GenomeLab GeXP Genetic Analysis System Gene Expression Workflow

Safety

Refer to the Safety Data Sheets (SDS), available at sciex.com/tech-regulatory, for information about the proper handling of materials and reagents. Always follow standard laboratory safety guidelines. For hazardous substance information, refer to the section: Hazardous Substance Information.
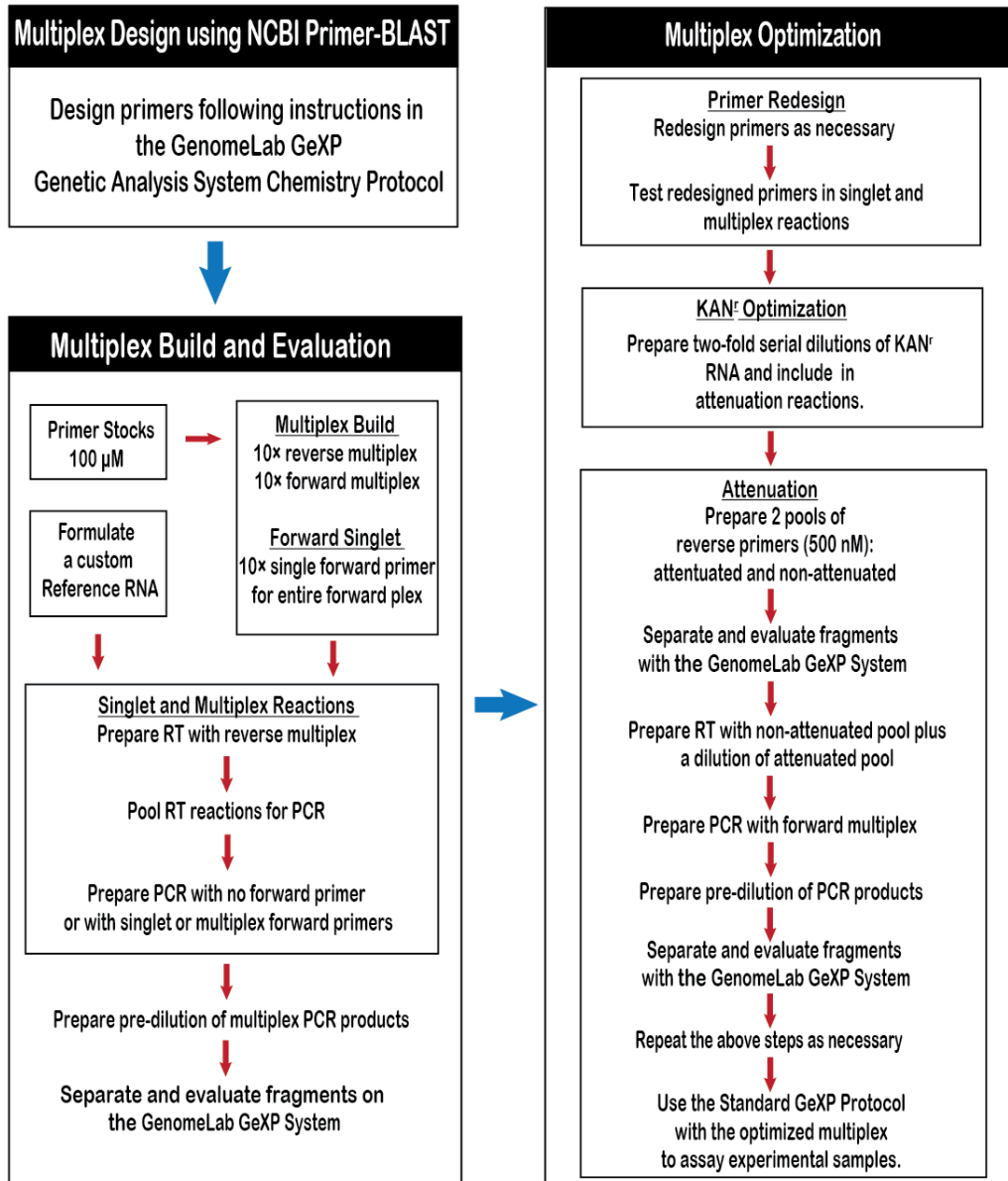
Gene Expression Workflow

The GenomeLab GeXP system uses a patented, highly-multiplexed PCR approach to efficiently examine the expression of multiple genes, with sensitivity and speed. Perform a multiplex gene expression analysis by completing the following procedures:

• Custom Multiplex Design and Optimization. Refer to Custom Multiplex Design and Optimization.

• Standard GenomeLab GeXP Protocol. Refer to Standard GeXP Protocol.

Custom Multiplex Design and Optimization

Figure 1 Custom Multiplex Design and Optimization Flowchart

Multiplex Design using NCBI Primer-BLAST

Design primers following instructions in the GenomeLab GeXP Genetic Analysis System Chemistry Protocol

Multiplex Build and Evaluation

Primer Stocks 100 µM

Multiplex Build
10× reverse multiplex
10× forward multiplex

Formulate a custom Reference RNA

Forward Singlet
10× single forward primer for entire forward plex

Singlet and Multiplex Reactions
Prepare RT with reverse multiplex

Pool RT reactions for PCR

Prepare PCR with no forward primer or with singlet or multiplex forward primers

Prepare pre-dilution of multiplex PCR products

Separate and evaluate fragments on the GenomeLab GeXP System

Multiplex Optimization

Primer Redesign
Redesign primers as necessary

Test redesigned primers in singlet and multiplex reactions

KAN^r Optimization
Prepare two-fold serial dilutions of KAN^r RNA and include in attenuation reactions.

Attenuation
Prepare 2 pools of reverse primers (500 nM): attenuated and non-attenuated

Separate and evaluate fragments with the GenomeLab GeXP System

Prepare RT with non-attenuated pool plus a dilution of attenuated pool

Prepare PCR with forward multiplex

Prepare pre-dilution of PCR products

Separate and evaluate fragments with the GenomeLab GeXP System

Repeat the above steps as necessary

Use the Standard GeXP Protocol with the optimized multiplex to assay experimental samples.

Design the Multiplex Primers Using NCBI Primer-BLAST

The NCBI Primer-BLAST tool is used to design an initial multiplex, using accession numbers of target and reference genes. The primer and amplicon information for the multiplex are saved in a

Microsoft Excel file. The multiplex primer and amplicon sequences are evaluated using BLAST analysis. If necessary, individual primers can be redesigned. Finally, the primer and amplicon information for the multiplex in the Microsoft Excel file is updated.

Note: Add an appropriate universal tag sequence at the 5′ end of the primers before ordering primers.

Pre-Design Considerations

1. Choose a correct accession number of each gene target.

2. For genes with transcript variants, decide how many variants to detect. Search for possible gene family members, pseudo genes for each gene target.

Primer Design Using NCBI Primer-Blast

1. Go to http://www.ncbi.nlm.nih.gov/tools/primer-blast/.

2. In the **PCR Template** field, type an accession number or a FASTA sequence.

3. In the Primer Parameters section:

   a. For **PCR product size Min**, enter **105**. For FFPE samples, enter **63**.

   b. For **PCR product size Max**, enter **350**. For FFPE samples, enter **150**.

   c. For **Primer melting temperatures**, accept the default settings. Make sure that the **Max T$_m$ difference** is not more than 5.

4. In the Exon/Intron selection section:

   a. In the **Exon junction span** list, select **Primer must span an exon-exon junction**.

   b. Select the **Intron Inclusion** check box.

   c. Modify the **Intron length range Min** as required.

5. In the Primer Pair Specificity Checking Parameters section:

   a. Select the **Enable search for primer pairs specific to the intended PCR template** check box.

   b. In the **Database** list, select **Refseq mRNA as Database**.

   c. In the **Organism** field, type the first few letters of the appropriate organism and then click the organism in the list that shows.

6. Click **Advanced parameters**, and then do the following:

   a. From the **Salt correction formula** list, select **Schildkraut and Lifson 1965**

   b. Select the **Primer binding site may not contain known SNP** check box.

     c.   From the **Table of thermodynamic parameter** list, select **Breslauer et al. 1986**.

7.   At the bottom on the page, click **Get Primers**.

     After a minute or two, the NCBI website will return search results that contain primer pair(s) information.

Post-Design Considerations

1.   Perform BLAST with each primer sequence to ensure it does not have significant homology to amplicon sequences for other genes in the multiplex.

2.   If a primer has significant homology to a region of an amplicon of another gene in the multiplex, redesign the primer.

3.   Before placing primer orders, add the forward universal tag to the 5′ end of forward primers. Add the reverse universal tag to the 5′ end of reverse primers.

Multiplex Build and Primer Evaluation

Task	Description
Make primer stocks	Suspend primers to a stock concentration of 100 µM in 10 mM Tris-HCl, pH 8.0.
Build multiplexes	Mix primers in a total of 1 mL with 10 mM Tris-HCl, pH 8.0 to create a 10× Forward Multiplex (200 nM) and a 10× Reverse Multiplex (500 nM).
Prepare forward singlet primers	Dilute individual forward primers in a total of 1 mL with 10 mM Tris-HCl, pH 8.0 to create 10× Forward Singlet Primer (200 nM) suspensions for forward singlet reactions
Formulate a custom reference RNA	Mix untreated and treated (or normal and tumor) sample total RNA to create a Reference RNA with all multiplex gene transcripts represented. Prepare a working stock at 100 ng/uL. 50 ng total RNA per reaction is the initial recommended concentration. Increase or decrease the total RNA for multiplex reactions based on the overall signal intensity.
Preliminary KAN^r concentration	Initially a 1:50 dilution of the KAN^r RNA with RI with RNA storage solution, TE buffer, or nuclease-free water is recommended.

Task	Description
Prepare singlet and multiplex evaluation reactions	a. Perform RT reaction with Reverse Multiplex and Reference RNA, including NTC and RT minus controls. b. Pool Standard RT reactions, then perform PCR with no forward primer or with singlet or multiplex forward primers
Perform evaluation	Dilute PCR reactions in 10 mM Tris-HCl, pH 8.0 as needed for detection within the linear range of the instrument. Perform the sample run. Evaluate results in the Fragment Analysis module.

Multiplex Optimization

Multiplex optimization manages redesigning of primers, performing KAN^r RNA with RI optimization, and attenuation.

Redesign Primers

1. Redesign a specific set(s) of primers using NCBI Primer-Blast to remove any significant undesigned peaks (UDPs) or co-migrating peaks from the multiplex.

2. Test redesigned primers in singlet and multiplex reactions.

Perform KAN^r Optimization

1. Adjust the concentration of KAN^r RNA with RI RNA per reaction such that the signal strength of the KAN^r peak is just below the median range of the multiplex.

2. The KAN^r optimization can be performed simultaneously with Attenuation. Once the optimal KAN^r RNA with RI RNA concentration is determined, use the same KAN^r RNA with RI RNA concentration for the entire study.

> **Note:** Every time a stock solution of KAN^r RNA with RI is thawed, make 10 µL aliquots immediately after thawing. Put the aliquots on dry ice during the process of making aliquots. When finished, store the aliquots at –80 °C. Use each aliquot only once.

Perform Attenuation

Bring the signal strength of high expressers down by adjusting the reverse primer concentration for each high signal peak.

1. Prepare two 500 nM pools of primers:

    a. Those to be attenuated.

      b.   Those to remain at the standard concentration (Non-attenuated).

2.   Prepare two-fold dilutions of the attenuated primer pool over 8 dilutions in 10 mM Tris-HCl, pH 8.0

3.   Combine 2 µL of the 10× non-attenuated primer pool with 2 µL of each 10× attenuated primer pool dilution in separate RT reactions. It is recommended that primer attenuation reactions be evaluated in duplicate (16 reactions total).

4.   Perform the PCR reactions with the optimized forward multiplex.

5.   Evaluate the products on the GenomeLab GeXP system to determine the best concentration for each reverse primer.

6.   Run duplicate reactions on inner and outer capillaries.

7.   Repeat the RT-PCR reactions with the fully attenuated reverse multiplex and the optimized KAN^r RNA concentration to verify the optimization.

8.   Re-attenuate or modify the KAN^r RNA with RI concentration as necessary.

Standard GenomeLab GeXP Protocol

Figure 2 Standard GenomeLab GeXP Protocol Flowchart
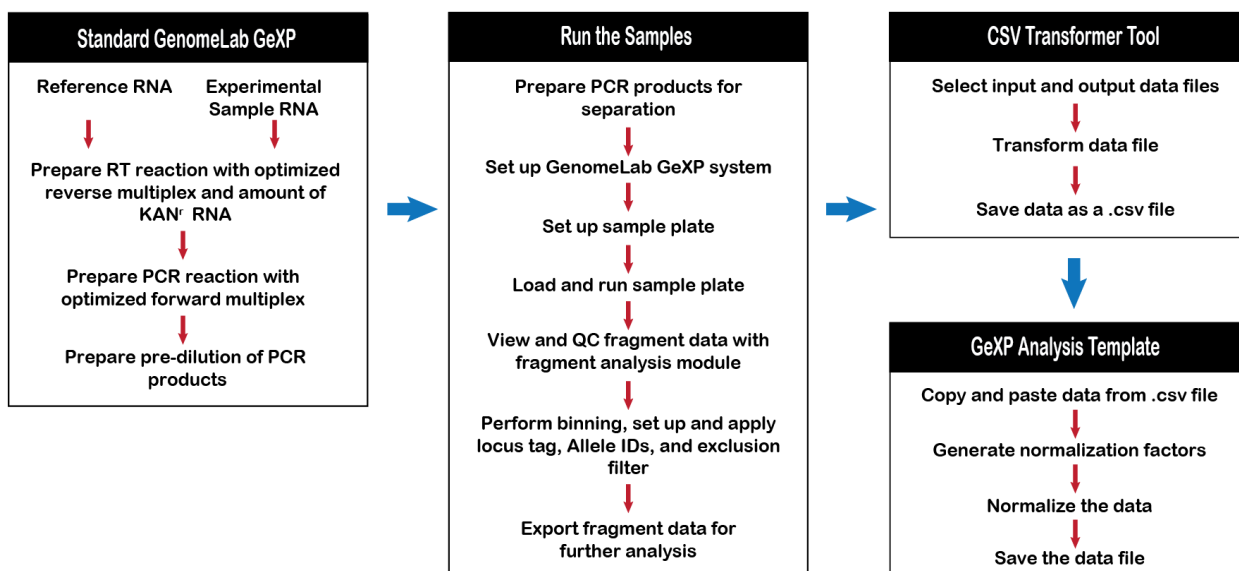
Standard GenomeLab GeXP Chemistry Protocol

1. To prepare the RT reaction, perform multiplex reactions with optimized reverse multiplex primers, the optimized KAN^r RNA with RI concentration and Reference RNA or Experimental sample RNA (5 ng to 100 ng total RNA). Include NTC and RT minus control reactions.

2. To prepare the PCR reaction, perform amplification with optimized forward multiplex primers and RT reaction.

3. For pre-dilution, dilute PCR reactions in 10 mM Tris-HCl, pH 8.0 as needed to bring the signal into linear range of detection.

GenomeLab GeXP System Run

Prepare PCR Samples

1. Combine 1 µL of the PCR reaction (or pre-dilution) into 38.5 µL SLS + 0.5 µL ss400 mix and cover with one drop of mineral oil.

2. Add 250 µL of GenomeLab GeXP Separation Buffer into each well of the buffer plate.

Set up the GenomeLab GeXP System

1.   Create a new project in the appropriate experimental database.

2.   Install capillary array and gel cartridge.

3.   Preheat capillaries to 50 °C.

4.   Perform Manifold Purge with 0.4 mL gel three times, and Gel Capillary Fill three times.

5.   Perform an Optical Alignment.

6.   Perform Monitor Baseline.

Set Up, Load, and Run the Sample Plate

1.   Name sample wells.

2.   Assign the **Frag-3** spearation method and **Sensitive GeXP Analysis Parameters** to each well. (Slope threshold = 1, Relative peak height threshold = 0%)

3.   Save the plate setup to the appropriate project.

4.   Refresh the wetting tray with DI water.

5.   Load sample and buffer plates and start the run.

View the Fragment Analysis Study

1.   Open the Fragment Analysis module and create a new study with analyzed results.

2.   In **Results Set View**, verify that all multiplex peaks are present in Reference RNA samples and all gene peaks are called as single peaks.

3.   Exclude any over-range peaks that are split into two called peaks, any data with abnormal current or poor resolution of fragments, and the NTC and RT minus controls.

Perform Binning and Set Up Locus Tag and Allele IDs

1.   Select **Analysis > New Binning Analysis**.

Figure 3 New Binning Analysis Option
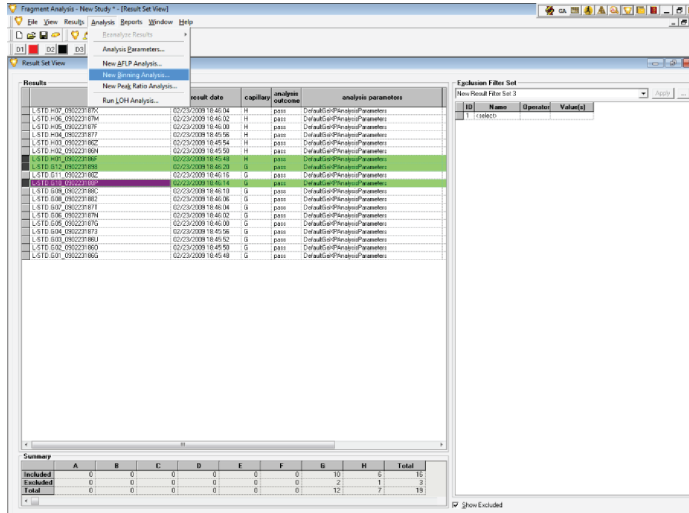
The New Binning Analysis window with default bin parameters opens.

Figure 4 New Binning Analysis Window with Default Bin Parameters
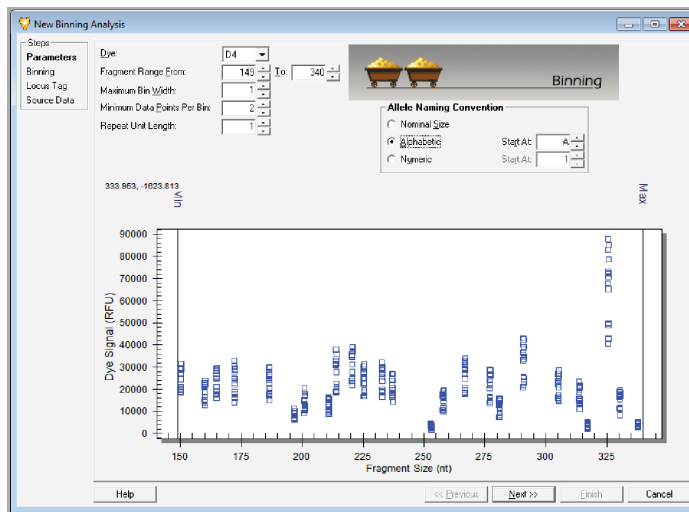
2. Modify the binning parameters. The following settings are recommended:

    • **Dye**: D4

    • **Fragment Range**: 149 to 340 (fragments size ranging from 150-338 nt)

    • **Maximum Bin Width**: 1

- • **Maximum Data Points Per Bin**: 2
- • **Repeat Unit Length of Allele**: 1
- • **Allele Naming Convention**: Alphabetic

3.   Adjust the **Minimal Relative Peak Height (Min Ref Peak Height)** to exclude any data points generated from small noise peaks.

Note: Excluded small peaks are shown as pink-colored dots at the bottom of the Bin View.

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4.   Screen for noise peaks, shoulders, and **-A** peaks. Delete them if present.

5.   Select **Sort by Nominal Size (nt)** so that the alleles is ordered by fragment size.

6.   Click the **Locus Tag** tab and type the identical name in the **Locus Tag** and **Locus Name** fields. If the gene is a housekeeping gene, then type **HK** followed by a space at the beginning of the gene name.

Note: Do not change any of the other fields in the Locus Information section.

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7.   Select **Allele ID Criteria** tab and **Overwrite system confidence interval**.

8.   Review the source data of all included samples, then click **Finish**.

9.   Highlight all the files in this study, right-click highlighted area,click **Reanalyze Results**, and then select **Using Additional/Edited Locus Tags**.

Figure 5 Select Allele Identification Type and STR Locus Tag
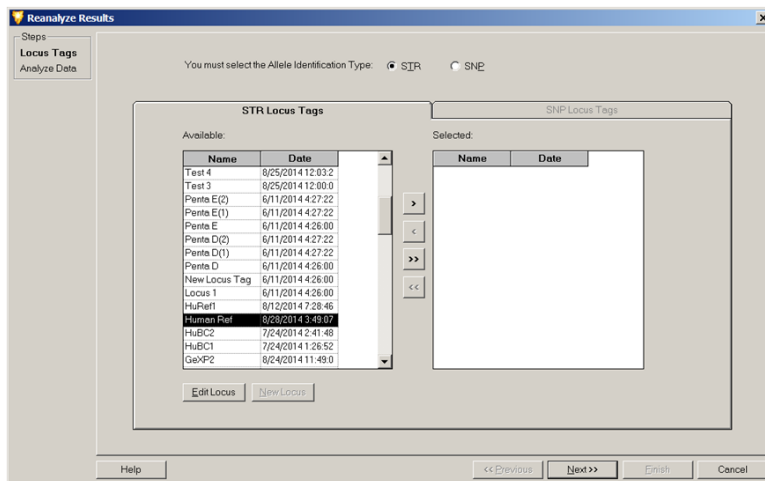
10.   Select the **Locus Tag** and click **Next**.

11. Click **Analyze**, and then click **Finish**.

12. In **Fragment List view**, set up and apply an exclusion filter: allele ID = (blank or empty space).

13. Click **File > Transfer Fragments for GeXP** to export the results. Make sure to select **csv** in the **Save as type** list.

# Normalize the Exported Fragment Data

After exporting the fragment data, it can be normalized using the GeXP Analysis Template. Before normalizing the data, it must be converted to the correct format using the CSV Transformer Tool.

Both tools are available on the GenomeLab System software DVD. If the DVD is not available, then contact SCIEX support at: sciex.com/request-support.

Convert the Exported Fragment Data

1. In File Explorer, double-click **CVS Transformer**.

2. Click **Transfer Format** and then select **GeXP Normalization Format**.

3. Click **Output By** and then select **Peak Area**.

4. Click **Select Input Files** and then select the file containing the data to be transformed.

    If required, additional files can be selected.

5. Click **Select Output Folder** and then select the location for the transformed file.

6. In the **Size difference range** field, type the difference between the lengths of the most closely spaced fragments in the multiplex.

Figure 6 CSV Transformer Tool
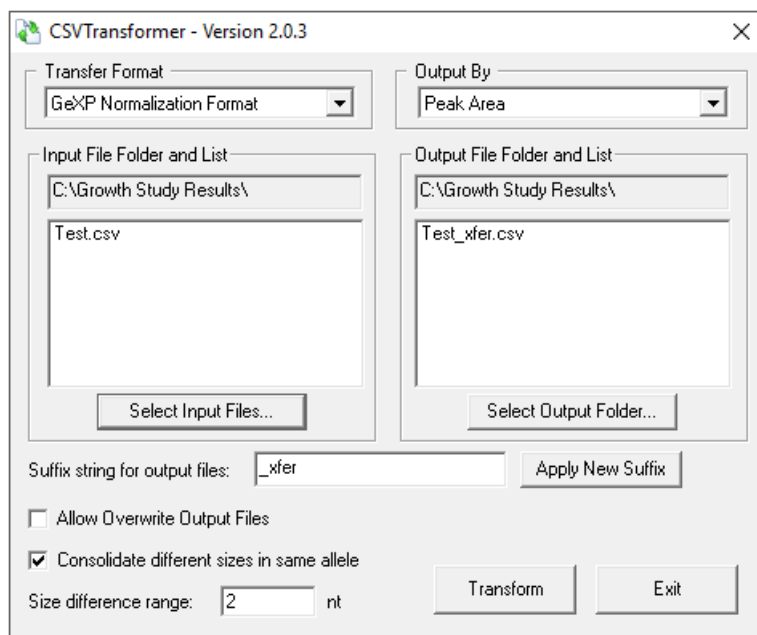
7.   (Optional) Type a different suffix in the **Suffix string for output files** field and then click **Apply New Suffix**.

The file name in the **Output File Folder and List** list updates with the new suffix.

8.   Click **Transform** and then click **OK** in the confirmation message.

The gene name, sizes, sample names, and peak areas of the fragments are extracted and saved to the output file.

Perform Gene Normalization

1.   In Excel, open the .csv file created by the CSV Transformer Tool.

2.   If neccesary, for each housekeeping gene, type **HK** followed by a space at the beginning of the gene name in the **Locus ID** field.

This may apply to a single gene, multiple genes, or every gene in the multiplex.

Note: To avoid editing the gene names in each exported file, add the HK to the gene names before exporting the fragment data from the GenomeLab System software. Refer to step 6 in Perform Binning and Set Up Locus Tag and Allele IDs.

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3.   In Excel, open the GeXP Analysis Template and then click the **Raw Data** tab.

4.   Copy the data from the transformed .csv file, and then paste the data in the B1 cell in GeXP Analysis Template.

Note: Do not copy the entire worksheet. Copy only the part of the worksheet with data.

Figure 7 Location to Paste Data in the GeXP Analysis Template
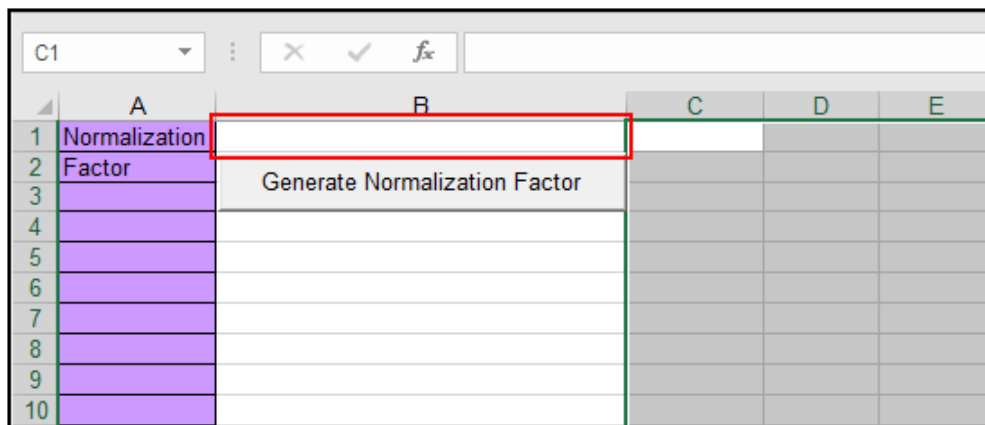
5.   Click **Generate Normalization Factor**, and then click **OK** in the two dialogs.

Column A in the worksheet is filled with the normalization factors for the data.

Figure 8 Normalization Factors

	A	B	C	D	E	F	G
1	Normalization		GENE A	GENE B	HK GENE C	GENE D	GENE E
2	Factor	Generate Normalization Factor	140	145	148	152	160
3							
4	5944	N9.A08_2102151109	8566	489	1506	3693	2642
5	5402.666667	N9.A05_210215110A	7163	444	1098	3119	2126
6	3351.333333	N9.A02_210215110A	4299	212	623	1949	1340
7	4702	N8.H07_210215110A	9529	5770	2272	9753	6421
8	9325	N8.H04_210215110A	20463	13212	5441	22049	14419
9	6154	N7.G07_210215110B	12106	516	1639	6437	1603
10	8929.333333	N7.G04_210215110B	14493	618	2004	8256	1979
11	4069.333333	N7.G01_210215110B	7603	402	1146	4334	962
12	16059.66667	N60.E09_210215110B	25156	1567	5477	17363	7502
13	38127.66667	N60.E06_210215110C	49534	3022	12807	35386	15669

6.   To normalize the data:

   a.   Click the **Normalized Data** tab.

b. Click **Insert Names** and then click **OK** in the two dialogs.

c. Click **Normalize Data** and then click **OK** in the two dialogs.

The normalized data is shown in the worksheet.

Figure 9 Normalized Results

	A	B	C	D	E	F	G
		K2					
1		Insert Names	GENE A	GENE B	HK GENE C	GENE D	GENE E
2		Normalize Data					
3							
4	N9		1.441117093	0.082267833	0.253364738	0.621298789	0.44448183
5	N9		1.325826752	0.082181639	0.203232971	0.577307502	0.393509378
6	N9		1.282773026	0.063258405	0.185896161	0.581559578	0.399840859
7	N8		2.026584432	1.227137388	0.483198639	2.074223735	1.365589111
8	N8		2.194423592	1.416836461	0.583485255	2.364504021	1.546273458
9	N7		1.967175821	0.083847904	0.266330842	1.04598635	0.260480988
10	N7		1.623077497	0.069210094	0.224428849	0.924593101	0.221629088
11	N7		1.868365007	0.09878768	0.281618611	1.065039318	0.236402359

7. Click **File > Save As**, type a name for the worksheet, and then click **Save**

Note: The GeXP Analysis Template file is in xls (Excel 97-2003) format and the file contains macros. Saving the file in xlsx (Excel Workbook) format deletes the macros from the file. To retain the macros, save the file in xlsm (Excel Macro-Enabled Workbook) format.

Hazardous Substance Information

The following information must be noted and the relevant safety measures taken. Refer to the respective safety data sheets for more information. These are available upon request or can be downloaded from our website sciex.com/tech-regulatory.

Hazard classification according to HCS 2012.

DNA Size Standard 400

DANGER! May damage fertility or the unborn child.

RT Buffer, 5×

WARNING! May be harmful if swallowed. Causes minor skin irritation.

Sample Loading Solution

 DANGER! May damage fertility or the unborn child.

Other Reagents

These components are not classified as hazardous:

- DNase/RNase-free distilled water
- KAN^r RNA with RI
- Mineral oil
- PCR Buffer 5×
- Reverse transcriptase
- Separation Buffer
- Separation gel
- Thermo-Start *Taq* DNA Polymerase

For reagents from other vendors, read the *Safety Data Sheet* from the vendor before use.

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