorm Output Charts.

1. Chart 1: Which are the most stably expressed reference genes in my system?



The chart generated indicates the average expression stability value M of reference genes at each step during stepwise exclusion of the least stable expressed reference gene. Starting from the **least stable gene at the left**, the genes are ranked according to increasing expression stability, ending with the two **most stable genes on the right**. In this example TOP1 and UBC are the two most stable genes.

2. Chart 2: How many reference genes do I need for optimal normalisation?



The second chart illustrates the levels of variation in average reference gene stability with the sequential addition of each reference gene to the equation (for calculation of the normalization factor). Starting with the two most stably expressed genes on the left, with the inclusion of a 3rd, 4th 5th gene etc. moving to the right.
This measure is known as the "pairwise variation *V*". We recommend a *V* score of below 0.15 as the ideal for your system. e.g. If a *V* score of 0.25 is achieved using two genes but a *V* score of 0.14 is achieved with three reference genes, then the average of the top three genes in your system would be the optimal normalisation factor for your future experiments. In the example above the two most stable genes would give high quality data, three would be ideal.

Note: Please bear in mind that the proposed 0.15 value must not be taken as a too strict cut-off. The second graph is only intended to be guidance for determination of the optimal number of reference genes. Sometimes, the observed trend (of changing *V* values when using additional genes) can be equally informative. Anyway, 'just' using the 3 best reference genes (and ignoring this second graph) is in most cases a valid normalization strategy, and results in much more accurate and reliable normalization compared to the use of only one single non-validated reference gene.

FAQ's

Q1: When I load geNorm, **the menu bar is not visible**
A1: Close all open instances of Microsoft Excel and reload geNorm

Q2: What should I do if I have an **empty cell**?
A2: Remove sample OR remove gene which contains an empty value, and recalculate. To remove the sample, click on the empty cell, and then click on the *Delete row* button. To remove the gene, click on the empty cell, and the click on the *Delete column* button.

Q3: **How many samples** should I analyze to determine which control genes are most stable?
A3: In principle, any number of samples higher than 2 would be sufficient. However, the more samples you use, the more reliable are the conclusions. We propose to use at least 10 samples.

Q4: **Do I always have to retest** and determine which genes are the most stable, and should be used for normalization?
A4: This depends on your experimental setup. Once you have determined which genes and how many are required for accurate and reliable normalization, you can use this information for future experiments, as long as no significant changes in the experimental setup have been introduced.
e.g. once you have determined that *HPRT1*, *GAPD* and *YWHAZ* are the most stable control genes for short term cultured human fibroblasts, you can use these genes for normalization of all future fibroblast samples, as long as you keep the culture conditions, harvesting procedures, etc. identical.

Q5: Whom should I **contact** if I have **further questions**?
A5: Technical support is available from PrimerDesign for all of our geNorm products provided the kit have been used according to the protocols presented in this manual. A geNorm discussion group is also available for those wishing to learn more about this approach. http://groups.yahoo.com/group/genorm

Q6: When using **replicated tubes** in the same run, should I **average first** the Ct values and then transform the data for input into geNorm, or vice versa?
A6: Either approach is satisfactory, although we recommend transforming the arithmetic mean of the replicate Ct values.

Q7: What should I do if my **negative control** well produces an amplification plot?
A7: If the signal is very late e.g. CT>35, and there is a >8CT value difference between the negative control and all the cDNA sample results, then ignore the no template signal and proceed with the analysis. It is likely due to a very low level of cross contamination during the set up of the plate. If a strong signal similar to the cDNA samples is obtained then analysis cannot be performed. One of your reagents has likely become significantly contaminated during your experiment set up.

Appendix 1: The geNorm Tool Bar

Load input data
loads Excel data file (see Requirements for data format)

Manual data input
provides possibility to type the data manually; indicate the number of samples and reference genes to be analyzed

Criteria settings
adjusts the expression stability threshold below which genes are included in the calculation of a normalization factor; genes (expression values) that are used for normalization are displayed in black, while genes in grey (inactive) are not used to calculate the normalization factors

Delete row
remove sample

Insert row
insert sample

Delete column
remove gene

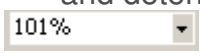
Insert column
insert gene

Show matrix
displays the pairwise variation *V* values for each gene with all other genes; click *Return* to leave the matrix view

Print/Save report

Save input data

Automated analysis
automatic ranking of control genes according to their expression stability (chart 1) and determination of optimal number of control genes (chart 2)



Zoom
user-adjustable zoom level, e.g. allows to view all genes and/or samples by highlighting the cells of interest, and selecting 'fit to selection'

Clear screen

About geNorm
brief information on contact address, method, input file, menu icons, and link to the most recent manual on the web

Exit geNorm
quit the application