Instructions For Use

GenomeLab GeXP Genetic Analysis System

GeXP Chemistry Protocol

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GeXP Chemistry Protocol
GenomeLab GeXP Genetic Analysis System

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Revision History

Fifth Revision, A29143AE, August 2014

AB SCIEX branding boiler copy added to Front Cover and Back page.

AB SCIEX consolidation with Beckman Coulter page added for SCIEX Separations.

Beckman Coulter changed to AB SCIEX (except for web addresses and RNA isolation Agencourt kits) on all pages except for Cover, Copyright, SCIEX Separations, and Revision History pages.

Numerous changes made throughout the manual due to the GeXP software being updated to work with Windows 7.

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Preface

1.1 Multiplex Gene Expression

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

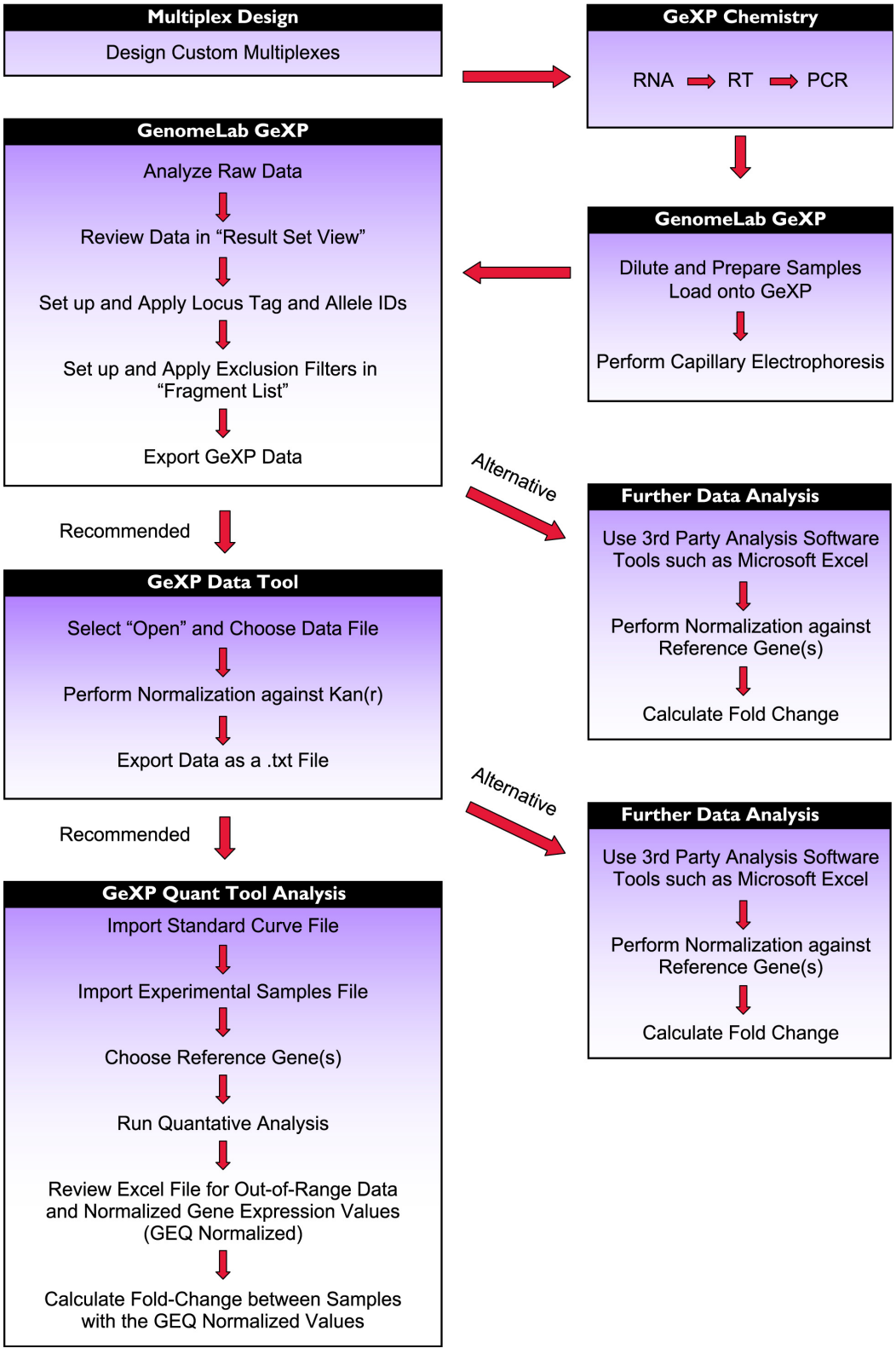
With a multiplexing capacity of analyzing up to 30 genes per reaction, the scalable GeXP can monitor tens to hundreds of genes for up to tens of thousands of samples.

GeXP 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 Reagent kits are optimized for effortless and robust, quantitative gene expression. The reagent kits are used for multiplexing user-defined genes. The pre-designed GenomeLab GeXP Human Reference*Plex* kit is available for assisting in the selection of optimal reference gene(s) for performing quantitative gene expression studies on human genes.

* *The PCR process is covered by patents owned by Roche Molecular Systems, Inc. and F. Hoffman La Roche, Ltd.*

The flow chart below depicts the Gene Expression workflow.



1.2 Preparing to Use the GeXP System

Reagents and Materials

- GenomeLab GeXP Human Reference*Plex* Kit (A54657)

OR

- Custom multiplex forward and reverse primers

- GenomeLab GeXP Start Kit (A85017), containing:
  - RT Buffer 5X, 480 µL
  - Reverse Transcriptase, 120 µL at 20 units/µL
  - PCR Buffer 5X, 480 µL
  - KAN^r RNA with RI, 600 µL
  - DNase/RNase Free H_2O, 1200 µL
  - DNA Size Standard-400, 55 µL
  - Mineral Oil, 5 mL
  - Sample Loading Solution, 6 mL

- Thermo-Start® DNA Polymerase, with separate 25 mM $MgCl_2$ (A85025)
- The RNA Storage Solution (Thermo Fisher Scientific, Inc. AM7000)
- Nuclease-Free H_2O, Non-DEPC Treated (Affymetrix 71786 or Thermo Fisher Scientific, Inc. 10977-015)
- 1M Tris-HCl pH 8.0 (Affymetrix 22638)
- GenomeLab Separation Buffer (608012)
- GenomeLab Separation Capillary Array (608087)
- GenomeLab Separation Gel, 20 mL (391438), or 10 mL (608010) for single-plate systems such as CEQ 8000
- Sample Microplates, 96-well (609801)
- 8-Well Cap Strips (BioRad TCS-0803 or Corning/Costar 0556)
- Buffer Microplates, 96-well (609844)
- 2.0 mL, 1.5 mL and 0.65 mL Microtubes
- Aerosol Resistant Tips for P10, P20, P100, P200 and P1000
- Regular tips for P10, P20, P100, P200 and P1000

Equipment

- GenomeLab GeXP Genetic Analysis System (A62684 for Single Rail System or A26572 for Dual Rail System)
- Pipettors, P10, P20, P100, P200, and P1000
- Microtube Centrifuge
- Microplate Centrifuge
- Thermal cycler with Heated Lid for 96-Well Plates
- Vortex Mixer
- Non-Frost-Free Freezers (-80°C and -20°C)

Workspace

To prevent the contamination of general reagents and ensure clean reverse transcriptase (RT) and PCR reactions, the following precautions and separated work areas are recommended when performing the procedures below.

Template-Free Area (Reagent Preparation Area)

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

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

Template-Addition Area (RNA Work Area)

Use this area for handling RNA samples such as Reference RNA or KAN^r RNA. This area will be used for adding RNA templates to cDNA reaction mixtures and cDNA templates to PCR reaction mixtures.

IMPORTANT Do not bring any PCR products into this area.

GeXP System Run Sample Preparation Area

This area should be used for preparing samples to run on the GeXP system. Manage the final PCR products in this area to prevent DNA contamination in the RNA work area.

Selecting the RNA Template for Mulitplex 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 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 reduce 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 50:50 ratio. If there is more than one treated sample, the 50% of treated sample in the starting 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 used to evaluate and validate primers, as well as create a standard curve.
- Aliquots (25-50uL) of a working stock of the Reference RNA should be stored at a concentration of 100ng/ul in a buffered RNA storage solution, such as **The RNA Storage Solution** (Thermo Fisher Scientific, Inc.), at -80°C.

RNA Isolation

The Beckman Coulter Agencourt RNA purification kits are recommended for total RNA isolation from FFPE samples (A33341), from Blood (A35603), from Cells (A47942), and from tissue (A32645).

Quantitation

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

Multiplex Design and Optimization

2.1 Overview

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
- Check 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 Genetic Analysis 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 increasing amounts of Reference RNA to create a Standard Curve
- Run optimized multiplex reactions with sample RNA templates

2.2 Multiplex Primer Design using NCBI Primer-BLAST

The NCBI Primer-BLAST tool is used to design an initial or "first-pass" 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 re-designed. Finally, the primer and amplicon information for the multiplex in the Microsoft Excel file is updated.

NOTE Add appropriate universal tag sequence at the 5' end of the primers before ordering primers.

Pre-Design Considerations

1. Determine which genes to be included in the multiplex for your study.
2. Select a correct Accession Number for each of the targeting genes.

   If possible, always use reference sequence (RefSeq) (NM_XXXXXX).

NOTE The Accession Numbers can be found on the National Center for Biotechnology Information (NCBI) web site at http://www.ncbi.nlm.nih.gov/.

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:

a. Correct Gene
   - Many genes have multiple names and aliases
   - Different genes can have similar names (e.g., IL2/IL2 receptor alpha/IL2 receptor beta/IL2 receptor gamma)
   - Verify that the gene is from the species of interest

b. Valid Sequence
   - 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 using the following page: www.ncbi.nlm.nih.gov/entrez/sutils/girevhist.cgi
   - RefSeq accession numbers starting with "NM_" are ideal choices for designing primers because they are from a curated, non-redundant database of known genes maintained by NCBI. The "NM_" accession prefix denotes mRNA sequences
   - Each RefSeq accession number correlates to an individual identified transcript variant. A gene may have more than one RefSeq identifiers
   - Be aware of and avoid pseudogenes by BLASTing the gene against the genome before primer design to avoid undesigned peaks (UDPs). See BLAST tool on the NCBI website (http://www.ncbi.nlm.nih.gov/)
   - Mutations or repeats can also influence results of primer design
   - Homologous genes, pseudogenes, or conserved domains should also be considered when choosing a gene sequence.

NOTE To aid in identifying a valid accession number, additional tools and information are available on the NCBI web site. Examples of these include Entrez Nucleotide. Entrez Gene, Homologene, Unigene, Blastn, or OMIM.

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

NOTE Be aware of transcript variants before primer design to avoid undesigned peaks. 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:

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

NOTE The ability to choose 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.

   a. Ideally, genes chosen for normalization purposes should be stably expressed in all samples under all conditions.

   b. In order to determine which genes are stably expressed, one can assay the reference genes in experimental samples with a multiplex such as the Human Reference*Plex* (A54657) or with one's own custom multiplex.

c. To monitor the stability of gene expression between samples, the peak areas of the reference genes should be normalized to Kan^r peak area in GeXP Data Tool and the resulting relative expression values compared between samples.

d. Those reference genes that have the most stable expression between samples should be chosen as normalization genes.

e. 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.

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

Primer Design using NCBI Primer-BLAST

1. Go to: http://www.ncbi.nlm.nih.gov/tools/primer-blast/
2. In "PCR Template" box, enter accession number or FASTA sequence.
3. Under "Primer Parameters," set PCR product size range as 105 to 350 nt.

NOTE 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.

NOTE For FFPE samples, set PCR product size range as 63 to 150.

4. In "Exon/Intron selection":
   - For "Exon junction span'" select "Primer must span an exon-exon junction."
   - Check the box for "Intron Inclusion" and modify Min. Intron length as needed.
5. For "Primer Pair Specificity Checking Parameters":
   - Check mark the box for "Specificity check."
   - Select Refseq mRNA as Database; select appropriate organism.
6. Click on "Advanced parameters" hyperlink:
   - Check-mark the box for "Primer binding site may not contain known SNP"
   - Change Salt correction formula to "Schildkraut and Lifson 1965"
   - Change "Table of thermodynamic parameter" to "Breslauer et al. 1986"
7. Click on the "Get Primers" button to submit your search request.
8. Within a minute or two, the NCBI web site will return search results that contain primer pair(s) information.

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 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 Tm for the forward primer, Tm 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. Save the changes to the Microsoft Excel worksheet.

NOTE Contact AB SCIEX Technical Support for sequences of universal tags.

Evaluating 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, re-design 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:
    - Examine each primer for nucleotide polymorphisms (SNP) 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.
    - Examine the amplicon sequence, generated by each set of primers, for repeat sequences.
        - Repeat sequences can lead to Taq polymerase slippage and result in stutter peaks.

- If 7 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.
- 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 an undesigned peak (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 GeXP Start Kit Chemistry.

2.3 Testing Individual Primer Pairs (Singlets)

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, Inc. (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 µM stock concentration in Resuspension Buffer (10 mM Tris-HCl, pH 8.0, DNase/RNase free). Consult with the manufacturers for specific information on resuspending and storage of the oligos.

NOTE For a list of reagents, materials and equipment, see "Preparing to Use the GeXP System" on page 3.

Testing the Forward Singlet -- Reverse Plex

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

1. Prepare a 10x (500 nM) reverse multiplex by combining each reverse primer in a total of 1 mL, with Resuspension Buffer (10 mM Tris-HCl, pH 8.0).
2. Prepare individual 10x (200 nM) working solutions for each forward primer, for a total of 1 mL in Resuspension Buffer.
3. Use the chemistry protocol for singlet reactions on page 12, 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. In addition, run a “no forward primer control” PCR reaction without any forward primer.
4. Evaluate the results of these singlet experiments for the following conditions:
    - Presence of an appropriately-sized PCR fragment
    - Absence of undesigned peaks 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 “no forward primer control” PCR reaction

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.

Testing 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 10x (500 nM) working solution of each reverse primer in Resuspension Buffer.
2. Prepare a 10x (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 these singlet experiments for the following conditions:
   - the presence of an appropriately-sized PCR fragment
   - the absence of extra peaks that would interfere with the quantitation of designed peaks in the multiplex
   - a sufficient signal above the background noise of the PCR reaction

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.

2.4 Chemistry Protocol for Singlet Reactions

Reverse Transcriptase Reaction

Calculating Reagent Volumes

Determine the number of Reverse Transcriptase (RT) reactions that need to be performed for the singlet primer evaluation and increase the number by 10%, rounding to the next reaction, to ensure adequate volume. Complete all singlet reactions in duplicate.

Preparing the No-Template Control (NTC) Reaction Mix

1. In the Template-Free area, add the following reaction components into a 1.5 mL. microcentrifuge tube labeled with "NTC."
2. Gently mix the components by pipetting up and down.

RT Minus Reaction Mix	Volume per Reaction
DNase/RNase Free H_2O	3 µL
RT Buffer 5X	4 µL
Reverse Transcriptase**	1 µL

**Add Reverse Transcriptase last to preserve enzyme activity

3. In the Template-Addition area, pre-dilute the KAN^r RNA with RI*** in DNase/RNase Free H_2O. The recommended initial 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:

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

*** Every time a stock solution of KAN^r RNA with RI is thawed, make 10 µl aliquots immediately after thawing. Place the aliquots on dry ice during the process of making aliquots. When finish, store aliquots at -80°C. Each aliquot should be used only once.

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

Preparing the RT Minus Control Reaction Mix

1. In the Template-Free area, add the following reaction components into a 1.5 mL microcentrifuge tube labeled with "RT Minus."
2. Gently mix the components by pipetting up and down.

RT Minus Reaction Mix	Volume per Reaction
DNase/RNase Free H_2O	4 µL
RT Buffer 5X	4 µL

3. In the Template-Addition area, pre-dilute the KAN^r RNA with RI in DNase/RNase Free H2O. 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:

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

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

Preparing the Standard Reaction Mix

1. In the Template-Free area, add the following reaction components into a 1.5 mL microcentrifuge tube labeled with "Standard."
2. Gently mix the components by pipetting up and down.

RT Plus Reaction Mix	Volume per Reaction	Volume per Mix (40 reactions)
DNase/RNase Free H_2O	3 µL	120 µL
RT Buffer 5X	4 µL	160 µL
Reverse Transcriptase**	1 µL	40 µL

**Add Reverse Transcriptase last to preserve enzyme activity.

3. In the Template-Addition area, pre-dilute the KAN^r RNA with RI in DNase/RNase Free H_2O. 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 Plus Reaction Mix	Volume per Reaction	Volume per Mix (40 reactions)
Pre-diluted KAN^r RNA with RI	5 µL	200 µL

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

Preparing the RT Reaction Sample Plates

NOTE To avoid edge effects, do not use the outer edge wells of the thermal cycler.

1. Add 2 µL of the 10x reverse multiplex (500 nM) **OR** 2 µL of an individual 10x 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 µL RT reaction.

2. Aliquot 13 µL of the RT Reaction mixes into the appropriate wells of a labeled 96-well plate. Use the 96-well plate coolers to keep the reagents cold or place them on ice.
3. Dilute the Reference RNA to 5-20 ng/µL with DNase/RNase Free H_2O. 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, see "Selecting the RNA Template for Mutiplex Optimization" in section 1.2.

4. Add 5 µL of the diluted Reference RNA (5-20 ng/µL) to each Standard and RT Minus well.
5. For each NTC well, add 5 µL of DNase/RNase Free H_2O in place of RNA template.
6. Gently mix the reaction components by pipetting up and down.

IMPORTANT Make sure that air bubbles are not induced.

7. Tightly cover the plate with the strip caps.
8. Consolidate the liquid to the bottom of the wells by briefly centrifuging (1 minute at 2,000 rpm).

Running the RT Reaction

1. Place 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 minute
42°C	60 minutes
95°C	5 minutes
4°C	hold

PCR Reaction

Calculating 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 ensure enough reagent volume. Complete all singlet evaluations in duplicate.

Preparing the PCR Reaction Mix

Add the following to a microcentrifuge tube labeled “PCRmix” and gently mix.

Reaction Mix	Volume per Reaction
25 mM $MgCl_2$	4.0 µL
PCR Buffer 5X	4.0 µL
ThermoStart DNA Polymerase**	0.7 µL
Total Volume	**8.7 µL**

**Add ThermoStart DNA Polymerase last to preserve enzyme activity.

Preparing Plates for PCR

NOTE To avoid edge effects, do not use the outer edge wells of the thermal cycler.

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

NOTE For the "no forward primer control" PCR reaction, add 2 µL of DNase, RNase free water.

2. Aliquot 8.7 µL of the PCR reaction mix into the appropriate wells of a 96-well PCR plate.
3. Briefly centrifuge the contents of the RT reactions to consolidate the solution to the bottom of the tube.
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. Pipet up and down to mix the reaction components thoroughly.

IMPORTANT Make sure that air bubbles are not induced.

6. Tightly cover the plate with the strip caps.

7. Consolidate the liquid to the bottom of the wells by briefly centrifuging (1 minute at 2,000 rpm).

Running the PCR Reaction

1. Place 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 minutes
2	94°C	30 seconds
3	55°C	30 seconds
4	68-70°C*	1 minute
5	N/A	Repeat steps 2-4 for an additional 34 cycles (total of 35 cycles).
6	4°C	Hold

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

3. For instruction on Loading and Running Samples on the GeXP System, see Section 3.3.

2.5 Testing and Optimizing Multiplex Primers

The singlet RT-PCR reactions that occur within the GeXP 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.

1. Assemble a 10x (500 nM) reverse multiplex primer mix by combining the reverse primers at 500 nanomolar each, in Resuspension Buffer (10 mM Tris-HCl pH 8.0).
2. Assemble a 10x (200 nM) forward multiplex primer mix by combining the forward primers at 200 nanomolar each, in Resuspension Buffer.
3. Assemble the multiplex RT and PCR reactions as described in the general procedure in section 3.3 for ‘performing Multiplex Gene Expression Analysis” and run them on the GeXP system.
4. Determine if all the primer sets in the multiplex produce a detectable signal.
5. Compare each gene's estimated fragment size in the singlet results to the corresponding peak in the multiplex results.

Troubleshooting 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. See "Pre-Design Considerations" on page 7. 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 GeXP system detector (130,000 RFU raw data, 120,000 RFU analyzed data) in the multiplex reaction will require pre-dilution and/or primer attenuation. For more information, refer to section 2.5 for "Attenuating 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 GeXP instrument. The attenuation process performed during multiplex optimization is described in "Determining the Reverse Primer Concentration for High-Expressing Genes" in Section 2.5.

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 an undesigned co-migrating peak 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 product, 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 undesigned peak.

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 on the GeXP system. Second, the binning of the multiplex fragments in the Peak Binning section of GenomeLab GeXP 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 3 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.

Attenuating GeXP Signals

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

Determining the Appropriate PCR Product Dilution

1. Run the standard GeXP 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:

PCR Pre-Dilution	SLS/SizeStd 400 Dilution	Total Dilution
Undiluted Reaction	1 µL into 39 µL SLS/SS (1:40)	1:40
2 µL into 8 µL 10 mM Tris-HCl pH 8.0* (1:5)	1 µL into 39 µL SLS/SS (1:40)	1:200
2 µL into 18 µL 10 mM Tris-HCl pH 8.0* (1:10)	1 µL into 39 µL SLS/SS (1:40)	1:400
2 µL into 38 µL 10 mM Tris-HCl pH 8.0* (1:40)	1 µL into 39 µL SLS/SS (1:40)	1:800

* Prepare 10 mM Tris-HCl pH 8.0 from 1M Tris-HCl pH 8.0 (Affymetrix 22638): Nuclease-Free H_20 (Affymetrix 71786)=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.

Determining the Reverse Primer Concentration for High-Expressing Genes

If one or several genes signals are close to or above the linear range of the 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 (10x non-attenuated primer pool).

NOTE To increase the signal intensity of one or more exceptionally low peaks, increased amounts of reverse primer (up to 1500nM) may be added to this mix for those gene peaks.

2. Generate a 500 nM reverse primer mix of the all of the primers that are to be attenuated (10x attenuated primer pool).
3. Serially dilute the 10x attenuated primer pool with two-fold dilutions over 8 concentrations (10x 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 H_2O	1 µL
RT Buffer (5X)	4 µL
10x non-attenuated primer pool	2 µL
10x attenuated primer pool (dilution)	2 µL
Reverse Transcriptase	1 µL
Pre-diluted KAN^r RNA with RI*	5 µL
Control Template	5 µL
Total	**20 µL**

*Determine optimal KAN^r RNA concentration using the protocol described in "Optimizing the KAN^r RNA Concentration" on page 20 and then pre-dilute KAN^r RNA in a buffered solution to stabilize RNA (e.g. Thermo Fisher Scientific Inc. RNA Storage Solution).

5. Perform the standard forward multiplex PCR protocol with cDNA generated from the RT reaction above.
6. Run the PCR products on the GeXP system using the PCR dilution determined above.

Refer to the GenomeLab Genetic Analysis System User's Guide (A29142), Chapters 2, 3 and 6, for detailed instructions on running samples on the GeXP System.

Analyzing 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 GeXP system detector and have sufficient dynamic range to measure changes in gene expression
    - The recommended range is approximately 2,000 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 µM in reverse multiplex).

Once optimum primer concentrations are determined, repeat *Determining the Appropriate PCR Product Dilution* on page 18 to determine the appropriate PCR dilution.

Optimizing the KAN^r RNA Concentration

The purpose of testing multiple concentrations of KAN^r RNA in the mutltiplex 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 5µL 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^r RNA concentration can be optimized together with reverse primer concentration optimization. The optimized KAN^r peak height should be about half the peak height of the target peak designated for primer concentration optimization.

1. Dilute KAN^r RNA over a series of dilutions, either lower or higher than the initial 1:50 dilution. See Table 2.1.

Table 2.1 KAN^r 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**

* Original GeXP Kan^r RNA with RI (BCI A21041) = 254 femtomolar

\# Diluent = The RNA Storage Solution (Thermo Fisher Scientific Inc.) or DNase/RNase-Free H_2O

2. Add 5µL of each dilution of KAN^r RNA to a separate RT reaction that contains the pre-determined optimal amount of Reference RNA (5-100ng) 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^r RNA dilution be assayed in duplicate or triplicate.

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

Confirming the PCR, Reverse Primer and KAN^r Dilutions on the GeXP System

Once the appropriate concentrations of reverse primers and KAN^r 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 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 and/or repeat *Determining the Appropriate PCR Product Dilution* on page 18 when necessary to ensure that all peaks are within range.

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

Using the Optimized Custom Multiplex

Once the reaction conditions for a custom multiplex are optimized, a standard curve is established with the Reference RNA and experimental samples are assayed using the standard multiplex gene expression analysis protocol. Refer to "Creating a Standard Curve" on page 23 or "Performing Multiplex Gene Expression Analysis" on page 24, for more information.

Multiplex Gene Expression Analysis

3.1 Overview

The following protocol is designed for performing multiplex gene expression analysis with the GenomeLab GeXP Start Kit, in combination with the GeXP Human Reference*Plex* Kit (A54657) or an optimized custom multiplex.

3.2 Creating a Standard Curve

Overview

The purpose of creating a high-quality standard curve with the Reference RNA for an optimized multiplex is to establish an equation for each gene from which accurate gene expression values for experimental (unknown) samples can be derived. The standard curve is created with increasing concentrations of Reference RNA, which translates to increasing concentrations of each gene product. The recommended range of Reference RNA for the standard curve is 10 data points from 1 ng to 500 ng. While this standard curve may be expanded in either direction, if necessary, for a multiplex with particularly high or low expressed genes, it should only need to be created one-time-per-multiplex on each GeXP system. The stored Standard Curve data will be used for all subsequent multiplex gene expression analyses performed with a particular multiplex.

Create a Standard Curve for multiplex gene expression analysis by completing the following procedures:

- Diluting the Reference RNA in series of two-fold dilutions
- Set up the RT reactions with diluted Reference RNA
- Running the RT Reaction
- Set up the PCR reaction
- Running the PCR Reaction
- Prepare samples for GeXP analysis
- Load, run, and analyze samples on the GenomeLab GeXP
- Normalize data to KAN^r using GeXP Data Tool
- Export a Standards file for use in GeXP Quant Tool

Diluting the Reference RNA

From the working stock of Reference RNA (100ng/uL) make a series of two-fold dilutions in either RNA Storage Solution (Thermo Fisher Scientific Inc.) or DNase/RNase-free dH_2O. See Table 3.1. It is recommended that enough of each dilution is prepared such that the standard curve is performed in triplicate reactions (at least three reactions per dilution). 5µL of each dilution of the Reference RNA will be needed per reaction.

Table 3.1 Two-fold serial dilutions of the Reference RNA to create a standard curve

Dilution	1*	2	3	4	5	6	7	8	9	10
Amount of RNA per reaction (ng)	500	250	125	62.5	31.3	15.6	7.8	3.9	2	1
Volume from previous dilution (µL)	50*	25	25	25	25	25	25	25	25	25
Volume from diluent# (µL)	0	25	25	25	25	25	25	25	25	25
Mixed volume transferred to the next dilution (µL)	25	25	25	25	25	25	25	25	25	25
Final volume (µL)	25	25	25	25	25	25	25	25	25	50
Final Concentration (ng/µL)	100	50	25	12.5	6.25	3.12	1.56	0.78	0.39	0.2

*100 ng/uL working stock of Reference RNA
#Diluent = RNA storage solution or Nuclease-free, non-DEPC water

Performing Reverse Transcriptase and PCR Reactions to Create a Standard Curve

See the reverse transcriptase and PCR chemistry protocols in 3.3 "Performing Multiplex Gene Expression Analysis" on page 24. Use 5 uL of each dilution of Reference RNA per reaction. It is recommended that the Standard Curve RT and PCR reactions be performed in triplicate, at least (three reactions-per-dilution).

NOTE Experimental RNA samples can be assayed simultaneously with the Standard Curve Reference RNA.

Loading and Running Standard Curve Reactions on the GeXP System

See "Loading and Running Samples on the GeXP System" on page 29.

IMPORTANT The naming convention for Standards and Experimental Samples, outlined in Table 3.2 is particularly crucial for downstream analysis.

3.3 Performing Multiplex Gene Expression Analysis

Overview

Perform a multiplex gene expression analysis of experimental samples by completing the following procedures:

- Set up the RT reactions
- Run the RT reactions
- Set up the PCR reaction
- Run the PCR reaction
- Prepare samples for GeXP analysis
- Load, run and analyze samples on the GenomeLab GeXP
- Analyze data using GenomeLab GeXP software, GeXP Data Tool, and GeXP Quant Tool software

NOTE For a list of reagents, materials and equipment, see "Preparing to Use the GeXP System" on page 3.

Reverse Transcriptase Reaction

Calculating Reagent Volumes

Determine the number of Reverse Transcriptase (RT) reactions that need to be performed and increase the number by 10%, rounding to the next reaction, to ensure adequate volume. For a full plate, 110 reactions provide an adequate volume for multichannel pipetting. Perform at least three technical replicates of the Standard Reaction for each biological sample.

Preparing the No-Template Control (NTC) Reaction Mix

1. In the Template-Free area, add the following reaction components into a 1.5 mL microcentrifuge tube labeled with "NTC." Gently mix the components by pipetting up and down.

NTC Reaction Mix	Volume per Reaction
DNase/RNase Free H_2O	3 µL
RT Buffer 5X	4 µL
RT Rev Primer Plex*	2 µL
Reverse Transcriptase**	1 µL

*User must design and prepare this primer plex when using the GeXP Start Kit. See "Multiplex Primer Design using NCBI Primer-BLAST" on page 7. Alternatively, use the Human Reference*Plex* kit.

**Add Reverse Transcriptase last to preserve enzyme activity.

2. 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 µL

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

Preparing the RT Minus Control Reaction Mix

1. In the Template-Free area, add the following reaction components into a 1.5 mL microcentrifuge tube labeled with "RT Minus." Gently mix the components by pipetting up and down.

RT Minus Reaction Mix	Volume per Reaction
DNase/RNase Free H_2O	4 µL
RT Buffer 5X	4 µL
RT Rev Primer Plex*	2 µL

*User must design and prepare this primer plex when using the GeXP Start Kit. See "Multiplex Primer Design using NCBI Primer-BLAST" on page 7. Alternatively, use the Human Reference*Plex* kit.

2. In the Template-Addition area, add the following to the mixture prepared above and gently mix:

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

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

Preparing the Standard Reaction Mix

1. In the Template-Free area, add the following reaction components into a 1.5 mL microcentrifuge tube labeled with "Standard." Gently mix the components by pipetting up and down.

RT Plus Reaction Mix	Volume per Reaction
DNase/RNase Free H_2O	3 µL
RT Buffer 5X	4 µL
RT Rev Primer Plex*	2 µL
Reverse Transcriptase**	1 µL

*User must design and prepare this primer plex when using the GeXP Start Kit. See "Multiplex Primer Design using NCBI Primer-BLAST" on page 7. Alternatively, use the Human Reference*Plex* kit.

**Add Reverse Transcriptase last to preserve enzyme activity.

2. 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

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

Preparing the RT Reaction Sample Plates

NOTE To avoid edge effects, do not use the outer edge wells of the thermal cycler.

1. Aliquot 15 µL of the RT Reaction mixes into the appropriate wells of a labeled 96-well plate. Use the 96-well plate coolers to keep the reagents cold or place them on ice.
2. Predilute the Control or Reference RNA templates to 5-10 ng/µL with DNase/RNase Free H_2O. Refer to the specific multiplex kit insert for the recommended working concentration.

NOTE The human reference control RNA template is supplied with the GeXP Human Reference*Plex* kit. For a custom multiplex Control RNA template, use the RNA mixture that was developed for multiplex optimization. See "Selecting the RNA Template for Mulitplex Optimization" on page 4, for more information.

3. Dilute the sample RNA to 5-20 ng/µL with DNase/RNase Free H_2O. 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 (5-20 ng/µL) or diluted Control RNA templates (5-10 ng/µL) to each Standard and RT Minus well.
5. For each NTC well, add 5 µL of DNase/RNase Free H_2O in place of RNA template.
6. Gently mix the reaction components by pipetting up and down.

IMPORTANT Make sure that air bubbles are not induced.

7. Tightly cover the plate with the strip caps.
8. Consolidate the liquid to the bottom of the wells by briefly centrifuging (1 minute at 2,000 rpm).

Running the RT Reaction

1. Place 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 minute
42°C	60 minutes
95°C	5 minutes
4°C	hold

PCR Reaction

Calculating 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 ensure enough reagent volume. For a full plate, 110 reactions provide an adequate volume for multichannel pipetting. Perform at least three technical replicates for each biological sample.

Preparing the PCR Reaction Mix

Add the following to a microcentrifuge tube labeled "PCRmix" and gently mix.

Reaction Mix	Volume per Reaction
25 mM $MgCl_2$	4.0 µL
PCR Buffer 5X	4.0 µL
PCR Fwd Primer Plex*	2.0 µL
ThermoStart DNA Polymerase**	0.7 µL
Total Volume	**10.7 µL**

*User must design and prepare this primer plex when using the GeXP Start Kit. See "Multiplex Primer Design using NCBI Primer-BLAST" on page 7. Alternatively, use the Human Reference*Plex* kit.

**Add ThermoStart DNA Polymerase last to preserve enzyme activity.

Preparing Plates for PCR

NOTE To avoid edge effects, do not use the outer edge wells of the thermal cycler.

1. Aliquot 10.7 µL of the PCR reaction mix into the appropriate wells of a 96-well PCR plate.
2. Briefly centrifuge the contents of the RT reactions to consolidate the solution to the bottom of the tube.
3. 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.
4. Pipet up and down to mix the reaction components thoroughly.

IMPORTANT Make sure that air bubbles are not induced.

5. Tightly cover the plate with the strip caps.
6. Consolidate the liquid to the bottom of the wells by briefly centrifuging (1 minute at 2,000 rpm).

Running the PCR Reaction

1. Place 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 minutes
2	94°C	30 seconds
3	55°C	30 seconds
4	68-70°C*	1 minute
5	N/A	Repeat steps 2-4 for an additional 34 cycles (total of 35 cycles).
6	4°C	Hold

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

Loading and Running Samples on the GeXP System

Differences in the quantity or quality of the RNA template, instrument sensitivity, or reaction efficiency can require loading a higher or lower dilution of the PCR product on the GeXP system. If the signal intensities from the 1:40 dilution used below exceed the linear detection range of the GeXP system, it will be necessary to pre-dilute the PCR product in 10 mM Tris-HCl pH 8.0 [prepared from 1M Tris-HCl pH 8.0 (Affymetrix 22638): Nuclease-Free H_20 (Affymetrix 71780) = 1:99 (v/v)].

See "Determining the Appropriate PCR Product Dilution" on page 18, before making the final dilution into SLS/SS 400 and loading it into the instrument. 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 GeXP system's linear range of detection while maintaining detection of the low gene signals.

Preparing Sample and Buffer Plates for GeXP Analysis

1. Thaw the following reagents to room temperature:
   - GenomeLab Separation Buffer (608012)
   - GenomeLab Sample Loading Solution
   - GenomeLab DNA Size Standard-400
2. Add 38.5 µL Sample Loading Solution and 0.5 µL of the DNA Size Standard-400 to each well in a new GeXP Sample Plate (609801).

NOTE Alternatively, a master mix of SLS and SS400 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 (un-diluted from the PCR plate or diluted from the pre-dilution plate) to each well of the GeXP Sample Plate.
4. Mix thoroughly by pipetting up and down.

5. Cover the plate with an aluminum foil lid or strip caps.
6. Consolidate the liquid to the bottom of the wells by briefly centrifuging the plate (1 minute at 2,000 rpm).
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 GeXP Buffer Microplate (609844) with approximately 250 µL of GenomeLab Separation Buffer (608012).

Running Samples on the GeXP System

NOTE Refer to the GenomeLab Genetic Analysis System User's Guide (A29142) for specific information on running samples on the GeXP system.

1. Launch the **Plate Setup** module on the GeXP system controller and set up a plate to run the samples using the Frag-3 protocol.

NOTE In order for GenomeLab GeXP software and GeXP Data Tool to properly sort the data and GeXP Quant Tool to recognize and differentiate the standard curve and experimental data, use the following naming convention when setting up sample names in the GeXP Genetic Analysis System:

Table 3.2 Naming convention for Standards and Experimental Samples in GeXP

Standards:	Correct	Incorrect
	STD<space>###ng	STD# (no space, no 'ng')
	STD 001ng	STD 1
	STD 010ng	STD10ng
	STD 100ng	
Experimental Samples (unknown):	**U<space>###** Note: An alphanumeric sample name can be inserted after the number.	SampleName # (without 'U' first or place-holding digits)
	U 001 Sample Name	U 1 SampleName
	U 010 Sample Name	SampleName U 10
	U 100 Sample Name	100 SampleName

2. Set the analysis parameters to the GeXP Sensitive Analysis Parameters.

   Edit the Peak Criteria of the Default GeXP Analysis Parameters to:

   Slope Threshold = 1

   Peak Height Threshold = 0%

   All other Default GeXP Analysis Parameters stay the same.

   Save As "GeXP Sensitive Analysis Parameters"
3. Launch the **Run** module.
4. Install the capillary array and LPA-1 separation gel on the GeXP system as described in the GenomeLab Genetic Analysis User's Guide.
5. Preheat the capillary array to 50°C for up to 15 minutes, before running the samples.

IMPORTANT Confirm that the **Wait for temperature to be reached** option is not selected.

6. Perform a **Purge Manifold** procedure three to nine times using 0.4 mL of gel each time.

7. Perform a **Gel Capillary Fill** procedure two to three times.
8. Run an **Optical Alignment** and **Monitor Baseline**.

NOTE If any capillary baseline is above 6000 RFU, clean the capillary window using a no-glue cotton swab moistened with deionized water, and then repeat the process starting with step 6.

9. Refresh the wetting tray(s) and load the sample plate and buffer plate.
10. Start the GeXP system run.

Analyzing Gene Expression Sample Data

Refer to Chapter 6 of the GenomeLab Genetic Analysis System User's Guide (A29142) for information on using the Fragment Analysis tool to analyze raw data. Then, refer to Chapter 7 of the same User's Guide for information on using the GeXP Data Tool to normalize the GeXP data to KAN^r. Once the Standard Curve and experimental data is normalized to KAN^r, the gene expression analysis is performed with the GeXP Quant Tool.

Troubleshooting

4.1 GeXP Reactions, Separations and Analysis

The following table shows problems that might be encountered while analyzing GeXP reactions on the GenomeLab Genetic Analysis System and identifies possible causes and what you can do to resolve the problem for each stated cause.

For more extensive troubleshooting procedures, refer to the GenomeLab GeXP Troubleshooting Guide (A53995).

Troubleshooting - GeXP Analysis

Problem	Cause	Solution
Low signal or absent gene peaks	Not enough RNA template	Recheck calculations for amount of input RNA (25 - 100 ng is recommended).
	Low expression	Increase the amount of RNA template used.
	Not enough enzyme activity	Check the expiration date of the Taq Polymerase.
	Not enough sample loaded on the GeXP system	Increase the amount of PCR product loaded on the GeXP system.
	Excessive exposure of PCR product to light	Rerun the samples. Do not expose the PCR product to light for an extended amount of time.
	No separation buffer loaded or excessive evaporation of buffer	Rerun samples. Add the required amount of buffer and keep the buffer evaporation cover over buffer plate when installed on instrument.
	Improper storage and handling of the RNA and/or reagents	Use fresh RNA and reagents. Always follow the proper storage and preparation instructions included with the reagents. Avoid excessive freeze-thaw cycles.
	Polymorphism within a priming site of a gene	Redesign the primers in a different region of the gene.
	Low starting RNA due to cell death prior to RNA isolation	Make fresh RNA preparation and check the RNA quality and quantity.
	Gene not expressed in the RNA sample	Choose a different RNA source.

Troubleshooting - GeXP Analysis

Problem	Cause	Solution
High signal (peak height) or any peak height greater than 130,000 fluorescent units in raw data	Too much PCR sample used for GeXP loading	Prepare a fresh sample for analysis by first diluting the PCR product in 10 mM Tris-HCl (pH 8.0) before adding the size standard and SLS mix, then rerun the sample.
	The reverse primer concentration is too high	Attenuate the reverse primer.
	Too much starting RNA	Reduce the amount of RNA.
Additional peaks present	Amplification is contaminated with another DNA template or DNA amplicons	Use aerosol-resistant (barrier) pipette tips to minimize contamination. Perform the PCR reaction again using controls to determine if the DNA template and/or reagents are contaminated.
	Pull-up. If the peak height (signal) is high (>135,000 RFUs), a small peak can be observed in an alternate dye channel either directly adjacent to, or underneath the over-ranged peak	Decrease the amount of sample loaded on the instrument.
Inconsistent fragment sizes or unusual peak shapes or low signal	The gel life has been exceeded	Check the on-board gel life. On-board gel life is verified for 3 days.
	The capillary array life has been exceeded	Check the capillary array life. Capillary array life has been verified for 100 runs and/or 30 days on instrument, whichever comes first.
	Failure to maintain proper capillary separation temperature	Check the run log for verification of separation temperature. Rerun samples.
Analysis error	Over-ranged/off-scale fluorescent signal at start of e-gram	Re-run the sample on the GeXP system. Refer to the pre-dilution instructions for High Signal on page 18.

Appendix A

Technical Bulletins

An up-to-date list of publications on Genetic Analysis can be found on our website: http://www.beckmancoulter.com/GenomeLab

Use the **Resource Center** drop-down list and select **Literature**.

Additional Technical Support

United States:
Contact AB SCIEX Technical Support

1.877.740.2129

Worldwide:
Contact your AB SCIEX Technical Support Associate.

Appendix A
Technical Bulletins

For Research Use Only. Not for use in diagnostic procedures.

www.sciex.com/ce

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