



GenomeLab GeXP Genetic Analysis System

Chemistry Protocol

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Multiplex Gene Expression

The GenomeLab GeXP system utilizes a patented, highly-multiplexed PCR approach to efficiently look at the expression of multiplexed gene sets with sensitivity and speed.

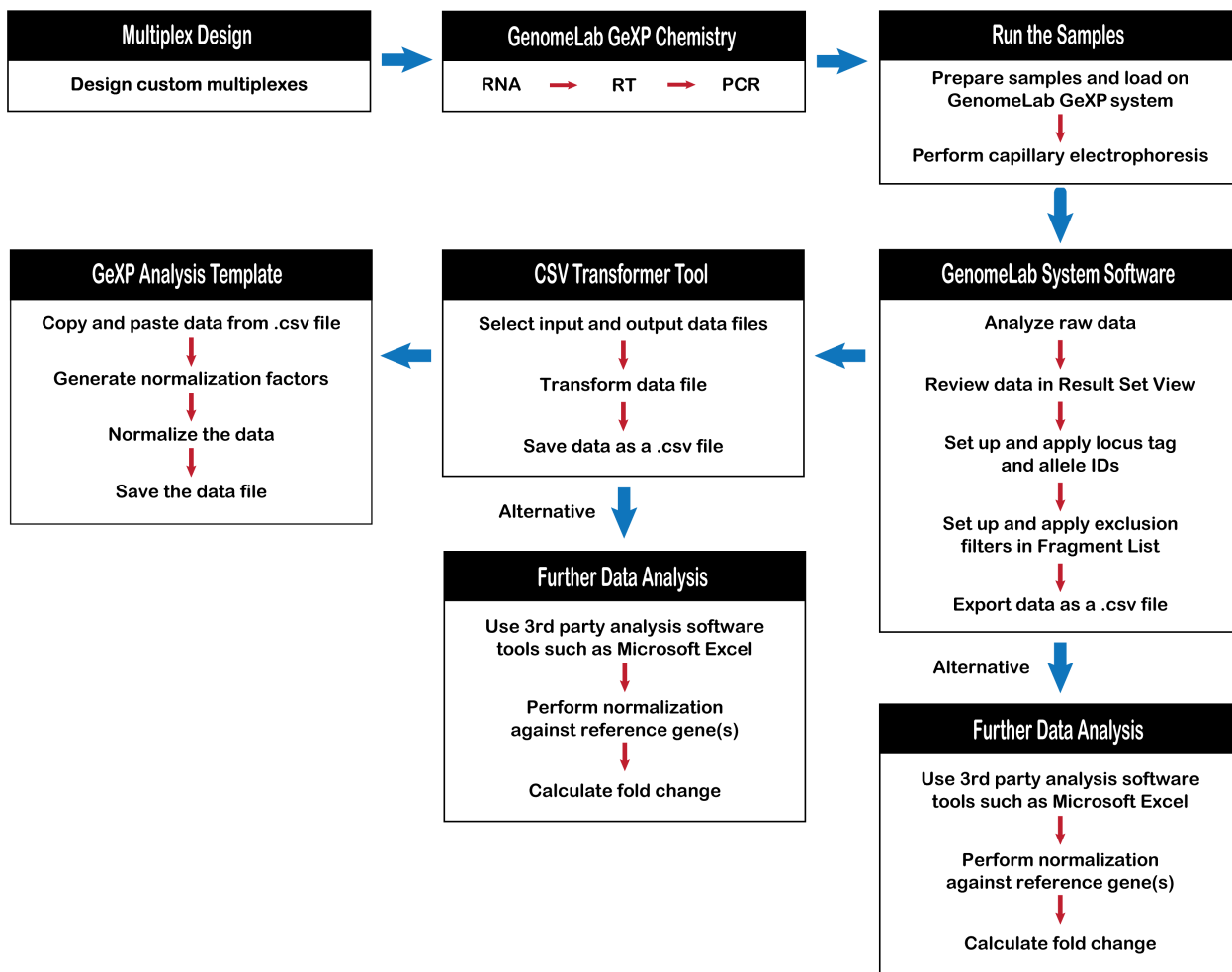
With a multiplexing capacity of analyzing up to 30 genes per reaction, the scalable GenomeLab GeXP system can monitor tens to hundreds of genes for up to tens of thousands of samples.

The GenomeLab GeXP system uses a combined gene-specific, universal priming strategy that converts multiplexed PCR to a two-primer process using universal primers. As a result, the gene ratio in RNA samples is maintained during the PCR process. This strategy overcomes the variations in amplification efficiency of different genes during the conventional amplification process without compromising the detection sensitivity.

Pre-formulated, ready-to-use GenomeLab GeXP system reagent kits are optimized for effortless and robust quantitative gene expression. The reagent kits are used for multiplexing user-defined genes.

The flow chart below depicts the gene expression workflow.

Figure 1-1 Gene Expression Workflow



Prepare to Use the GenomeLab GeXP System

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](#).

Reagents and Materials

Table 1-1 Required Materials from SCIEX

Material	Part Number
GenomeLab GeXP Start Kit, which contains the following: <ul style="list-style-type: none"> • RT Buffer 5×, 480 µL (green) • Reverse transcriptase, 120 µL at 20 units/µL (red) • PCR Buffer 5×, 480 µL (amber) • KAN^r RNA with RI, 600 µL (yellow) • DNase/RNase-free distilled water, 1,200 µL (white) • DNA Size Standard 400, 55 µL • Mineral oil, 5 mL • GenomeLab Sample Loading Solution, 6 mL 	A85017
Separation gel, 10 mL, for single-rail systems	608010
Separation gel, 20 mL, for dual-rail systems	391438
Buffer microplates, 96-well	609844
DNA separation capillary array	608087
GenomeLab Separation Buffer	608012
Sample microtiter plate, 96-well	609801
Thermo-Start <i>Taq</i> DNA Polymerase	A85022

Table 1-2 Additional Required Materials

Material	Manufacturer	Part Number
1 M Tris-HCl, pH 8.0	Thermo Fisher	15568025
Custom multiplex forward and reverse primers	Preferred supplier	N/A
DNase/RNase-free distilled water	Thermo Fisher	10977015
THE RNA Storage Solution	Thermo Fisher	AM7000

Customer-Supplied Equipment and Supplies

- Aerosol-resistant tips for P10, P20, P100, P200, and P1000 pipettors
- Aluminum foil seal
- Microplate centrifuge
- Microtube centrifuge
- Microtubes: 2.0 mL, 1.5 mL, and 0.65 mL
- Non-frost-free freezers (–80 °C and –20 °C)
- Optically clear flat cap strips for RT-PCR tubes
 - Optical Flat 8-Cap Strips for PCR Tubes (BioRad TCS0803)
 - Thermowell GOLD PCR 1 x 8 Optically Clear Flat Cap Strips, for RT-PCR (Corning 3742)
- Pipettors: P10, P20, P100, P200, and P1000
- Regular tips for P10, P20, P100, P200, and P1000 pipettors
- Thermal cycler with heated lid for 96-well plates
- Vortex mixer

Workspace

To prevent the contamination of general reagents and make sure that the reverse transcriptase (RT) and PCR reactions are clean, separated work areas and the following precautions are recommended.

Template-Free Area (Reagent Preparation Work Area)

Use this area to assemble general reagents into reaction mixtures in a nuclease-free and nucleic acid-free environment.

IMPORTANT:

Except for primers and nucleotides, do not use RNA, cDNA, DNA samples, or PCR products in this work area.

Template-Addition Area (RNA Work Area)

Use this area to do work with RNA samples such as Reference RNA or KAN^r RNA. Use this area to add RNA templates to cDNA reaction mixtures and cDNA templates to PCR reaction mixtures.

IMPORTANT:

Do not use PCR products in this work area.

GenomeLab GeXP System Run Sample Preparation Work Area

Use this area to prepare samples to run on the GenomeLab GeXP system. Do work with the final PCR products in this area to prevent DNA contamination in the RNA work area.

Select the RNA Template for Multiplex Optimization

It is important to consider the expected expression level of each gene in any particular tissue or RNA sample when running the initial tests of the designed primers. Failure to detect an appropriate PCR product can indicate that the primer design is not optimal or that the gene is not represented in the RNA sample tested. Knowledge of the target gene's expression profile in various tissues is essential when interpreting these initial test results. This information is available for many genes in the National Center for Biotechnology Information (NCBI) UniGene database at <http://www.ncbi.nlm.nih.gov/unigene>.

Mixtures of total RNAs from different tissues or cell lines are commercially available. These mixtures can decrease the bias present when using a single tissue or cell line's RNA composition. Alternatively, one can use a mixture of total RNA from the tissues or cell lines that will be analyzed individually with the final multiplex.

For example, the reference RNA can be a mixture of control and treated sample mixed at a 50:50 ratio. If there is more than one treated sample, the 50% of treated sample in the initial RNA should be a combination of equal contributions from all treated samples. Start by using 50 ng total RNA per reaction. Increase or decrease the total RNA for multiplex reactions based on the overall signal intensity. The overall goal is to reach a median state of expression for all of the genes represented in the multiplex.

A high-quality reference RNA consists of a mixture of all the experimental samples (control and treated, normal and disease, etc.) that will be assayed by the optimized multiplex.

- The mixture should contain all the gene transcripts represented in the multiplex.
- This mixture aims to achieve a moderate level of gene expression for each gene represented in the multiplex, which results in an optimal range for detection of gene expression for each gene.
- This is the same RNA that is used to evaluate and validate primers.
- Aliquots (25 μ L to 50 μ L) of a working stock of the reference RNA should be stored at a concentration of 100 ng/ μ L in a buffered RNA storage solution, such as THE RNA Storage Solution, at -80 °C.

RNA Isolation

The Beckman Agencourt RNA purification kits are recommended for total RNA isolation from FFPE samples (C16675), blood (A35603), cells (A47942), and tissue (A32645).

Preface

Quantitation

The quantity of RNA can be determined by $OD_{260\text{ nm}}$, whereas the quality of RNA can be assessed by the value of $OD_{260\text{ nm}}/OD_{280\text{ nm}}$. The acceptable range of $OD_{260\text{ nm}}/OD_{280\text{ nm}}$ is 1.8 to 2.0.

Multiplex Design and Optimization

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This protocol defines the process by which a custom multiplex is designed and optimized for gene expression analysis. The following procedures are designed for use with the GenomeLab GeXP Start Kit.

- Design primers using NCBI Primer-BLAST
- Evaluate primer and amplicon sequences
- Order primers
- Test single primer sets (singlets) on reference RNA templates
- Test the multiplex on reference RNA templates
- Analyze singlets and the multiplex reactions on the GenomeLab GeXP system
- Perform signal attenuation by reverse primer dilution to balance the gene expression profile
- Perform optimization of KAN^r RNA concentration
- Run optimized multiplex reactions with sample RNA templates

Multiplex Primer Design 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. Determine which genes to include in the multiplex for the study.
2. Select a correct accession number for each of the targeting genes. If possible, always use reference sequence (RefSeq) (NM_XXXXXX).

Multiplex Design and Optimization

Since the sequence represented by the accession number will be used to generate the multiplex primers, certain considerations should be made when selecting accession numbers. A single gene can be represented in the GenBank database by multiple accession numbers such as those that refer to genomic sequences, partial sequences, mutations, mRNA, ESTs, alternate transcripts, splice variants, or pseudogenes.

Ensure the following when selecting an accession number:

Correct gene	<ul style="list-style-type: none">• Many genes have multiple names and aliases.• Different genes can have similar names (such as IL2, IL2 receptor alpha, IL2 receptor beta, and IL2 receptor gamma).• Verify that the gene is from the species of interest.
Valid sequence	<ul style="list-style-type: none">• Verify that the sequence contains only the letters A, T, G, C, or N.• Verify that the sequence is for mRNA (cDNA).• Avoid genomic DNA sequences with introns.• Verify that the sequence is still active by reviewing the accession number's revision history. Use the following page: www.ncbi.nlm.nih.gov/entrez/sutils/girevhist.cgi .• RefSeq accession numbers that start with <i>NM_</i> are ideal choices for designing primers because they are from a curated, non-redundant database of known genes maintained by NCBI. The <i>NM_</i> accession prefix denotes mRNA sequences.• Each RefSeq accession number correlates to an individual identified transcript variant. A gene may have more than one RefSeq identifier.• Be aware of and avoid pseudogenes by BLASTing the gene against the genome before primer design to avoid undesigned peaks (UDPs). See the BLAST tool on the NCBI website (www.ncbi.nlm.nih.gov/Blast.cgi).• Mutations or repeats can also influence the results of primer design.• Homologous genes, pseudogenes, or conserved domains should also be considered when choosing a gene sequence.

Note: Accession numbers can be found on the NCBI website at www.ncbi.nlm.nih.gov. Use the tools and information (such as Entrez Nucleotide, Entrez Gene, Homologene, Unigene, Blastn, or OMIM) to aid in identifying a valid accession number.

3. For genes with transcript variants, decide how many variants to detect.

Note: Be aware of transcript variants before primer design to avoid UDPs. See Tools on the NCBI website (<http://www.ncbi.nlm.nih.gov/gene>).

4. Select regions that do not have high homology to gene family members or regions without pseudogenes.
5. Choose reference genes.
 - Ideally, genes selected for normalization purposes should be expressed stably in all samples under all conditions.
 - To determine which genes are expressed stably, assay the reference genes in experimental samples with a custom multiplex.
 - To monitor the stability of gene expression between samples, normalize the peak areas of the reference genes, at minimum, to the KAN^r peak area in the CSV Transformer Tool and compare the resulting relative expression values between samples.
 - Select the reference genes with the most stable expression between samples as the normalization genes.
 - The greater the number of normalization genes used, the lower the chance of any one of those genes affecting the normalization factor. Therefore, if there is some fluctuation in expression of one reference gene, the effect will be diluted by the presence of the other reference genes.

Note: Several reference genes can be used as internal controls for normalizing the gene expression level across samples.

Note: The ability to select multiple reference genes and use the calculated geometric mean of expression for these genes to normalize expression in each well is a powerful tool in the XP-PCR process.

6. Design amplicons with sizes in the range of 105 nt to 350 nt (without universal tags).
7. Make sure that the spacing between amplicons in the same multiplex is at least 5 nucleotides.

Primer Design Using NCBI Primer-BLAST

1. Go to the website: <http://www.ncbi.nlm.nih.gov/tools/primer-blast/>.
2. In the PCR Template section, enter the accession number or FASTA sequence.
3. In the Primer Parameters section:
 - a. For **PCR product size Min**, enter **105**. For FFPE samples, enter **63**.

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- b. For **PCR product size Max**, enter **350**. For FFPE samples, enter **150**.
 - c. For **Primer melting temperatures**, accept the default settings. Ensure that the Max T_m difference between the forward and the reverse primers is no more than 5.
4. In the Exon/Intron selection section:
 - a. From the **Exon junction span** list, select **Primer must span an exon-exon junction**.
 - b. Select the **Intron inclusion** check box.
 - c. Modify **Intron length range Min** as needed.
5. In the Primer Pair Specificity Checking Parameters section:
 - a. Select the **Specificity check** check box.
 - b. In the **Database** list, select **Refseq mRNA**.
 - 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**.
7. In the Primer Parameters section:
 - a. From the **Salt correction formula** list, select **Schildkraut and Lifson 1965**.
 - b. From the **Table of thermodynamic parameters** list, select **Breslauer et al. 1986**.
 - c. Select the **SNP handling** check box.
8. Click **Get Primers** to submit your search request.

The NCBI website will return search results that contain information about the primer pair(s).

Assemble a Multiplex

1. Start with designing primers for genes that require targeting to a specific region in order to detect desired number of transcript variants or to avoid pseudogene or highly homologous region among gene family members. Set PCR product size range as 105 nt to 350 nt to generate multiple primer pairs. Select the best primer pair based on evaluation of primer and amplicon sequences. In a Microsoft Excel worksheet, record the gene name, the primer sequence, primer positions, the T_m for the forward primer, T_m for the reverse primer, and the amplicon size (without universal tags) for the chosen primer pair. Save this file with the name of the multiplex.
2. Include primers that were previously validated for a gene target or a reference gene. Record primer and amplicon information as in step 1.
3. For the remaining gene targets in the multiplex, design amplicons with smaller sizes first, then, work toward larger amplicon sizes. To ensure a desired amplicon size is assigned for

a particular gene target, set the minimum and the maximum PCR product size at the same value.

For example, if 133 nt is the desired amplicon size, enter **133** both as the minimum PCR product size, and as the maximum PCR product size during primer design. Record primer and amplicon information as in step 1 for each gene target. Ensure the amplicons are at least 5 nt apart.

4. In the Microsoft Excel worksheet, create a new column named **Amplicon size with Universal tags**.

The amplicon size with universal tags is calculated by adding 37 nt to the amplicon size without universal tags.

5. In the Microsoft Excel worksheet, create a column named **reverse primer with tag**, copy and paste the reverse universal tag sequence at the 5' end of gene-specific reverse primer sequence.
6. In the Microsoft Excel worksheet, create a column named **forward primer with tag**, copy and paste the forward universal tag sequence at the 5' end of gene-specific forward primer sequence.
7. Click **Save**.

Note: Contact SCIEX Technical Support for sequences of universal tags.

Evaluate the Primer and Amplicon Sequences Using NCBI BLAST Searches

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. Use NCBI BLAST with species-specific searches to determine if the amplicon for each primer set contains homologous regions and if so, where those regions lie. BLASTing against the RefRNA database is helpful for homology.
 - If the homologous regions are within the reverse primer sequence, redesign the primer to target a region of low homology, particularly at the 3' end of the primer.
 - If there is extensive homology between two genes within the same multiplex, be sure to design primers to unique regions of each gene in order to prevent cross amplification.
4. If primers were designed using primer 3, not using NCBI Primer-BLAST, evaluate primer and amplicon sequences by performing the following:

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- a. Examine each primer for single nucleotide polymorphisms (SNPs) with BLAST-SNP, especially at the 3' end, which can lead to the preferential amplification of one allele over another. If a polymorphism is found, redesign the primer to a more conserved region.
- b. Examine the amplicon sequence, generated by each set of primers, for repeat sequences.
 - Repeat sequences can lead to Thermo-Start *Taq* DNA Polymerase slippage and result in stutter peaks.
 - If 5 or more consecutive single or di-nucleotide repeats or a series of such repeats in close proximity to each other are present, redesign the primers to target an amplicon without repeats.
- c. BLASTing against a genomic database will help determine if any pseudogene expression may be detected.

Any set of primers that has the potential to generate a UDP within the size range of multiplex should be redesigned to prevent the production of a UDP.

Once the primer and amplicon sequences have been reviewed, update the Microsoft Excel worksheet for the multiplex. Proceed with ordering and testing the multiplex primers. Order the primers with the appropriate universal tag sequence.

IMPORTANT:

Do not order the KAN^r primers. They are included in the GenomeLab GeXP Start Kit.

Test Individual Primer Pairs

After the gene-specific primer sequences are determined to be suitable multiplex primers, order the corresponding primers with universal primer tags from an oligo manufacturer, such as Sigma or Integrated DNA Technologies (IDT). Order the smallest scale for primer synthesis. This amount is usually adequate for primer evaluation. Standard desalted, deprotected oligos are typically of sufficient purity to use in the multiplex format. Once received, resuspend each primer at 100 μ M stock concentration in resuspension buffer (10 mM Tris-HCl, pH 8.0, DNase/RNase-free distilled water). Consult with the manufacturers for specific information on resuspending and storage of the oligos.

Note: For a list of reagents, materials and equipment, refer to the section: [Prepare to Use the GenomeLab GeXP System](#).

Test the Forward Singlet: Reverse Plex

IMPORTANT:

This process is essential for evaluating the primers and optimizing the multiplex.

1. Prepare a 10× (500 nM) reverse multiplex by combining each reverse primer in a total of 1 mL with resuspension buffer.
2. Prepare individual 10× (200 nM) working solutions for each forward primer, for a total of 1 mL in resuspension buffer.
3. To run each forward primer in a PCR reaction on cDNA produced by the complete reverse multiplex to assess any unintended PCR products produced by primer pairs across genes, use the chemistry protocol for singlet reactions in the section: [Chemistry Protocol for Singlet Reactions](#). In addition, run a PCR reaction without any forward primer.
4. Evaluate the results of the singlet experiments for the following:
 - Presence of an appropriately-sized PCR fragment
 - Absence of UDPs that would interfere with the quantitation of designed peaks in the multiplex
 - Sufficient signal above the background noise of the PCR reaction
 - No UDP present in the PCR reaction without forward primer

If any of the primer pairs fail to meet the criteria above, consider a different RNA template (ideally in a sample where the gene is expected to be upregulated) or redesign the primers in a different region of the sequence.

Test the Forward Singlet: Reverse Singlet

Note: This process is optional and especially useful for troubleshooting primer designs.

Each gene's primer pair can be tested in an independent two-step RT-PCR reaction to verify that the primers produce the expected fragment and assess the number and sizes of any unintended PCR products.

1. Prepare a 10× (500 nM) working solution of each reverse primer in resuspension buffer.
2. Prepare a 10× (200 nM) working solution of each forward primer in resuspension buffer.
3. Use the chemistry protocol for singlet reactions below to run each forward primer in a PCR reaction on cDNA produced by the complementary reverse primer, to assess if the intended PCR fragment is produced.
4. Evaluate the results of the singlet experiments for the following:
 - Presence of an appropriately-sized PCR fragment
 - Absence of UDPs that would interfere with the quantitation of designed peaks in the multiplex
 - Sufficient signal above the background noise of the PCR reaction

- No UDP present in the PCR reaction without forward primer

If any of the primer pairs fail to meet the criteria above, consider a different RNA template (ideally in a sample where the gene is expected to be upregulated) or redesign the primers in a different region of the sequence.

Chemistry Protocol for Singlet Reactions

RT Reaction

Calculate Reagent Volumes

Determine the number of RT reactions that need to be performed. Increase the number of reactions by 10%, rounding up to the next reaction, to make sure that the volume is sufficient. Complete all singlet reactions in duplicate.

Prepare the No-Template Control (NTC) Reaction Mix

1. In the template-free area, add the following reaction components to a 1.5 mL microcentrifuge tube labeled with **NTC**.

Note: Add the reverse transcriptase last to preserve enzyme activity.

NTC Reaction Mix	Volume per Reaction
DNase/RNase-free distilled water	3 μ L
RT Buffer 5 \times	4 μ L
Reverse transcriptase	1 μ L

2. Gently mix the components by pipetting up and down.
3. In the template-addition area, pre-dilute the KAN^f RNA with RI in DNase/RNase-free distilled water. The recommended initial dilution is 1:50 with a range from 1:10 to 1:100. The final KAN^f concentration will be optimized in later steps.

Note: Every time a stock solution of KAN^f RNA with RI is thawed, make 10 μ L 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.

4. Add the pre-diluted KAN^f solution to the RT Minus Reaction Mix prepared above and gently mix.

NTC Reaction Mix	Volume per Reaction
Pre-diluted KAN ^r RNA with RI	5 µL

- Keep this mixture on ice until ready to proceed.

Prepare the RT Minus Reaction Mix

- In the template-free area, add the following reaction components to a 1.5 mL microcentrifuge tube labeled with **RT Minus**.

RT Minus Reaction Mix	Volume per Reaction
DNase/RNase-free distilled water	4 µL
RT Buffer 5×	4 µL

- Gently mix the components by pipetting up and down.
- In the template-addition area, pre-dilute the KAN^r RNA with RI in DNase/RNase-free distilled water. The recommended starting dilution is 1:50 with a range from 1:10 to 1:100. The final KAN^r concentration will be optimized in later steps.
- Add the pre-diluted KAN^r solution to the RT Minus Reaction Mix prepared above and gently mix.

RT Minus Reaction Mix	Volume per Reaction	Volume per Mix (3 reactions)
Pre-diluted KAN ^r RNA with RI	5 µL	15 µL

- Keep this mixture on ice until ready to proceed.

Prepare the Standard Reaction Mix

- In the template-free area, add the following reaction components to a 1.5 mL microcentrifuge tube labeled with **Standard**.

Note: Add the reverse transcriptase last to preserve enzyme activity.

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RT Plus Reaction Mix	Volume per Reaction	Volume per Mix (40 reactions)
DNase/RNase-free distilled water	3 μ L	120 μ L
RT Buffer 5 \times	4 μ L	160 μ L
Reverse transcriptase	1 μ L	40 μ L

2. Gently mix the components by pipetting up and down.
3. In the template-addition area, pre-dilute the KAN^f RNA with RI in DNase/RNase-free distilled water. The recommended starting dilution is 1:50 with a range from 1:10 to 1:100. The final KAN^f concentration will be optimized in later steps.
4. Add the pre-diluted KAN^f solution to the RT Plus Reaction Mix prepared above and gently mix.

RT Plus Reaction Mix	Volume per Reaction	Volume per Mix (40 reactions)
Pre-diluted KAN ^f RNA with RI	5 μ L	200 μ L

5. Keep the mixture on ice until ready to proceed.

Prepare the RT Reaction Sample Plates

Note: To avoid edge effects, do not use the wells on the outer edge of the thermal cycler.

1. Add 2 μ L of the 10 \times reverse multiplex (500 nM) **or** 2 μ L of an individual 10 \times reverse singlet primer (500 nM) directly to the appropriate well of a labeled 96-well plate.

Note: The final standard concentration for each reverse primer is 50 nM in the 20 μ L RT reaction.

2. Aliquot 13 μ L of the RT reaction mixes to the appropriate wells of a labeled 96-well plate. Use the 96-well plate coolers to keep the reagents cold or put them on ice.
3. Dilute the reference RNA to 5 ng/ μ L to 20 ng/ μ L with DNase/RNase-free distilled water. The amount of reference RNA per reaction can range from 25 ng to 100 ng depending on the gene expression levels in the samples.

Note: For instructions on how to formulate a reference RNA for singlet reactions, refer to the section: [Select the RNA Template for Multiplex Optimization](#).

4. Add 5 μL of the diluted reference RNA (5 $\text{ng}/\mu\text{L}$ to 20 $\text{ng}/\mu\text{L}$) to each Standard and RT Minus well.
5. For each NTC well, add 5 μL of DNase/RNase-free distilled water in place of the RNA template.
6. Gently mix the components by pipetting up and down.

Note: Do not create air bubbles.

7. Tightly cover the plate with the strip caps.
8. Using a centrifuge, spin the plate for 1 min at 2,000 rpm to bring the contents to the bottom of the wells.

Run the RT Reaction

1. Put the RT reaction sample plate in the thermal cycler and close the lid.
2. Run the following incubation program with the correct reaction volume (20 μL) and a heated lid:

Temperature	Time
48 °C	1 min
42 °C	60 min
95 °C	5 min
4 °C	Hold

PCR Reaction

Calculate Reagent Volumes

Determine the number of PCR reactions that need to be performed. Increase the number of reactions by 10%, rounding up to the next reaction, to make sure that the volume is sufficient. Complete all singlet reactions in duplicate.

Prepare the PCR Reaction Mix

Add the following to a microcentrifuge tube labeled **PCR Mix** and gently mix.

Note: Add the Thermo-Start *Taq* DNA Polymerase last to preserve enzyme activity.

Multiplex Design and Optimization

PCR Reaction Mix	Volume per Reaction
25 mM MgCl ₂	4.0 µL
PCR Buffer 5×	4.0 µL
Thermo-Start <i>Taq</i> DNA Polymerase	0.7 µL
Total Volume	8.7 µL

Prepare the PCR Reaction Sample Plates

Note: To avoid edge effects, do not use the wells on the outer edge of the thermal cycler.

1. Aliquot 2 µL of each 10× forward singlet primer (200 nM) to the appropriate wells of a 96-well PCR plate.

Note: For the PCR reaction without forward primer, add 2 µL of DNase/RNase-free distilled water.

2. Aliquot 8.7 µL of the PCR reaction mix to the appropriate wells of a 96-well PCR plate.
3. Using a centrifuge, spin the plate for 1 min at 2,000 rpm to bring the contents to the bottom of the wells.
4. Transfer 9.3 µL of the template cDNA samples (completed RT reactions) from the RT reaction plate to the corresponding wells on the PCR plate.
5. Gently mix the components by pipetting up and down.

Note: Do not create air bubbles.

6. Tightly cover the plate with the strip caps.
7. Using a centrifuge, spin the plate for 1 min at 2,000 rpm to bring the contents to the bottom of the wells.

Run the PCR Reaction

1. Put the sample plate in the thermal cycler.
2. Run the following thermal cycling program with the correct reaction volume (20 µL) and a heated lid:

Step	Temperature	Time
1	95 °C	10 min
2	94 °C	30 s
3	55 °C	30 s
4	68 °C to 70 °C (see Note)	1 min
5	N/A	Repeat steps 2 through 4 for an additional 34 cycles (total of 35 cycles)
6	4 °C	Hold

Note: The optimal extension temperature can vary depending on the multiplex. Refer to the multiplex kit insert for specific instructions. For custom multiplexes, the recommended starting temperature is 70 °C.

- For instruction on loading and running samples, refer to the section: [Load and Run Samples](#).

Test and Optimize Multiplex Primers

The singlet RT-PCR reactions that occur within the PCR process can differ from those in multiplexed reactions. Signals that were high in singlet reactions do not necessarily result in high signals in the multiplex. Therefore, it is important to characterize the products of the primer pairs in a multiplexed reaction.

- Assemble a 10× (500 nM) reverse multiplex primer mix by combining the reverse primers at 500 nM each in resuspension buffer.
- Assemble a 10× (200 nM) forward multiplex primer mix by combining the forward primers at 200 nM each in resuspension buffer.
- Assemble the multiplex RT and PCR reactions as described in the section: [Perform Multiplex Gene Expression Analysis](#).
- Run the multiplex RT and PCR reactions on the GenomeLab GeXP system.
- Determine if all the primer sets in the multiplex produce a detectable signal.
- For each gene, compare the estimated fragment size in the singlet results to the corresponding peak in the multiplex results.

Troubleshoot Primer Design for Multiplex Optimization

A multitude of factors affect the efficiency of PCR primers in a multiplexed format. Unintended results can be minimized by following the guidelines that were previously suggested. Refer to the section: [Pre-Design Considerations](#). Additional troubleshooting could be required to improve the quality of the multiplex assay.

High Signals

Any gene signals that are close to or above the linear detection limit of the GenomeLab GeXP system detector (130,000 RFU raw data or 120,000 RFU analyzed data) in the multiplex reaction will require pre-dilution and/or primer attenuation. For more information, refer to the section: [Attenuate the GeXP Signals](#).

Missing Signals

A lack of signal can be caused by interference from another primer in the multiplex. To test for primer interference, perform duplex reactions by combining the affected primer set with each of the remaining primer sets in the multiplex to determine which primer set is causing the interference.

Alternatively, the gene could be suppressed or not expressed in the RNA sample that was used to evaluate the multiplex, test other RNA samples to verify a gene product. Some genes can be undetectable unless induced in a biological system such as an animal model or cell line. Choose RNA in which the gene in question has higher representation.

A single gene peak of the appropriate size detected in a singlet reaction indicates that a primer pair is capable of amplifying a specific gene target. However, if the signal of the gene peak is significantly lower than other genes in the multiplex profile and it is not a low expresser, something may be interfering with detection of this gene in multiplex format.

The attenuation of high expressers can sometimes remedy this problem. Attenuation attempts to balance the signals from high expressers and low expressers within the linear range of detection for the GenomeLab GeXP system. The attenuation process performed during multiplex optimization is described in the section: [Determine the Reverse Primer Concentration for High-Expressing Genes](#).

Co-Migrating Peaks

If two or more different PCR products of similar size are indistinguishable in capillary electrophoresis, it is because they have co-migrated and the result is an additive effect on the peak area. This will affect the relative quantitation accuracy for the gene assigned to the particular fragment size.

Results from the singlet experiments above should alert the designer to any potential for co-migrating peaks. Another method to confirm the source of a co-migrating UDP is to run a Plex-1 (plex minus one) experiment in which the multiplex is run without the primer pair suspected of contributing the co-migrating peak. If the co-migrating peak is still present in the absence of the suspect primer pair, review the singlet experiments or run a variety of follow-up experiments to identify the primer pair responsible for the undesigned peak, then redesign the primer(s).

Often, the most direct resolution is to redesign the primer(s) for the designed peak so that this peak no longer co-migrates with the UDP.

Unintended Peaks

The unintended amplification of sequences, other than the selected target gene, can occur due to high homology and conserved sequences among various genes, undiscovered gene sequences, pseudogenes or alternate transcripts.

Since peak identification is based on the target gene's fragment size and relative quantitation of gene expression is based on peak area, the elimination of the unintended signals is integral to the process at several stages. First, exclusion filters can be applied to the list of identified fragments in Fragment Analysis module on the GenomeLab GeXP system. Second, the binning of the multiplex fragments in the Peak Binning section of GenomeLab System software excludes any signals outside of the user-adjustable bins.

To discriminate between genes with highly conserved regions and/or high homology to other members within the same gene family, obtain an alignment of the highly homologous genes including the gene of interest. Several online tools are available for generating alignments such as ClustalW[EBI, UK]: <http://www.ebi.ac.uk/Tools/clustalw/index.html>. By designing primers with one or two 3' mismatched bases to the unwanted sequence, discrimination between similar sequences improves.

Many highly homologous sequences can contain dissimilar 5' or 3' untranslated regions. Primer design can be directed to these regions.

If an unintended peak co-migrates within three nucleotides of a designed peak in the multiplex, has high signal, and/or affects the quantitation of another gene peak, then redesign primers that cause this unintended peak. Assess the significance of an unintended peak in the singlet reaction with the primers that cause the unintended peak when the peak height of the designed peak is below 120,000 RFU.

Attenuate the GeXP Signals

To keep the overall fluorescent signal within the linear range of detection, pre-dilute the PCR products before analysis on the GenomeLab GeXP system. To create balance with the rest of the multiplex profile, attenuate the signal of high expressers and KAN^r, as necessary.

Determine the Appropriate PCR Product Dilution

1. Run the standard GenomeLab GeXP system multiplex protocol using 50 nM of each of the gene-specific reverse primers in each reaction.
2. Generate the following dilutions of the resulting PCR products:

Multiplex Design and Optimization

PCR Pre-Dilution	Sample Loading Solution / DNA Size Standard 400 Dilution	Total Dilution
Undiluted reaction	1 μ L to 39 μ L (1:40)	1:40
2 μ L to 8 μ L 10 mM Tris-HCl, pH 8.0 (1:5) (see Note)	1 μ L to 39 μ L (1:40)	1:200
2 μ L to 18 μ L 10 mM Tris-HCl, pH 8.0 (1:10) (see Note)	1 μ L to 39 μ L (1:40)	1:400
2 μ L to 38 μ L 10 mM Tris-HCl, pH 8.0 (1:40) (see Note)	1 μ L to 39 μ L (1:40)	1:800

Note: Prepare 10 mM Tris-HCl, pH 8.0 from 1 M Tris-HCl, pH 8.0 : DNase/RNase-free distilled water = 1:99 (v/v).

3. Determine the optimal PCR dilution at which the:

- Lowest gene signal is still detectable
- Background noise or baseline is minimized
- Highest gene signal has sufficient potential dynamic range

IMPORTANT:

If none of the dilutions allow for sufficient dynamic range, appropriate reverse primer attenuation must be performed. Continue by following the attenuation procedure to satisfy requirements for the lowest gene signal and background noise as referenced above, then revisit the dilution factor parameter once the proper attenuation has been determined.

Typically, as signals from high expressers are attenuated down, the weaker signals increase. Although the dilution factor can bring the weak signals close to the baseline, the risk of losing the signal below the minimum detectable level is low.

Determine the Reverse Primer Concentration for High-Expressing Genes

If one or several genes signals are close to or above the linear range of the GenomeLab GeXP system detector (> 130,000 RFU in raw data or > 120,000 RFU in analyzed data), when run at the dilution factor determined above, they will require attenuation by lowering the reverse primer concentration relative to the other reverse primers in the multiplex.

Choose a target gene peak from the multiplex profile that represents the median peak height of the multiplex. The target peak serves as a benchmark by which a balanced multiplex profile is designed through optimization of reverse primer concentrations. The reverse primers of all peaks with signals greater than the target peak should be attenuated. Gene peaks with signals significantly less than the target peak should be considered for increased reverse primer concentration.

Run serial dilutions of the reverse primers of the genes that will be attenuated with the remaining reverse primers at the normal 50 nM per RT reaction as follows:

1. Generate a 500 nM reverse primer mix without the primers of the attenuated genes (10× non-attenuated primer pool).

Note: To increase the signal intensity of one or more exceptionally low peaks, increased amounts of reverse primer (up to 1,500 nM) may be added to this mix for those gene peaks.

2. Generate a 500 nM reverse primer mix of all of the primers that are to be attenuated (10× attenuated primer pool).
3. Serially dilute the 10× attenuated primer pool with two-fold dilutions over 8 concentrations (10× concentrations of 500 nM to 3.9 nM).
4. Assemble and run the RT reaction as follows (for each reaction):

Reaction Mix	Volume
DNase/RNase-free distilled water	1 µL
RT Buffer 5×	4 µL
10× non-attenuated primer pool	2 µL
10× attenuated primer pool (dilution)	2 µL
Reverse transcriptase	1 µL
Pre-diluted KAN ^r RNA with RI (see Note)	5 µL
Control template	5 µL
Total	20 µL

Note: Determine the optimal KAN^r RNA concentration using the protocol described in the section: [Optimize the KAN^r RNA Concentration](#). Then, pre-dilute the KAN^r RNA in a buffered solution (such as THE RNA Storage Solution) to stabilize the RNA.

5. Perform the standard forward multiplex PCR protocol with cDNA generated from the RT reaction above.

Multiplex Design and Optimization

6. Run the PCR products on the GenomeLab GeXP system using the PCR dilution determined above.

For detailed instructions on running samples, refer to the document: *GenomeLab GeXP Genetic Analysis System User Guide*.

Analyze the Attenuation Results

Observe the signal levels of all the multiplex products at each reverse primer concentration. Evaluate the results to determine if the:

- Signals of the attenuated genes fall within the linear range of the GenomeLab GeXP system detector and have sufficient dynamic range to measure changes in gene expression
 - The recommended range is approximately 2,000 RFU to 50,000 RFU for most genes over all eight capillaries
- Signal levels of other genes in the multiplex remain in the linear range and have sufficient dynamic range

In many cases, optimal attenuation occurs at different primer concentrations for different genes. For each gene, choose the concentration at which the gene peak is approximately equal in signal to the target gene peak. Also, attenuation of one gene signal can raise a non-attenuated gene signal to a level above the linear range. A systematic, repetitive approach to attenuation and primer dilution optimization is often necessary to bring all signals to the desired levels.

More often than not, the attenuation of high expressers will result in an increased signal of the low expressers. However, if the attenuation does not result in an adequate increase of the low expresser signal, the reverse primer concentration for those genes can be raised up to 150 nM per reaction (1.5 μ M in reverse multiplex).

After the optimum primer concentrations are known, determine the appropriate PCR dilution. Refer to the section: [Determine the Appropriate PCR Product Dilution](#).

Optimize the KAN^r RNA Concentration

The purpose of testing multiple concentrations of KAN^r RNA in the multiplex reaction is to optimize the KAN^r peak height relative to the gene peaks such that it can be used for calculating the relative signals for the other genes in the multiplex. The ideal peak height for the KAN^r peak is approximately equal to or slightly below the median signal range of the multiplex.

First, make an initial 1:50 dilution of the KAN^r RNA in water to assay with the initial multiplex evaluation reactions in order to determine if the amount of KAN^r RNA should be titrated up or down. Dilute just enough KAN^r RNA to perform these initial, evaluation reactions and use 5 μ L of the 1:50 dilution per reaction.

Based on the initial results with the 1:50 dilution, titrate up or down the amount of KAN^r RNA used in subsequent reactions. For example, if the KAN^r peak is too low relative to the gene peaks, use more KAN^r RNA per reaction. If the KAN^r peak is too high, use less.

Note: The KAN^f RNA concentration can be optimized together with reverse primer concentration optimization. The optimized KAN^f peak height should be about half the peak height of the target peak designated for primer concentration optimization.

1. Dilute KAN^f RNA over a series of dilutions, lower or higher than the initial 1:50 dilution. For the diluent, use THE RNA Storage Solution or DNase/RNase-free distilled water.

The original vial contains 254 femtomolar of KAN^f RNA with RI.

Table 2-1 KAN^f RNA Dilution Series

Dilution	Original Vial	1	2	3	4	5	6
Volume from previous dilution (μL)	-	4	25	25	25	25	25
Volume of diluent (μL)	-	46	25	25	25	25	25
Final concentration (femtomolar)	254	20.3	10.2	5.1	2.5	1.3	0.6
Mixed volume transferred to the next dilution (μL)	4	25	25	25	25	25	25
Final volume (μL)	-	25	25	25	25	25	25
Final dilution factor	Undiluted	1:12.5	1:25	1:50	1:100	1:200	1:400

2. Add 5 μL of each dilution of KAN^f RNA to a separate RT reaction that contains the pre-determined optimal amount of reference RNA (5 ng to 100 ng) per reaction. This is the same amount of sample RNA that will be used for experimental multiplex reactions.

Note: It is recommended that each KAN^f RNA dilution be assayed in duplicate or triplicate.

Once the optimal KAN^f RNA dilution is determined for a particular multiplex, use the same KAN^f RNA concentration for all future reactions with the optimized multiplex.

Confirm the PCR, Reverse Primer, and KAN^f Dilutions

Once the appropriate concentrations of reverse primers and KAN^f RNA have been determined for a given reference RNA sample, assemble and run the optimized multiplex against a panel of RNA samples from various sources. This will verify the optimized multiplex conditions for RNA samples that can have varying gene expression profiles. Additional total RNA templates from various species, tissues, and cell lines are available from a number of suppliers. Expected expression

Multiplex Design and Optimization

levels, cell and tissue types, experimental conditions, and quality of the RNA (no contaminating genomic DNA) should be considered when assembling the panel of RNA samples.

Even with different expression levels between RNA samples, all of the multiplex signals should remain within the linear range of detection. If one or more gene signals exceeds the linear range of detection, the high gene signal(s) must be further attenuated and retested against the panel of RNA samples.

Adjust the amount of total RNA or determine the PCR product dilution again to make sure that all peaks are within range. Refer to the section: [Determine the Appropriate PCR Product Dilution](#).

IMPORTANT:

Once the parameters are optimized for a particular multiplex, they should remain fixed throughout a study.

Use the Optimized Custom Multiplex

Once the reaction conditions for a custom multiplex are optimized, experimental samples are assayed using the standard multiplex gene expression analysis protocol. Refer to the section: [Perform Multiplex Gene Expression Analysis](#).

Multiplex Gene Expression Analysis

3

The following protocol is designed for performing multiplex gene expression analysis with the GenomeLab GeXP Start Kit in combination with an optimized custom multiplex.

Perform Multiplex Gene Expression Analysis

Do the following to perform a multiplex gene expression analysis of experimental samples:

1. Set up the RT reactions.
2. Run the RT reactions.
3. Set up the PCR reactions.
4. Run the PCR reactions.
5. Prepare samples for analysis.
6. Load, run, and analyze samples on the GenomeLab GeXP system.
7. Analyze the data using the GenomeLab GeXP software, CSV Transformer Tool, and GeXP Analysis Template.

Note: For a list of reagents, materials, and equipment, refer to the sections: [Reagents and Materials](#) and [Customer-Supplied Equipment and Supplies](#).

RT Reaction

Calculate Reagent Volumes

Determine the number of RT reactions that need to be performed. Increase the number of reactions by 10%, rounding up to the next reaction, to make sure that the volume is sufficient. For a full plate, 110 reactions supply sufficient volume to do multichannel pipetting. Perform at least three technical replicates of the standard reaction for each biological sample.

Prepare the No-Template Control (NTC) Reaction Mix

1. In the template-free area, add the following reaction components to a 1.5 mL microcentrifuge tube labeled with **NTC**.

Multiplex Gene Expression Analysis

Note: Add the reverse transcriptase last to preserve enzyme activity.

NTC Reaction Mix	Volume per Reaction
DNase/RNase-free distilled water	3 μ L
RT Buffer 5 \times	4 μ L
RT Reverse Primer Plex (see Note)	2 μ L
Reverse transcriptase	1 μ L

Note: The user must design and prepare this primer plex when using the GenomeLab GeXP Start Kit. Refer to the section: [Multiplex Primer Design Using NCBI Primer-BLAST](#).

2. Gently mix the components by pipetting up and down.
3. In the template-addition area, add the following to the mixture prepared above and gently mix.

NTC Reaction Mix	Volume per Reaction
Pre-diluted KAN ^r RNA with RI	5 μ L

4. Keep this mixture on ice until ready to proceed.

Prepare the RT Minus Reaction Mix

1. In the template-free area, add the following reaction components to a 1.5 mL microcentrifuge tube labeled with **RT Minus**.

RT Minus Reaction Mix	Volume per Reaction
DNase/RNase-free distilled water	4 μ L
RT Buffer 5 \times	4 μ L
RT Reverse Primer Plex (see Note)	2 μ L

Note: The user must design and prepare this primer plex when using the GenomeLab GeXP Start Kit. Refer to the section: [Multiplex Primer Design Using NCBI Primer-BLAST](#).

- Gently mix the components by pipetting up and down.
- In the template-addition area, add the following to the mixture prepared above and gently mix.

RT Minus Reaction Mix	Volume per Reaction
Pre-diluted KAN ^r RNA with RI	5 µL

- Keep this mixture on ice until ready to proceed.

Prepare the Standard Reaction Mix

- In the template-free area, add the following reaction components to a 1.5 mL microcentrifuge tube labeled with **Standard**.

Note: Add the reverse transcriptase last to preserve enzyme activity.

RT Plus Reaction Mix	Volume per Reaction
DNase/RNase-free distilled water	3 µL
RT Buffer 5×	4 µL
RT Reverse Primer Plex (see Note)	2 µL
Reverse transcriptase	1 µL

Note: The user must design and prepare this primer plex when using the GenomeLab GeXP Start Kit. Refer to the section: [Multiplex Primer Design Using NCBI Primer-BLAST](#).

- Gently mix the components by pipetting up and down.
- In the template-addition area, add the following to the mixture prepared above and gently mix.

RT Plus Reaction Mix	Volume per Reaction
Pre-diluted KAN ^r RNA with RI	5 µL

- Keep this mixture on ice until ready to proceed.

Multiplex Gene Expression Analysis

Prepare the RT Reaction Sample Plates

Note: To avoid edge effects, do not use the wells on the outer edge of the thermal cycler.

1. Aliquot 15 μL of the RT reaction mixes to the appropriate wells of a labeled 96-well plate. Use the 96-well plate coolers to keep the reagents cold or put them on ice.
2. Dilute the control or reference RNA templates to 5 ng/ μL to 10 ng/ μL with DNase/RNase-free distilled water. Refer to the specific multiplex kit insert for the recommended working concentration.

Note: For a custom multiplex control RNA template, use the RNA mixture that was developed for multiplex optimization. For more information, refer to the section: [Select the RNA Template for Multiplex Optimization](#).

3. Dilute the sample RNA to 5 ng/ μL to 20 ng/ μL with DNase/RNase-free distilled water. The amount of sample RNA per reaction can range from 25 ng to 100 ng depending on the gene expression levels in the samples.
4. Add 5 μL of the desired sample RNA (5 ng/ μL to 20 ng/ μL) or diluted control RNA templates (5 ng/ μL to 10 ng/ μL) to each Standard and RT Minus well.
5. For each NTC well, add 5 μL of DNase/RNase-free distilled water in place of the RNA template.
6. Gently mix the components by pipetting up and down.

Note: Do not create air bubbles.

7. Tightly cover the plate with the strip caps.
8. Using a centrifuge, spin the plate for 1 min at 2,000 rpm to bring the contents to the bottom of the wells.

Run the RT Reaction

1. Put the RT reaction sample plate in the thermal cycler and close the lid.
2. Run the following incubation program with the correct reaction volume (20 μL) and a heated lid:

Temperature	Time
48 °C	1 min
42 °C	60 min

Temperature	Time
95 °C	5 min
4 °C	Hold

PCR Reaction

Calculate Reagent Volumes

Determine the number of PCR reactions that need to be performed. Increase the number of reactions by 10%, rounding up to the next reaction, to make sure that the volume is sufficient. For a full plate, 110 reactions supply sufficient volume to do multichannel pipetting. Perform at least three technical replicates for each biological sample.

Prepare the PCR Reaction Mix

Add the following to a microcentrifuge tube labeled **PCR Mix** and gently mix.

Note: Add the Thermo-Start *Taq* DNA Polymerase last to preserve enzyme activity.

PCR Reaction Mix	Volume per Reaction
25 mM MgCl ₂	4.0 µL
PCR Buffer 5×	4.0 µL
PCR Forward Primer Plex (see Note)	2.0 µL
Thermo-Start <i>Taq</i> DNA Polymerase	0.7 µL
Total Volume	10.7 µL

Note: The user must design and prepare this primer plex when using the GenomeLab GeXP Start Kit. Refer to the section: [Multiplex Primer Design Using NCBI Primer-BLAST](#).

Prepare the PCR Reaction Sample Plates

Note: To avoid edge effects, do not use the wells on the outer edge of the thermal cycler.

1. Aliquot 10.7 µL of the PCR reaction mix to the appropriate wells of a 96-well PCR plate.
2. Using a centrifuge, spin the plate for 1 min at 2,000 rpm to bring the contents to the bottom of the wells.

Multiplex Gene Expression Analysis

- Transfer 9.3 μL of the template cDNA samples (completed RT reactions) from the RT reaction plate to the corresponding wells on the PCR plate.
- Gently mix the components by pipetting up and down.

Note: Do not create air bubbles.

- Tightly cover the plate with the strip caps.
- Using a centrifuge, spin the plate for 1 min at 2,000 rpm to bring the contents to the bottom of the wells.

Run the PCR Reaction

- Put the sample plate in the thermal cycler.
- Run the following thermal cycling program with the correct reaction volume (20 μL) and a heated lid:

Step	Temperature	Time
1	95 °C	10 min
2	94 °C	30 s
3	55 °C	30 s
4	68 °C to 70 °C (see Note)	1 min
5	N/A	Repeat steps 2 through 4 for an additional 34 cycles (total of 35 cycles)
6	4 °C	Hold

Note: The optimal extension temperature can vary depending on the multiplex. Refer to the multiplex kit insert for specific instructions. For custom multiplexes, the recommended starting temperature is 70 °C.

- For instruction on loading and running samples, refer to the section: [Load and Run Samples](#).

Load and Run Samples

Differences in the quantity or quality of the RNA template, GenomeLab GeXP system sensitivity, or reaction efficiency can require loading a higher or lower dilution of the PCR product. If the signal intensities from the 1:40 dilution used below exceed the linear detection range of the GenomeLab

GeXP system, it will be necessary to pre-dilute the PCR product in 10 mM Tris-HCl, pH 8.0 [prepared from 1 M Tris-HCl, pH 8.0 : DNase/RNase-free distilled water = 1:99 (v/v)].

Before making the final dilution into Sample Loading Solution/DNA Size Standard 400 and loading it into the GenomeLab GeXP system, refer to the section: [Determine the Appropriate PCR Product Dilution](#). Dilution of the PCR product can influence the detection of the genes with lower signals. The goal of determining the correct dilution factor is to keep the higher gene signals under the upper limit of the GenomeLab GeXP system linear range of detection while maintaining detection of the low gene signals.

Prepare Sample and Buffer Plates for Analysis

1. Thaw the separation buffer, Sample Loading Solution, and DNA Size Standard 400 to room temperature.
2. Add 38.5 μL of Sample Loading Solution and 0.5 μL of DNA Size Standard 400 to each well in a new sample plate.

Note: Alternatively, a master mix of Sample Loading Solution and DNA Size Standard 400 can be made by increasing the volume by 10% based on the number of samples. Aliquot 39 μL of master mix per well for each sample.

3. Transfer 1 μL of each PCR reaction sample (undiluted from the PCR plate or diluted from the pre-dilution plate) to each well of the sample plate.
4. Gently mix the components by pipetting up and down.
5. Cover the plate with an aluminum foil lid or strip caps.
6. Using a centrifuge, spin the plate for 1 min at 2,000 rpm to bring the contents to the bottom of the wells.
7. Remove the foil lid from the sample plate.
8. Overlay each well with one drop of mineral oil.
9. Fill the appropriate number of columns of a new buffer microplate with approximately 250 μL of separation buffer.

Run Samples

For more information about the following procedure, refer to the document: *GenomeLab GeXP Genetic Analysis System User Guide*.

1. On the GenomeLab GeXP system controller, launch the Sample Setup module and set up a plate to run the samples using the Frag-3 separation method.
2. Select **Sensitive GeXP Analysis Parameters**.

Change the following **Default GeXP Analysis Parameters**:

Multiplex Gene Expression Analysis

- a. For **Slope Threshold**, enter **1**.
- b. For **Peak Height Threshold**, enter **0%**.
3. Open the Run module.
4. Install the capillary array and fresh separation gel on the GenomeLab GeXP system.
5. Preheat the capillary array to 50 °C for up to 15 min before running the samples.
IMPORTANT:
Make sure that the **Wait for temperature to be reached** option is cleared.
6. Purge the manifold three to nine times using 0.4 mL of gel each time.
7. Replenish the capillaries with gel two to three times.
8. Perform an optical alignment and monitor the baseline.
9. If any capillary baseline is above 6,000 RFU, clean the capillary window using a no-glue cotton swab moistened with deionized water, and then start the process again with step 6.
10. Replenish the wetting tray(s).
11. Load the sample plate and buffer plate.
12. Start the run.

Analyze Gene Expression Sample Data

The user can export fragments/genotypes data as a csv document that contains a list of fragments with ID tags, and then analyze the data using the CSV Transformer Tool and the GeXP Analysis Template. For more information about using the Fragment Analysis module to analyze raw data and the CSV Transformer Tool to normalize the data to KAN^f, refer to the document: *GenomeLab GeXP Genetic Analysis System User Guide*.

GeXP Reactions, Separations, and Analysis

The following table shows problems that might be encountered while analyzing reactions on the GenomeLab GeXP system, identifies possible causes, and makes recommendations about how to resolve the problem for each stated cause.

For more extensive troubleshooting procedures, refer to the document: *GenomeLab GeXP Genetic Analysis System Troubleshooting Guide*.

Troubleshooting

Table 4-1 Troubleshooting - GeXP Analysis

Problem	Cause	Solution
Low signal or absent gene peaks	<ol style="list-style-type: none"> 1. There was not enough RNA template, or expression was low. 2. Enzyme activity was too low. 3. The amount of sample loaded was not sufficient. 4. The PCR product was exposed to too much light. 5. The amount of separation buffer loaded was not sufficient, or too much of the separation buffer evaporated. 6. Storage and handling of the RNA and/or reagents was incorrect. 7. There was a polymorphism in the priming site. 8. Because of cell death before RNA isolation, the initial RNA concentration was too low. 9. A gene was not expressed in the RNA sample. 10. The Sample Loading Solution is contaminated. 	<ol style="list-style-type: none"> 1. Use the recommended amount of initial RNA (25 ng to 100 ng). 2. Make sure that the Thermo-Start <i>Taq</i> DNA Polymerase has not passed its expiration date. 3. Load more PCR product on the GenomeLab GeXP system. 4. Run the samples again. Do not expose the PCR product to light for an extended amount of time. 5. Add the required amount of buffer. Run the samples again. Keep the buffer evaporation cover over the buffer plate when the plate is on the GenomeLab GeXP system. 6. Use fresh RNA and reagents. Follow the storage and preparation instructions included with the reagents. Avoid excessive freeze-thaw cycles. 7. Redesign the primers in a different region of the gene. 8. Prepare the RNA again. Make sure that the RNA quality and quantity are correct. 9. Select a different RNA source. 10. Use fresh Sample Loading Solution.

Table 4-1 Troubleshooting - GeXP Analysis (continued)

Problem	Cause	Solution
High signal (peak height) or any peak height greater than 130,000 RFU in the raw data	<ol style="list-style-type: none"> 1. There was too much PCR sample loaded. 2. The reverse primer concentration was too high. 3. There was too much initial RNA. 	<ol style="list-style-type: none"> 1. Prepare a fresh sample for analysis by diluting the PCR product in 10 mM Tris-HCl, pH 8.0 before adding the Sample Loading Solution/DNA Size Standard 400 mix. Run the samples again. 2. Attenuate the reverse primer. 3. Decrease the amount of initial RNA.
Additional peaks present	<ol style="list-style-type: none"> 1. Amplification was contaminated with another DNA template or DNA amplicons. 2. Dye-channel pull-up (a small dye peak underneath the over-ranged peak) occurs. 	<ol style="list-style-type: none"> 1. Use aerosol-resistant (barrier) pipette tips to minimize contamination. Perform the PCR reaction again, using controls to determine if the DNA template or reagents are contaminated. 2. Decrease the amount of sample loaded on the GenomeLab GeXP system.

Troubleshooting

Table 4-1 Troubleshooting - GeXP Analysis (continued)

Problem	Cause	Solution
Inconsistent fragment sizes or unusual peak shapes or low signal	<ol style="list-style-type: none">1. The gel has expired.2. The capillary array has expired.3. The correct temperature for capillary separation was not maintained.	<ol style="list-style-type: none">1. Verify that the 3-day life of the on-board gel has not expired. If it has, replace the on-board gel.2. Verify that the life of the capillary array has not expired. Capillary life is 100 runs or 30 days on the GenomeLab GeXP system, whichever occurs first. If the capillary array is beyond its life time, replace it.3. Look at the run log to make sure that the separation temperature is correct. Run the samples again.
Analysis error	There was an over-range/off-scale fluorescent signal at the start of the electropherogram.	Refer to the section: Determine the Appropriate PCR Product Dilution . Run the samples again.

Hazardous Substance Information

A

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

Hazardous Substance Information

- Thermo-Start *Taq* DNA Polymerase

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

Additional information on genetic analysis applications can be found at <https://sciex.com/genomelab> in the Resources area.

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SCIEX and its representatives maintain a staff of fully-trained service and technical specialists located throughout the world. They can answer questions about the system or any technical issues that might arise. For more information, visit the SCIEX website at sciex.com or contact us in one of the following ways:

- sciex.com/contact-us
- sciex.com/request-support

CyberSecurity

For the latest guidance on cybersecurity for SCIEX products, visit sciex.com/productsecurity.

Documentation

This version of the document supercedes all previous versions of this document.

To view this document electronically, Adobe Acrobat Reader is required. To download the latest version, go to <https://get.adobe.com/reader>.

To find software product documentation, refer to the release notes or software installation guide that comes with the software.

To find hardware product documentation, refer to the *Customer Reference* DVD that comes with the system or component.

The latest versions of the documentation are available on the SCIEX website, at sciex.com/customer-documents.

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