Instructions For Use

GenomeLab GeXP
Genetic Analysis System

GeXP Troubleshooting Guide

A53995AD
August 2014

BECKMAN COULTER

GeXP Troubleshooting Guide
GenomeLab GeXP Genetic Analysis System

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Revision History

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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) on some pages.

Numerous changes made throughout the manual due to the GeXP product being updated to Windows 7.

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Introduction

Overview

The GeXP Troubleshooting Guide has been designed to help you identify and correct the issues most commonly encountered with the gene expression application of the GenomeLab GeXP Genetic Analysis System. Read through the entire GeXP Troubleshooting Guide first, before performing troubleshooting experiments.

Begin with General Diagnostics, which will inform you on the data you will need and where it can be found. Next, Instrumentation & Chemistry will provide a systematic approach to enable you to effectively diagnose the problem. See Figure 1.1 on page 2 for the GeXP Gene Expression Troubleshooting Workflow. This serves as a visual aide for the troubleshooting process.

Finally, the Reference Tables and Questions & Answers sections are particularly helpful for resolving specific problems. These sections can also aid in rapid identification of the most likely suspects for poor results.

1.1 Gene Expression Troubleshooting Workflow

View the data

- **Is the problem with the GeXP instrument?** — Yes → Run the Size Standard-400
- **I'm not sure where the problem is.** → Run the Size Standard-400

Run the Size Standard-400

- **Are the peak resolution and current okay?**
  - Yes → Peak resolution and current are okay.
  - No → Are there current abnormalities?
- **Are there current abnormalities?**
  - Yes → Run the Sequencing Test Sample (PN 608070)
  - No → I have poor peak resolution.

I have poor peak resolution. → Run the Sequencing Test Sample (PN 608070)

Run the Sequencing Test Sample (PN 608070)
- **Is the current okay?**
  - Yes → Peak resolution and current are okay.
  - No → I have current abnormalities.

I have current abnormalities. → Contact your AB SCiex Field Service Representative

Peak resolution and current are okay. → Go to Chemistry Troubleshooting

Use the GeXP Start Kit and control multiplex gene set kit to perform GeXP reactions
- **Do you have a KAN^r peak?**
  - No → I don't have a KAN^r peak.
  - Yes → I have KAN^r with all multiplex peaks.

I don't have a KAN^r peak.
- Review GeXP protocol
- Test third-party products
- Test a new kit

→ **Do you have a KAN^r peak?**
  - No → I still don't have a KAN^r peak. → Contact your AB Sciex Technical Support Representative

I have KAN^r with all multiplex peaks. → Kit and process are okay. → Test experimental RNA and primers
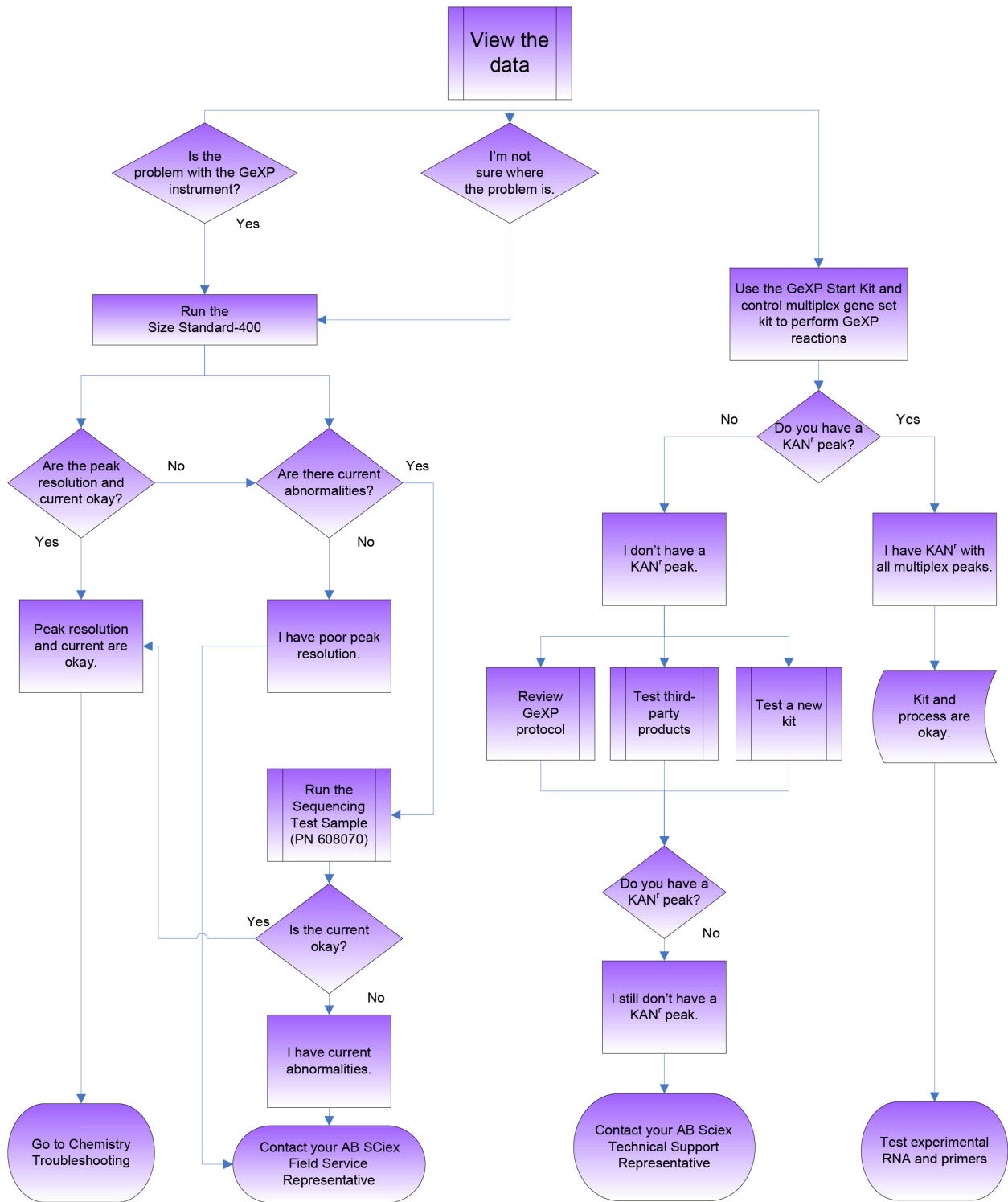
Figure 1.1 Gene Expression Troubleshooting Workflow

General Diagnostic Guidelines

Overview

The GenomeLab GeXP™ provides software functions that will help you to identify and resolve GeXP problems. Two key features in this regard are the Raw Data and Current Profile.

NOTE The Analyzed Data can also be used for troubleshooting, but only after the Raw Data and Current Profile have been shown to be acceptable.

Use the raw data, current profile and analyzed data to determine whether the issue is caused by GeXP instrumentation or chemistry or possibly both. Be sure to note the following aspects of each type of data for all of the affected samples and appropriate controls:

Raw and Analyzed Data

- Baseline for D4 (blue) and D1 (red)
- Signal strength
- Signal profile (level or drop-off)
- Landmarks
  - unincorporated primers (raw only)
  - multiplex peaks
  - KAN^r peak
  - size standard peaks

See for more information.

Current Profile

- Ramping profile
- Maximum separation current (µA)
- Level of current throughout separation

See for more information.

2.1 Evaluating the Raw Data

The raw data generated by the GeXP is displayed in the Data Monitor window of the Run Control module, during the actual separation. The data is also accessible by using Fragment Analysis and Sequencing Analysis modules.

Examining Signal Strength and Multiplex Profile

Refer to the signal strength and multiplex profile as the first step in diagnosing problems, when looking at raw data.

- Although peak heights vary for different multiplex fragments, the signal strength of D4-dye (blue) labeled multiplex peaks should be fairly even across the separation after the initial unincorporated primer dye front
  - One exception is the positive control, 325 nucleotide kanamycin resistance (KAN^r) peak, which is usually over-range in most GeXP reactions
- All of the D1-dye (red) labeled size standard peaks should be appropriately spaced and approximately the same height
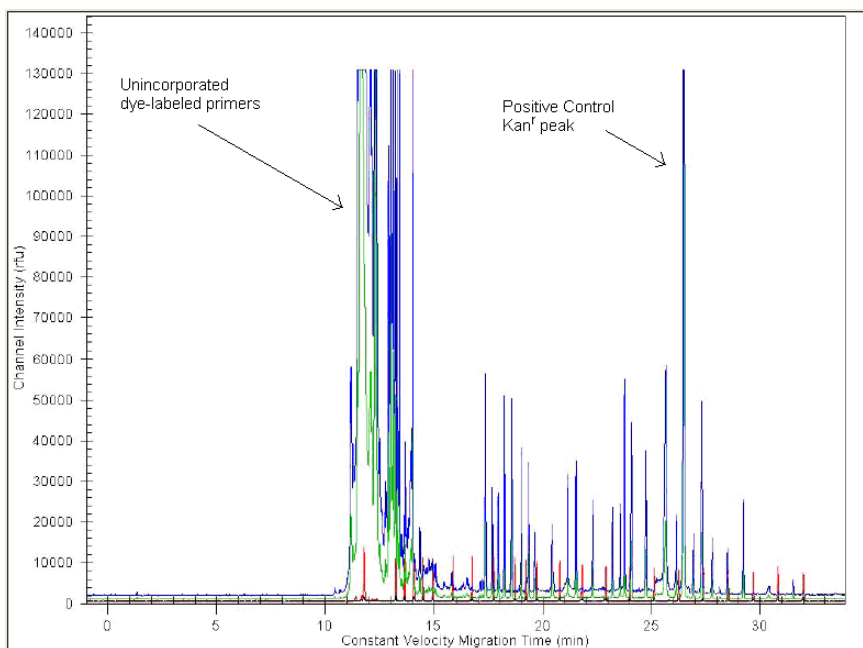
Figure 2.1 GeXP Raw Data

Checking the Raw Data

The electropherogram shown in **Figure 2.1** shows a typical GeXP separation with a start time at approximately 11 minutes for the Frag-3 separation method. The signal strength at the beginning of the separation is over-range due to excess primers remaining in the PCR reaction. This is common for GeXP reactions. The multiplex fragments begin appearing at approximately 17.5 minutes for this particular sample. The positive control KAN[r] fragment appears at approximately 26.5 minutes. The D1-dye (red) labeled Size Standard-400 peaks are appropriately spaced and even in height.

The data is displayed as signal intensity, measured in relative fluorescence units (RFU) vs. time. The above example shows excellent signal strength. However, it is not necessary for each sample to have such high signal in order to obtain accurate peak area measurements. More important is that all the peaks fall within the limits of detection (below 130,000 RFU in the raw data) and the electropherogram shows a balanced profile with no signal drop-off.

Signal Drop-Off

The example of analyzed data in **Figure 2.2** shows a balanced profile, whereas the analyzed data in **Figure 2.3** shows signal drop-off. Signal drop-off is characterized by relatively high signal for shorter fragments and significantly lower signal for the longer fragments. The result is a multiplex profile with a downward slope toward the larger fragments, while the size standard peaks are even and appear normal. The KAN[r] peak may or may not appear to be affected by signal drop-off.

Irregularities in PCR cycling conditions, particularly the extension temperature, are the most likely cause of signal drop-off. This is often seen in the edge wells of a thermal cycler. Ensure that the thermal cycler is calibrated and consider using only the non-edge wells to avoid signal drop-off. See **"GeXP Chemistry" on page 20** for more information.

NOTE Signal drop-off can be caused by using the wrong DNA Polymerase. ThermoStart DNA Polymerase (A85025) has been validated for use with the GenomeLab GeXP system.
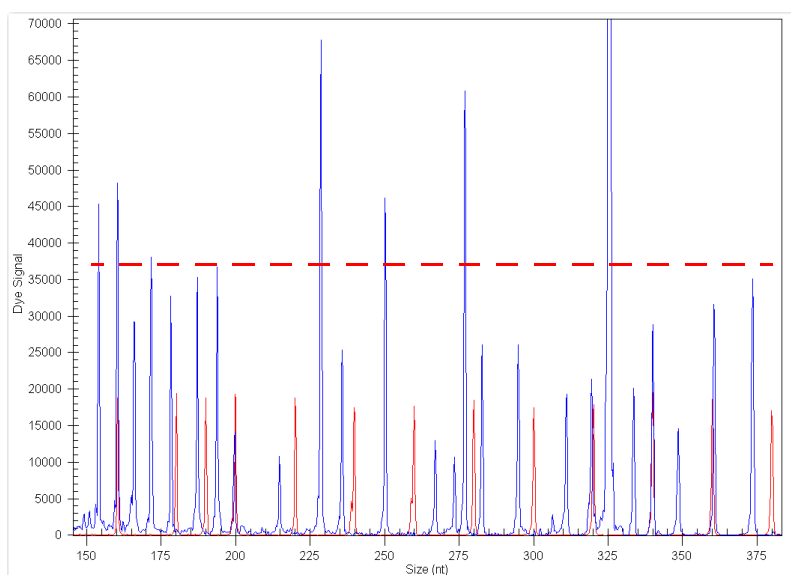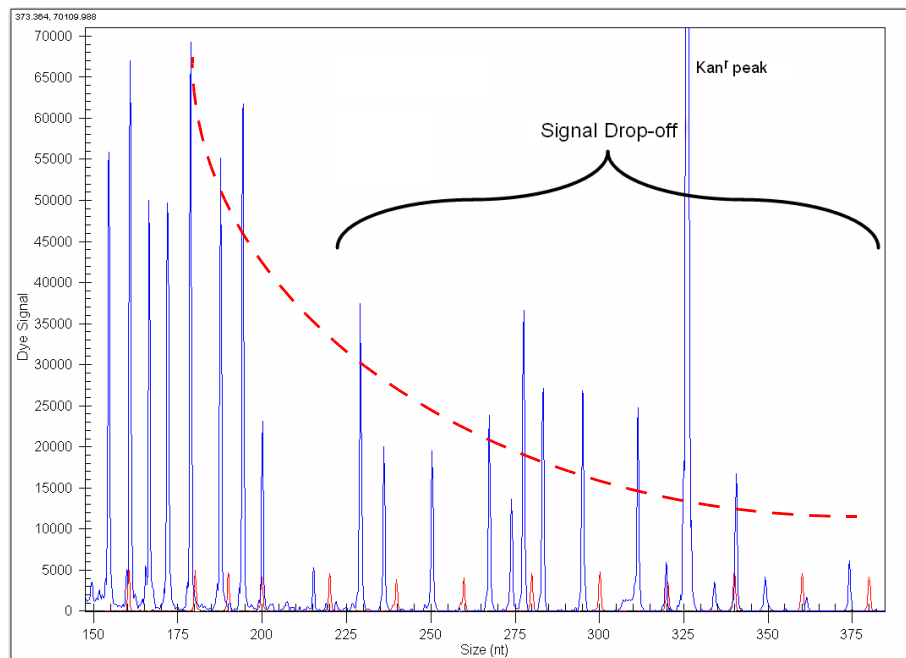

Figure 2.2 GeXP Analyzed Data: Normal Peak Profile

Figure 2.3 GeXP Analyzed Data: Peak Profile with Signal Drop-off

IMPORTANT Signal drop-off can lead to high %CV in analyzed data. Do not use the results of these wells for data analysis.

## 2.2 Evaluating the Current Profiles

The GeXP instrument continuously monitors the current in each of the eight capillaries, while the system is running. The current profile can be extremely useful in diagnosing certain issues. The current profile is displayed in the Data Monitor window of the Run Module, during the actual separation, and is also accessible by using Fragment Analysis.

Confirming the Separation Current

The figures below display a comparison of raw data from the same sample separated with normal current vs. an abnormal current.

Normal Current

The current should ramp up to the final level in a single stage and then plateau. The plateau level is determined by the separation voltage, which is set in the separation method (Frag-3) and is maintained throughout the separation. The final level should be approximately 7-11 µA. The current profile should look similar to the figure below.
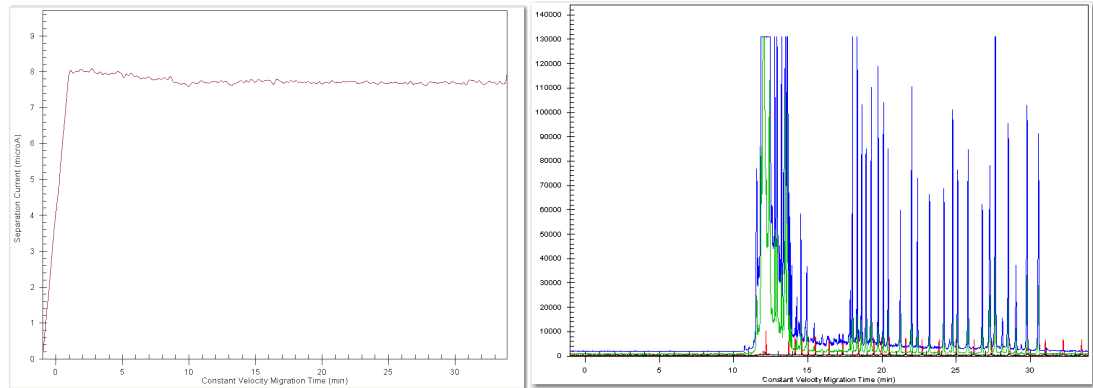
Figure 2.4 Normal Capillary Current Profile and corresponding Raw Data

Abnormal Current

A number of characteristics can be used to determine a current failure for GeXP. The list below represents the most commonly observed characteristics for a failed current:

- ramps in two or more stages
- does not maintain the separation current at a steady level
- a change in current level of more than 10%

A current failure, as shown in **Figure 2.5**, will cause the GeXP fragments to do the following:

- separate abnormally with delayed peaks (**Figure 2.5**)
- have reduced peak resolution
- have low signal

IMPORTANT If a capillary has current failure, do not use data from these wells because the peak area calculation is affected by abnormal current.
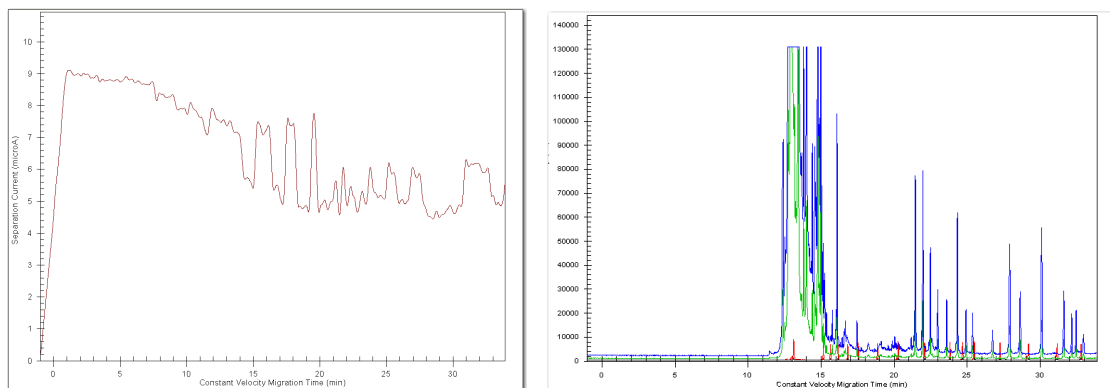
Figure 2.5 Abnormal Capillary Current Profile and corresponding Raw Data

Causes of Current Abnormalities

Current profile abnormalities can be caused by the following:

- a bubble in the manifold
- no separation buffer
- impurities or debris in the sample

See "Performing Instrument Diagnostics" on page 9, for instructions on how to verify the cause of the problem.

Instrumentation & Chemistry

Overview

Issues encountered with the GeXP process can be divided into two areas: instrumentation and chemistry.

A systematic approach to troubleshooting is described below, to enable the user to effectively diagnose a problem with the GeXP process.

First, use the raw data, current profile and analyzed data to determine whether the issue is caused by one of the following:

- GeXP instrument
- GeXP chemistry
- GeXP instrument and chemistry

Initial Indications

- If the size standard peaks and/or current are abnormal, then begin by troubleshooting the instrument. See "Performing Instrument Diagnostics" on page 9.
- If the size standard peaks and current are normal, then begin by troubleshooting the Chemistry. See "Testing the Chemistry" on page 12.
- If you are unsure where to start, begin by troubleshooting the GeXP instrument.

3.1 Performing Instrument Diagnostics

The separation of Size Standard-400, in the absence of GeXP reaction products, is performed to test the integrity of the GeXP instrument and the various reagents used by the system. These reagents include:

- separation gel
- sample loading solution
- capillary array

Running Size Standard-400

1. Thaw Size Standard-400 (PN 608098) and Sample Loading Solution (PN 608082) at room temperature.

NOTE It is recommended that the lot numbers of all consumables be recorded during troubleshooting.

2. Combine 13 µL of ss-400 with 1027 µL of SLS in a microcentrifuge tube and mix.

IMPORTANT Use only Beckman Coulter SLS. Do not substitute SLS with fomamide from a third-party.

IMPORTANT Use non-barrier pipette tips when pipetting SLS.

3. Pipette 40 µL of the ss-400 + SLS mixture into each of 24 wells of a sample plate (three rows total).

4. Place a drop of Beckman Coulter mineral oil (PN 608114) over the ss-400 + SLS mixture in each well.

5. Add Separation Buffer to each corresponding well of a buffer tray, filling each well about ¾ full (250 µL).

6. Perform Sample Plate Setup.

7. Select the **Frag-3** separation method for all three rows.

8. Edit the **Default Fragment Analysis Parameters** so that **AE-Ver1** dye mobility calibration is selected in the **Advanced** option of Analysis Parameters.

9. Select the edited method for automatic analysis of each well.

10. Run the samples according to the standard GeXP procedures.

11. Review the data as described in "General Diagnostic Guidelines" on page 3.

Interpreting the Results

The Size Standard-400 provides acceptable results by meeting the following criteria:

- all size standards peaks are present and called
- the peaks are well resolved
- the current is normal

- If this is the case, the GeXP instrument and reagents are fine and further diagnostics of the chemistry are needed.

- If any of the above criteria are not met, try testing the system consumables such as the separation gel, SLS, the capillary array and another lot of the Size Standard-400. The individual components should be tested in separate experiments to identify the faulty component.

- If current failure is the problem, proceed with using the Sequencing Test Sample to assess the instrument and consumable reagents. See "Running the Sequencing Test Sample" below for more information.

Running the Sequencing Test Sample

The Sequencing Test Sample is used to test the integrity of the GeXP instrument and the consumable reagents used by the system, particularly when there is a problem with separation current.

1. Thaw three vials of Sequencing Test Sample (PN 608070) at room temperature.

NOTE It is recommended that the lot numbers of all consumables be recorded during troubleshooting.

2. Pipette 40 µl of Sequencing Test Sample directly into each of 24 wells of a sample plate (three rows total).

IMPORTANT Use non-barrier pipette tips when pipetting Sequencing Test Sample, which contains SLS.

3. Place a drop of Beckman Coulter mineral oil over the Sequencing Test Sample in each well.

4. Add Separation Buffer to each corresponding well of a buffer tray, filling each well about ¾ full (250 µL).

5. Perform sample plate Setup and select the **LFR-a** separation method, and modify it by increasing the "pause" time to 5 minutes.

6. Run the samples according to the standard GeXP procedures.

7. Assess the current profile of data obtained with Sequencing Test Sample in the Sequencing Analysis module. Click **File | Open | Sample Data tab** then select sample files from the appropriate project and click **OK**.

8. Review the current profile. The current should ramp in one stage and plateau at 5-9 µA. The current profile should look similar to the current in the example in the figure below.
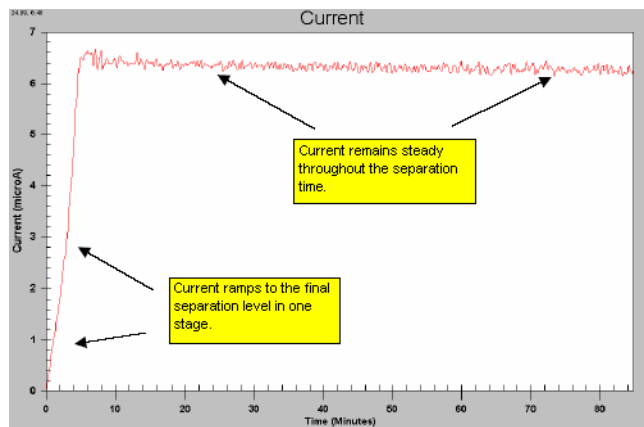
Figure 3.1 Sequencing Current

Interpreting the Results

The Sequencing Test Sample provides acceptable results by meeting the following criteria:

- good raw data signal
- normal current profile
- meets the system specifications of 98% accuracy at 700 bases, when the LFR-1 separation method is used

- If this is the case, the GeXP instrument and reagents are fine and further diagnostics of the chemistry are needed.

NOTE For more information on Sequence Analysis and troubleshooting problems with separation current, refer to the Sequence Analysis Troubleshooting Guide (390216) which can be downloaded from www.BeckmanCoulter.com/Genomelab

- If the Sequencing Test Sample does not yield an acceptable result try testing the other system consumables such as the separation gel, the capillary array and another lot of the Sequencing Test Sample. The individual components should be tested in separate experiments to identify the faulty component.

- If an acceptable result is still not achieved after substituting all new components contact your AB SCIEX Field Service Representative for service on the GeXP system.

3.2   Testing the Chemistry

Once it is determined that the GenomeLab GeXP instrument is functioning properly, then proceed with troubleshooting the following components of GeXP chemistry:

- GeXP Start Kit
- experimental process
- third-party reagents
- experimental RNA
- custom multiplex primers

Testing the GeXP Start Kit and Experimental Process

Test the components of the GeXP Start Kit (A85017) and the user's experimental process by performing standard GeXP reactions with control reagents.

1. Perform GeXP reactions with Control RNA and a set of GeXP multiplex primers previously demonstrated to generate size-specific amplicons, such as those provided in the GenomeLab GeXP Human Reference*Plex* (A54657) kit from AB SCIEX.

2. Analyze the reactions with the GeXP instrument and examine the analyzed results.

Interpreting the Results

- If all of the multiplex peaks and one KAN[r] peak are present, this confirms the functional quality of the GeXP Start Kit reagents and third-party-supplied materials. These results also confirm that the user's experimental process is satisfactory.

    - These results **do not** confirm the functional quality of any custom-designed multiplex primers and RNA template. See "Testing the Experimental RNA and Custom Multiplex Primer" on page 14 and continue in the troubleshooting process with these reagents.

- If the test does not yield all multiplex peaks and one KAN[r] peak, with good raw data signal and normal current profiles, perform the test again using a new GeXP Start Kit.

    - Failure to yield all multiplex peaks and one KAN[r] peak with a second kit would indicate that third-party reagents or the experimental process are suspect. See "Examining the Experimental Process" below and "Testing Third-Party Products" on page 13.

Examining the Experimental Process

The following are items to review as part of a successful GeXP Experimental Process:

- Reagent handling and storage
- RNA handling and storage
- Accurate pipetting
- Protocol Steps

See the GeXP Chemistry Protocol (A29143) for additional instructions on performing this process.

3.3   Testing Third-Party Products

Some reagents and plasticware, supplied by third-parties can have a negative impact on the GeXP RT-PCR reaction, raw data signal and the current profile. Third-party products includes the chemicals and plasticware used for suspension and dilution of RNA and primers, the RT-PCR reactions and the pre-dilution of the PCR reaction products.

Third-Party Products: Do's & Don'ts

- Use high-quality, nuclease-free plasticware.
- Suspend primer multiplexes in 10 mM Tris-HCl, pH 8.0.
- Store stock solutions of RNA samples in a buffered solution, such as The RNA Storage Solution (Thermo Fisher Scientific, Inc. PN AM7000).
- Store stock solutions of RNA in small aliquots at -80°C to preserve RNA integrity.
- Suspend working concentrations of RNA in nuclease-free, non-DEPC treated water, as supplied in the GenomeLab GeXP Start Kit or from Affymetrix (PN 71786) or Thermo Fisher Scientific, Inc. (PN 10977-015).

IMPORTANT Do not use DEPC-treated water or plasticware with the GeXP process. Residual DEPC can inhibit the PCR reaction and result in low signal strength and a high baseline in the electropherogram.

- Use aerosol-resistant barrier pipette tips with a dedicated set of pipettes for the setup of reverse transcription (RT) and PCR reactions to prevent cross-contamination of samples.
- Do not allow amplified product to enter the area of RT and PCR reaction setup (amplicon-free zone).
- Use **non-barrier** pipette tips and a separate set of pipettes, when handling the PCR products, Sample Loading Solution (SLS) and Size Standard-400 in a PCR amplicon zone. The SLS reagent may dislodge filter particles of aerosol-resistant barrier tip into the sample and this contamination can cause current failure in the GeXP System.
- Perform pre-dilution of the PCR reaction with 10 mM Tris-HCl, pH 8.0

Interpreting the Results

The common symptoms for poor quality reagents or the wrong reagent concentration are low raw data signal, high baseline and erratic current profiles.

- If the GenomeLab GeXP Human Reference*Plex* (A54657) kit reactions with Control RNA as outlined in "Testing the GeXP Start Kit and Experimental Process" on page 12, yield all the multiplex peaks and one KAN^r peak and the raw data signal baseline and current profiles look good, then third-party reagents are satisfactory.
- If the control multiplex reactions did not yield all of the multiplex peaks or the raw data or current profile were abnormal, then replace each third-party reagent in a systematic approach (one at a time) to identify the faulty reagent. Perform GeXP control reactions as described in "Testing the GeXP Start Kit and Experimental Process" on page 12, and analyze them with the GenomeLab GeXP.
- If the experimental RNA and custom multiplex primers still do not yield acceptable results, and a specific third-party product was not identified as the cause, proceed to the next section.

Testing the Experimental RNA and Custom Multiplex Primer

After confirming that the GenomeLab GeXP Human Reference*Plex* (A54657) kit and Control RNA function correctly with the GeXP instrument, test the experimental RNA and custom multiplex primers. Test the reagents independently. This is the most efficient means of identifying the source of the problem.

Testing the Experimental RNA

NOTE Use high quality experimental RNA that has a 28S/18S ratio greater than 1.0 with GeXP. Confirm that the ribosomal RNA 28S and 18S bands are readily visible and predominant when analyzed on an agarose gel.

Use a set of control GeXP multiplex primers that are known to produce size-specific amplicons from the source of experimental RNA, in order to test the functional integrity of the experimental RNA. For example, experimental human RNA sample(s) can be tested with multiplex primers from the GenomeLab GeXP Human Reference*Plex* Kit (PN A54657) which contains 24 sets of primers that target human housekeeping and other reference genes.

See "Interpreting the Results" on page 14 for more information.

Testing the Custom Multiplex Primers

NOTE Order custom multiplex primers with the universal tag sequences fused to the gene-specific sequence. The oligos should be of standard desalted, deprotected processing.

During the initial evaluation of the multiplex, the chimeric primers must be evaluated for their ability to produce the expected size amplicon with Control RNA (RNA template known to contain the target transcript). For example, the Human Reference Control RNA from the GenomeLab GeXP Human ReferencePlex Kit (PN A54657) can be used as a template to test custom multiplex primers that target human transcripts.

NOTE While the Human Reference Control RNA provides broad gene coverage, not all the gene transcripts are necessarily present. It is highly advisable to have a thorough understanding of the gene expression patterns for the transcripts detected by the custom multiplex primers.

- Develop a custom Control RNA by mixing RNA from several sources to achieve full transcript representation. Generally, this mixture will contain 50% of normal and 50% treated RNA.

NOTE If more than one treatment is being studied, combine the treatments so that each one is equally represented in the 50% treated RNA.

- Evaluate reverse primers in a multiplex context.

- Evaluate forward primers in both multiplex and singlet reactions. See the GeXP Chemistry Protocol (PN A29143) for more information.

Interpreting the Results

- If all of the multiplex peaks and one KAN^r peak are present, with no significant undesigned peaks (UDPs), the functional quality of the experimental RNA and custom multiplex primers are confirmed. See "Reference Tables" on page 17 for more information on UDPs.

- If a reaction containing experimental RNA and control multiplex primers yields a KAN^r peak, but no multiplex peaks or multiplex peaks with low signal, then it is likely that the quality of the RNA is poor. Re-evaluate the RNA source or purification process to yield higher quality RNA.

- If a reaction containing custom multiplex primers and Control RNA yields a KAN[r] peak, but is missing one or more peaks in the multiplex, then it is likely that redesign is required for the primer(s) of the missing peak(s). See "Multiplex Primer Design" on page 17 for more information on troubleshooting this issue.

- If a reaction containing custom multiplex primers and Control RNA yields a KAN[r] peak and all the multiplex peaks, but has significant UDPs, then primer redesign is necessary. See "Multiplex Primer Design" on page 17 for more information on troubleshooting this issue.

- If no reaction yields a KAN[r] peak (positive control), then see "Testing the GeXP Start Kit and Experimental Process" on page 12 to re-evaluate the start kit components and experimental process.

By using the previously described process, you should have been able to identify many of the most common causes of GeXP problems associated with instrumentation and chemistry in capillary electrophoresis-based gene expression profiling.

Refer to the "Reference Tables" on page 17 or the "Questions & Answers" on page 33 for additional causes and corrective actions for troubleshooting purposes.

Reference Tables

The following tables list issues that might be encountered while analyzing GeXP multiplex reactions with the GenomeLab GeXP Genetic Analysis System.

- Multiplex Primer Design on page 17
- GeXP Chemistry on page 20
- GeXP Separation and Fragment Analysis on page 25

For specific solutions or examples, look for references to sample figures in "Appendix A" on page 39.

4.1   Multiplex Primer Design

Issue	Cause	Solution
Having difficulties connecting to NCBI Primer-BLAST site.	• Internet connection is down. • The NCBI website is offline for maintenance (rarely occurs).	• Work with your local IT help desk to restore internet connection. • In a very rare case, if you can connect to other websites, but not NCBI Primer-BLAST site, report the issue via e-mail to blast help team at blast-help@ncbi.nlm.nih.gov for assistance. If you would like to use another primer design tool when you cannot access the NCBI Primer-BLAST site, you may use Primer 3 at http://primer3.ut.ee/. Please note that primers designed with Primer3 need to be evaluated using blast search to ensure there is no SNP sites in primer region and there is no low-complexity sequences in the amplicon.
The content of the help file for each search parameter could not be displayed when the "help" button ⓘ is clicked. In addition, the "Advanced parameters" are not available.	The web browser version is too old.	Use a more updated browser version or use a different browser. Use browsers that do automatic updates.
No results for primer searches when using the default values of "1000 to 1000000" for "Intron length range".	The intron size in the target gene is smaller than 1000.	Reduce the minimum intron length based on the sizes of the introns in the target gene.

Issue	Cause	Solution
I used to see exon numbers displayed in the "Graphical view of primer pairs" when I designed primers using NCBI Primer-BLAST. I no longer see the exon numbers. The exons are still displayed.	NCBI made some policy changes. Exon numbers are no longer provided in RefSeq transcript records. For details, please refer to the article published on Nucleic Acids Research, 2014, Volume 42, Database issue, D756-D763.	Instead of using exon numbers, describe an exon by referencing its location in the genomic sequence.
Multiple peaks appear for one set of primers in reverse singlet + forward singlet reaction	mRNA isoforms or homologous sequence	The gene sequence used for primer design may have led to amplification of alternative transcripts (isoforms) or a homologous sequence in another gene. • Be sure to choose mRNA or cDNA, and not genomic DNA, sequence files for primer design. • BLAST designed primer sequences to determine if more than one product will be amplified. • Use sequence alignment software to find a region of the gene that does not share homology with any other gene. Specifically target that region in the Primer Design option. • For transcript variants, design primers to bridge a specific exon-exon junction such that a specific transcript is amplified.
Undesigned peak(s) (UDP) is present in singlet or multiplex reactions	Non-specific amplification	If a UDP migrates within 3nt of a designed peak in the multiplex or affects the calculation of a designed gene peak area, then perform singlet reactions to identify the primer(s) causing the UDP. See **Figure A.1 on page 39**. Redesign the offending primer by targeting a different sequence or move the affected amplicon(s) to a different location in plex (different size), away from the UDP.
One or more designed gene peaks is absent from multiplex profile but KAN[r] peak is present	Poor primer design	Perform singlet reactions to determine if each set of primers amplifies the expected fragment size. Redesign primers for those genes that do not yield an acceptable singlet profile.
	RNA sample does not contain the transcript	Use an RNA sample that contains the transcript

Issue	Cause	Solution
A gene peak disappears from the multiplex profile when it is expected to be present in the RNA sample	Poor primer design	If the peak is present in some wells, but not others prepared from the same RNA sample and Master Mix reagents (RT, PCR), then the primer may not have good binding specificity. Polymorphism(s) in the primer sequence, especially at the 3' end, may result in amplification from one sample RNA but not another. Redesign the primer set to a better region of the gene (not too close to either the 5' or 3' end of the transcript, no repeat sequences, no GC rich regions, no homologous regions, no polymorphisms).
Low or no signal for a particular peak in a multiplex reaction with RNA expected to contain the transcript, using primers that generate a peak in a singlet reaction.	Primers for high expressing genes need attenuation or Primer interference	Reduce the concentration of reverse primers for the high expressing genes. It is most efficient to redesign the affected primer(s). However, if it is necessary to determine which primer is the interfering primer, perform duplex reactions containing the affected primer with each of the other primer sets in the multiplex, then redesign the interfering primer.
A particular amplicon has two or more shoulders or stutter peaks.	Repeats in amplicon sequence cause polymerase slippage See **Figure A.2 on page 40**	Check the designed amplicon for repeat sequences. Redesign the primers to a region of the gene that does not contain repeat sequences.
Significantly higher %CV in biological replicates compared to technical replicates	There is a SNP in the 3' end of primer sequence	Check the primers for single nucleotide polymorphisms (SNPs). Redesign the primer if any SNPs are present. SNPs can have profound impact on the priming efficiency depending on how much of the instability is introduced. A biological sample with the perfect match to the primers will generate a higher signal than a sample with a mismatch due to the presence of SNPs. This will result in high %CV in biological replicates, but not technical replicates. Biological replicates generally have slightly higher %CV than technical replicates, due to the natural variation between organisms or the variation that was introduced during sample preparation.

4.2   GeXP Chemistry

Issue	Cause	Solution
A variation in multiplex raw data is seen from build-to-build of custom primer plexes for the same RNA sample	The multiplex formulation has changed	Once optimized, do not change the multiplex formulation (primer concentrations). Carefully assemble each multiplex build for consistency. Remake the multiplex consistent with the optimized formula. Make sure to use the same primer plex formulation throughout a study.
Multiple designed gene peaks are present in reverse multiplex + forward singlet reactions	Primer contamination	The reverse primer stocks or reverse primer multiplex is contaminated with forward primers. Alternatively, forward primer stocks are cross-contaminated with other forward primers. - Decontaminate the lab bench, pipettes, etc. with a nucleic acid-destroying solution - Use fresh reagents and remake primer stocks and/or reverse multiplex - Use aerosol-barrier filter tips for making multiplexes and assembling RT and PCR reactions

Issue	Cause	Solution
Low signal	Primer Design	See this topic in **"Multiplex Primer Design" on page 17**.
	DEPC interference	**IMPORTANT** Do not use DEPC-treated water for GeXP as residual DEPC can interfere with PCR amplification. Use Nuclease-free water (Affymetrix 71786 or Thermo Fisher Scientific, Inc. 10977-015) when making Resuspension Buffer (10 mM Tris-HCl, pH 8) for primer plexes.
	Primer degradation	Use 10mM Tris-HCl, pH 8 (Resuspension Buffer) for making primer plexes. Store multiplex primers at -20°C.
	Primer quality	Primers can contain residual amounts of organic solvent. Order new primers from well-respected oligo vendors, such as Integrated DNA Technologies (IDT).
	Reagents - expired and/or improper storage/handling	• Check reagent storage conditions and expiration dates. All kit components, except RNA, should be stored at -20°C. • Avoid excessive freeze-thaw cycles. Control RNA and KAN^r RNA should be aliquoted into single use volumes after the first thaw and always stored at -80°C. • Use fresh reagents that have been properly stored.
		Vortex the 5x RT Buffer containing DTT and the 25 mM MgCl$_2$ to dissolve any precipitant before use.
	RNA template - quality, quantity	Verify that the RNA is of high quality and adequate quantity. Ribosomal RNA 28S and 18S bands should be prominent on agarose gel and 28S/18S ratio > 1.0. Recheck calculations for amount of input total RNA (25-100 ng is recommended). Increase the amount of RNA template used.
	Thermal cycler	Check thermal cycler calibration and protocol cycling temperatures. Raise the PCR extension temperature to 70°C to overcome variation in thermal cycler wells.
		Use a thermal cycler with a heated lid to prevent evaporation and verify that the lid temperature is the same temperature as the incubation chamber.

Issue	Cause	Solution
Low Signal	Sample degradation or Photobleach	• RT reactions can be stored at -20°C for up to one month. • Do not expose 5x PCR buffer or PCR reactions to light for an extended period of time. • Store PCR reactions at -20°C in the dark (wrapped in foil) for up to one month. • Diluted PCR sample may degrade faster in unbuffered water. It is recommended to use fresh 10mM Tris-HCl, pH 8 for pre-dilutions of PCR reactions.
	Capillary electrophoresis	See this topic in **"GeXP Separation and Fragment Analysis" on page 25**.
High Signal (peak height is greater than 120,000 RFU in analyzed data for one or more fragments)	Reverse primer concentration is too high See **Figure A.3 on page 40**	Attenuate the reverse primer(s) by reducing the concentration in the reverse multiplex
	Too much RNA	Reduce the amount of RNA.
	Too much PCR product loaded	See this topic in **"GeXP Separation and Fragment Analysis" on page 25**.

Issue	Cause	Solution
One or more designed gene peaks are absent from multiplex profile but KAN[r] peak is present	Poor primer design	See this topic in **"Multiplex Primer Design" on page 17**.
	RNA	The RNA sample may not contain the transcript(s) or the gene is downregulated in that sample. • Use more RNA in the reverse transcription reaction (up to 100ng per reaction) • Use an RNA source known to contain the gene transcript of interest to validate the primer design • Combine RNA samples/sources such that all genes in multiplex are represented at a relatively moderate detectable level and use this RNA as a positive control for gene detection and multiplex optimization
		RNA may be degraded. Verify the RNA quality (Ribosomal RNA 28S/18S ratio should be > 1.0). Store stock RNA samples in The RNA Storage Solution (Thermo Fisher Scientific, Inc. AM#7000), at -80°. Aliquot to working volumes, and thaw aliquots only once to ensure RNA integrity.
	Primer concentration	Increase reverse primer up to 150 nM per reaction (1.5 μM in the reverse multiplex) for each low expresser. Additionally, decreasing the high signal of high expressers through attenuation may bring up the signal of low expressers.
Peaks are present in RT minus reactions.	Genomic DNA contamination See **Figure A.5 on page 41**	Treat RNA with RNase-free DNase during RNA purification.
	Amplification contaminated with another DNA template or DNA amplicons	• Use aerosol-resistant barrier tips during RT and PCR reaction preparation to minimize contamination from external sources. • Use fresh tips for each step in preparing the GeXP reactions • Separate pre- and post-PCR work areas. • Do not bring amplified product into pre-PCR area.
	Well-to-well contamination	Contamination across wells can occur during set up for PCR or separation, especially with single channel pipetting. Use multichannel pipette to reduce pipetting error and contamination.

Issue	Cause	Solution
Low or no signal for a particular peak in a multiplex reaction using primers that were validated in a singlet reaction	Primer interference	See this topic in **"Multiplex Primer Design" on page 17**.
Undesigned peak(s) (UDP) is present in multiplex reactions	Non-specific amplification	See this topic in **"Multiplex Primer Design" on page 17**.
Sloping gene expression profile or Smaller fragments have much higher signal than larger fragments but size standard peaks look normal.	Signal drop-off See **Figure A.6 on page 42**	Thermal cycler temperature fluctuation during PCR extension leads to a disproportionate amplification of shorter fragments over long fragments, called signal drop-off. This commonly occurs in the edge wells of a thermal cycler that is not performing well. • Repair and/or calibrate the thermal cycler • Use the center wells of a thermal cycler • Alternatively, optimize the extension temperature; generally by raising the extension temperature by a degree or two **IMPORTANT** Signal drop-off can lead to high %CV in analyzed data and results of these wells should not be used for data analysis.
		Wrong DNA Polymerase was used. AB SCIEX has validated ThermoStart DNA Polymerase (A85022) for use with the GeXP System.
Unbalanced profile or Reference (housekeeping) genes are not generating the expected high peaks relative to other genes in the plex.	Multiplex is not optimized See **Figure A.3 on page 40**	Attenuate the concentration of reverse primers for high signal peaks and high expressers. The purpose of attenuation is to bring down the peak height of high expressers to the level of moderate expressers in the same plex. It is an approach that balances relative signal strength in the optimal detection range. • Select a moderate expresser peak in a moderate signal range (10,000 - 50,000 RFU) as reference upon which the optimal attenuation factor for high expressers will be decided. Then bring down the high expresser peaks to the level of the moderate expresser reference peak by reducing reverse primer concentration. It is not necessary, nor correct, to consider absolute peak height for this approach. • Increase the concentration of reverse primers for low expressers up to 150 nM per reaction to increase signal strength relative to the moderate expresser gene(s).

4.3   GeXP Separation and Fragment Analysis

Issue	Cause	Solution
Current failure	Air bubble in the manifold	Perform extensive purging of the GeXP instrument, with fresh separation gel before each run. Perform a Manifold Purge three times with 0.4 mL gel. Then perform three capillary fills.
	No separation buffer or excessive separation buffer	Rerun PCR samples on GeXP. Add the recommended amount of separation buffer (250 ul) to each well of the buffer plate that corresponds to a filled well of the sample plate. Keep the buffer evaporation cover over the buffer plate when installed on instrument.
	Excessive amount of salt in sample	Use fresh 10 mM Tris-HCl, pH 8 to pre-dilute PCR reactions, if necessary, before adding the diluted sample to Sample Loading Solution with Size Standard 400.
Low signal or no signal	No separation buffer or excessive separation buffer	Rerun PCR samples on GeXP. Add the recommended amount of separation buffer to each well of the buffer plate that corresponds to a filled well of the sample plate. Keep the buffer evaporation cover over the buffer plate when installed on instrument.
	Poor injection due to excess salt	Too much salt in a sample can lead to poor injection of amplicons which leads to low signal. Use fresh 10 mM Tris-HCl, pH 8 to pre-dilute PCR reactions, if necessary, before adding the diluted sample to Sample Loading Solution with Size Standard 400.
	Chemistry	See this topic in **"GeXP Chemistry" on page 20**.
High Signal (peak height is >120,000 RFU in analyzed data for one or more fragments)	Too much RNA	Reduce the amount of RNA used in the RT reaction.
	Too much PCR sample was used for loading See **Figure A.4 on page 41**	Prepare serial dilutions (1:5, 1:10, 1:20) in 10 mM Tris HCl, pH 8 of the PCR products. Mix 1 uL of the pre-diluted sample to Sample Loading Solution with Size Standard 400 and test each dilution in all capillaries to find the best dilution for analysis. The linear range of detection lies between 370 - 120,000 RFU in analyzed data. It is recommended that data outside this range not be used for analysis.
Additional peaks from different dye-channels are present in the data	Too much PCR sample was used for loading See **Figure A.7 on page 42**	If the peak height (signal) is too high (>120,000 RFU), a small peak may 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 or pre-dilute the PCR product in 10mM Tris-HCl, pH 8.

Issue	Cause	Solution
Unresolved fragment in a particular size range is present in standard (STD) and no template control (NTC) reactions.	Protein-nucleic acid complex See **Figure A.8 on page 43**	A non-specific, protein-nucleic acid complex will migrate in a particular size region of electropherogram. This complex is of consistent size, specific to a multiplex and does not affect analysis unless it overlaps with a designed peak. Redesign the primers of any gene peaks that comigrate with the complex.
Valid peaks were not analyzed or called in Fragment Analysis	Analysis parameters	The wrong analysis parameters can lead to poor analysis. Use the Default GeXP Analysis Parameters: Slope Threshold = 10%, Relative Peak Height Threshold = 1%, Confidence level = 95%, SizeStd 400, Cubic Model, Dye Mobility Calibration ON = PA ver.1, Calculated Dye Spectra. The Slope and Peak Height Thresholds can be reduced to pick up small peaks.
	Exclusion filter	An exclusion filter in the Fragment List tab of Fragment Analysis module may be active.
Size shift in designed gene peaks and/or KAN^r peak	Analysis parameters	Use the Default GeXP Analysis Parameters: Slope Threshold = 10%, Relative Peak Height Threshold = 1%, Confidence level = 95%, SizeStd 400, Cubic Model, Dye Mobility Calibration ON = PA ver.1, Calculated Dye Spectra.
	Wrong size standard	Verify which Size Standard chemistry was used. GeXP Analysis Parameters are designed to be used with Size Standard-400. If Size Standard-600 was used, the fragment sizing will be different for samples analyzed with Default GeXP Analysis Parameters. Edit the GeXP Analysis Parameters so that Size Standard-600 and Quartic model are selected and then reanalyze the data. Save this revised Analysis Parameter with a new name (i.e. GeXPss600).
The peak for a low expresser or uninduced gene was not detected.	Analysis parameters See **Figure A.9 on page 44** and **Figure A.10 on page 44**	When GeXP analysis parameters are lowered to Slope Threshold = 1 and Peak Height Threshold = 0, nearly every peak, can be detected. If a very small peak (~370 RFU) with the exact same fragment size as the induced gene is detected, it can be treated as real peak in the uninduced sample. It is best if this very small, uninduced peak resides in an area with a clean baseline (no UDPs or excessive noise) of the multiplex profile so the peak call is accurate. See **Figure A.10 on page 44**.
Too many small, undesigned peaks called	Analysis Parameters	Set up and apply locus tag and allele IDs through binning. Then, apply an exclusion filter in "Fragment List View": allele ID = (empty space or blank). Alternatively, exclude the small insignificant peaks by establishing an exclusion filter in the Fragment List of Fragment Analysis. Then the peaks below a particular peak height will not be called.

Issue	Cause	Solution
Analysis error	Over-range data	The data is above the instrument's linear range of detection and the software cannot analyze the data properly. Any over-range data should not be used for expression analysis. • Dilute PCR samples in 10 mM Tris-HCl, pH 8 to bring data into the linear range; below 120,000 RFU in analyzed data. • Attenuate high expressers if necessary, to balance the profile.
Split peaks with extremely high signals in analyzed data	Over-range data See **Figure A.11 on page 45**	Check the raw data to confirm the results. Do not use over-range data for expression analysis. Dilute PCR sample in 10 mM Tris-HCl, pH 8 to bring data in linear range of detection, less than 120,000 RFU in analyzed data. Attenuate the high expressers if necessary to balance profile.
Inconsistent fragment sizes or unusual peak shapes or low signal	Gel life exceeded	Check the on-board gel life. Gel life is verified for 72 hours on the instrument.
	Capillary array life exceeded	Check capillary array life. Capillary array life has been verified for 100 runs 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 the samples. If the problem continues, contact your AB SCIEX Field Service Representative.

4.4   Gene Expression Analysis

Issue	Cause	Solution
High %CV	Sample size	Perform at least three technical replicates per RNA sample. Generally, more technical replicates of the same RNA sample or RT reaction reduces the %CV.
	Sample type	A small number of biological replicates can lead to a high %CV due to inherent diversity between organisms. Low expresser genes tend to have higher %CV.
	Reference Gene	Review the choice of reference gene to verify that relative expression of this gene is constant across all samples. If the reference gene expression fluctuates greatly between samples, this could influence the %CV. Choose a reference gene that has constant expression under all conditions that will be examined in the study. The GenomeLab Human Reference*Plex* Kit (A54657) is a validated assay designed to determine the best references gene for human RNA samples. **NOTE** Normally, the KAN^r peak signal is usually out of range and should therefor not be used as a reference gene for normalization of experimental genes. If it is desired to use KAN^r as a reference gene for normalization, the amount of KAN^r RNA needs to be reduced so that the peak height of KAN^r is similar to that of the median-expressers in the multiplex.
	Signal drop-off	See this topic in **"GeXP Chemistry" on page 20**.
	SNPs in the primer(s)	See this topic in **"Multiplex Primer Design" on page 17**.
Third-party software for gene expression analysis	Data analysis	The following analyses can be performed on GeXP data, using Microsoft Excel: • multiple reference gene normalization • calculation of fold-change • statistical analysis Additional software programs for use with GeXP-generated data are GeNorm, DecisionSite, PartekGS and Prism. Visit the websites below for information on these software programs. Third-party software information: http://primerdesign.co.uk/genorm_licence.asp http://www.spotfire.com http://www.partek.com http://www.graphpad.com/prism/Prism.htm

4.5   GeXP Data Tool

Issue	Cause	Solution
Error message "The file format is incorrect" appears when opening the input file.	1. The input file was created using older versions (version 10 or older) of GeXP software. 2. The input file was created using "Export Fragments/Genotypes..." or "Export Grid..." instead of using the "Transfer Fragments for GeXP..." function. 3. The original export file from Fragment Analysis module was modified in Microsoft Excel. The double quotes inside the input file was removed by Excel. The GeXP Data Tool will not be able to accept this modified input file even if it was saved in .csv format.	Re-create the input file in the correct format using the "Transfer Fragments for GeXP..." export function of the GeXP software (version 11.0 or newer). Do not modify the input file in Excel. If it is necessary to modify the sample names, perform the name change for the raw data files in the "Database" module of the GeXP software. Then, repeat the analysis and data export.
No further processing of the data file.	Sample names have more than 10 replicates.	Limit the number of replicates to 10.
	There is more than one value for a gene within the same result (replicate).	During the binning process in fragment analysis module, ensure the allele ID names for different genes are unique, and no more than 8 characters long.
Some sample data were excluded from the export file.	There was no replicate data (ND) for this sample.	Use only optimized primer multiplex for generating standard curve data.
	If there was no data for one of the genes at a particular standard concentration, the corresponding row for all genes at this standard concentration will be excluded from the export file.	Ensure all genes in the multiplex are expressed in the RNA sample used for generating the standard curve so all genes are detected at the desired standard concentrations. Make sure replicate data is included in the input file

NOTE Please refer to the on-line Help file in the GeXP Data Tool for additional troubleshooting information.

4.6   GeXP Quant Tool

Issue	Cause	Solution
Cannot find the GeXP report file or GeXP Data Tool export file in the folder where the exported data was saved.	The exported data file was renamed and the file extension ".txt" was removed.	Keep the file extension ".txt" when renaming the exported data file.
Standards and samples have mismatched genes.	Standard and samples are not from the same multiplex or the ".txt" file was modified to remove some genes or data for genes.	Match the multiplex for standards and samples.
Error message: Standard or Samples names may not be correctly formatted.	Standard or sample was not named correctly.	Follow the instructions for naming standard and experimental samples. Standard names must start with "Std", followed by a space character and the standard concentration in nanograms in the following format: "Std xxxng"; where xxx is any whole or decimal number. Sample names must start with "U", followed by a space character and other information in the following format: "U HuBCRNA".
Error message "Problem building samples for gene ..." appears during Quant Tool analysis process.	An internal processing error occurred due to unexpected contents (not data) or formatting in the input files.	Click "Ignore" to resume analysis process. Check results for the affected gene in the analysis report generated. If data points are missing for this gene at certain standard concentrations, remove data points for ALL genes at these standard concentrations in the .txt file and repeat the analysis in GeXP Quant Tool.
The R^2 value of this gene cannot be returned by the Quant Tool.	No data entry for this gene at certain standard concentrations.	Start with a good reference RNA, or load more PCR samples for CE separation, make sure that every gene can be called at least once, even at the lowest standard concentration. Alternatively, remove data points for ALL genes at the standard concentrations where there was no data for the affected gene in the .txt file. Repeat the analysis in GeXP Quant Tool.

Issue	Cause	Solution
#DIV/0! Error	Formula used is dividing by zero or empty cells; likely cause is a missing GEOMEAN value on the **Normalization_Values** worksheet. No data points in the replicate wells will also cause this error.	Choose different normalization gene(s) or rerun sample.
#NUM! Error	Formula has invalid numeric values. Likely cause is a GEOMEAN calculation using a negative GEQ replicate value.	Delete the formula from the normalized GEQ replicate cell with the error message. Select a different normalization gene.
Gene names are in red text on Summary worksheet Negative or erroneous GEQ values on gene worksheets	Gene has sample values that are outside the range of the standard curve.	Increase the range (up and/or down) of the standard curve to cover all sample values. Repeat experiments with the new standard curve. Alternatively, for samples below the range of the standard curve, create a linear standard curve from the three lowest concentrations of RNA (y-axis) and signal level (x-axis) and force the y-intercept to zero. From the equation of the trend line, calculate the GEQ value for each sample replicate that was below the range of the original standard curve.
Empty GEOMEAN formula cell in the Normalization_Values worksheet	GEQ value for one or more of normalization genes is missing.	A GEOMEAN cannot be calculated when a GEQ is missing for one or more of the selected normalization genes. Rerun the analysis with different normalization genes.
R^2 value below 0.99	Poor standard curve due to: 1) One or more standard data points deviating from the trend line (curve). 2) One or more replicates deviate from the others (outlier). 3) Not enough standard data points. 4) Poor primer design.	Remove outlier data from standard curve calculation (When deleting a standard, delete all of the standard's cells -- the concentration, the replicates and the statistics. Do not delete the entire row, since this may also delete the standard curve formula cells). Increase the number of standard data points. Redesign primers. Rerun the standard curve.

NOTE Please refer to the on-line Help file in the GeXP Quant Tool for additional troubleshooting information.

Questions & Answers

5.1   Multiplex Primer Design

Question	Answer
What are the optimal conditions to use when I manually design or redesign primers for use with GeXP?	Although these are the optimal conditions for primers, individual primers within a multiplex will vary. • Approximately 20 nt in length, without universal tag • 50% G+C content • Tm = 60°C (Range: 57-63°C) • Ensure that the Max Tm difference between the forward and the reverse primers is no more than 5°C • The last 5 nucleotides at 3' end should contain at least 2 As or Ts and not contain any polymorphisms The primer length can be vary from 20 nt as long as it meets the other conditions.
Can I design primers to detect alternative transcripts?	Yes. First, define the mRNA isoform(s) of interest and design primers specifically to include or exclude exons or to bridge unique exon-exon junctions.

5.2   GeXP Chemistry and Fragment Analysis

Question	Answer
What is the difference between the pre- and post-PCR areas?	The pre-PCR area is used to set up both the reverse transcription (RT) and PCR reactions. The post-PCR area is where the PCR reaction goes after amplification. The pre-PCR area is divided into two zones: • No Template Zone: Assemble and aliquot the master mixes • Template Addition Zone: Add RNA for the RT reaction or add cDNA to the PCR reaction **IMPORTANT** Amplified (PCR) product should never be brought into the pre-PCR area. Use the thermal cycler for PCR in the post-PCR area. Any area that is exposed to amplified PCR product should be considered as an area that contains amplified template in the environment.
What can happen if amplified PCR product is brought into the pre-PCR area?	One symptom of PCR product contamination of the pre-PCR area is peaks in the RT minus and No Template Control reactions of GeXP. The contamination of GeXP RT-PCR sample wells with exogenous template will compromise the relative quantitation of GeXP. To clean up a pre-PCR area contaminated with amplified product or other nucleic acid template, use a 5% bleach solution or commercially available decontaminant such as DNA Zap (Thermo Fisher Scientific, Inc.) or DNA AWAY (Molecular BioProducts), to wipe down all surfaces and equipment.
What RNA should I use to evaluate and optimize my custom multiplex?	Initially test the multiplex on a Control RNA that consists of a mixture of the RNA samples that will eventually be tested individually with the multiplex (e.g. untreated + treated or normal + disease). All the gene transcripts must be present in the Control RNA for initial evaluation and validation of multiplex primers. For optimization of the multiplex, ratios of each RNA sample in the Control RNA should reflect a moderate level of expression for most individual genes. **NOTE** Levels of expression are relative for each gene.
Are undesigned peaks (UDPs) in singlet reactions cause for concern?	If UDPs: • migrates at the same size as a designed peak in the multiplex • affect the quantitation of a designed gene peak then redesign primers that cause this UDP. It is best to assess the significance of a UDP in a singlet reaction when the peak height of the designed peak is below 120,000 RFU.
What do I do if an undesigned peak (UDP) co-migrates with a designed peak in a multiplex?	If it is possible to determine which primer is causing the UDP from singlet reactions, then redesign this primer by targeting a different sequence. Alternatively, the designed peak can be moved to a different location in the plex by redesigning the primers. This may simply involve moving the primer position a few nucleotides in one direction or the other to shift the designed peak away from the UDP.

What is attenuation and how will it affect my results?	Attenuation is the process by which the reverse primer concentration of high expressers is reduced. This brings the high expresser gene signals into range of the moderate expressers. Attenuation is used to balance the signal of the gene expression profile within the linear range of detection. The GeXP instrument has a lower and upper limit in its range of detection (370 - 120,000 RFU in the analyzed data). During multiplex optimization, it is important to ensure that all peaks fall within that limit, ideally within a range between 2,000 - 50,000 RFU. Attenuation performed during the optimization of a particular multiplex has no effect on results, because this optimization is carried out on Control RNA. The goal is to establish the baseline levels of expression and gene specific reverse primer concentration with Control RNA before testing other RNAs. Attenuation affects only the detection of mRNA of the particular transcript. This is usually a high expresser, such as a housekeeping gene that needs to be brought into the linear range of detection. The concentration of reverse gene-specific primers are changed only during the plex optimization stage. Once concentrations for all primers in a reverse multiplex have been optimized, the primer concentrations remain fixed for all subsequent experiments. Thus, the relative quantitation of gene expression for any particular gene will remain constant in a particular sample. Further the fold change in expression for this gene between treatments is always calculated using the relative quantitation. For more information on Attenuation, visit our website: www.beckmancoulter.com/genomelab and obtain a copy of the Applications Information Bulletin A- 2049A Multiplexed, Quantitative Gene Expression Analysis for Lettuce Seed Germination on GenomeLab GeXP Genetic Analysis System.
What is the best way to detect the peak area of an uninduced or low expresser gene after primer optimization?	To detect the peak area of an uninduced/low expresser gene: Change the GeXP Analysis parameters in Fragment Analysis to Slope Threshold = 1 and Peak Height Threshold = 0, and reanalyze. With these settings, nearly every peak, can be detected. If a very small peak (~370 rfu) with the exact same fragment size as the induced gene is detected, it can be treated as real peak in the uninduced sample. For the most accurate peak call, it is best if this very small, uninduced peak resides in an area with a clean baseline (no UDPs or excessive noise) of the multiplex profile.

What is the best way to detect a large fold induction with GeXP?	Induced genes will usually have a relatively low signal (small peak) in the uninduced or basal state and high signal (tall peaks) in the induced state. Attenuate the reverse primer concentration and pre-dilute the PCR products such that uninduced samples generate a very small, yet detectable peak and induced samples generate a peak within linear range of detection (370 - 120,000 RFU in analyzed data). Separate PCR reactions from uninduced RNA samples in middle CEQ capillaries (C, D, E, F) and reactions from induced samples in the outer capillaries (A,B, G, H). Detection of approximately 1000-fold change in expression can be achieved with this method.
What is the minimum and maximum relative limit in fluorescence units (RFU) for a designed peak to remain in the linear range of detection?	370 - 120,000 RFU in the analyzed data. This is the recommended signal range for quantitation with GeXP. In order to detect variations in gene expression from sample to sample, design gene peaks within the range of approximately 2000 - 50,000 RFU, during multiplex optimization.
Can I use the KAN^r peak as a reference gene for normalization?	Yes, under certain conditions. KAN^r RNA is an independent template that is designed to serve as a positive, internal control for the RT and PCR reactions. Normally, the KAN^r peak signal is out of range and should not be used as a reference gene for normalization of experimental genes. If it is desired to use KAN^r as a reference gene for normalization, the amount of KAN^r RNA needs to be reduced so that the peak height of KAN^r is similar to that of the median-expressers in the multiplex.

5.3   Gene Expression Analysis

Question	Answer
Are there any third-party software programs that I can use with GeXP-generated data for gene expression analysis?	Yes. The following analysis can be performed on GeXP data, using Microsoft Excel: • multiple reference gene normalization • calculation of fold-change • statistical analysis Additional software programs for use with GeXP-generated data are GeNorm, DecisionSite, PartekGS and Prism. Visit the websites below for information on these software programs. Third-party software information: http://primerdesign.co.uk/genorm_licence.asp http://www.spotfire.com http://www.partek.com http://www.graphpad.com/prism/Prism.htm

Appendix A

A.1   Reference Figures

The following images are for use with the **"Reference Tables" on page 17** and **"Questions & Answers" on page 33** of this guide.

Undesigned Peak (UDP)

A UDP that co-migrates with a designed peak will affect quantitation of the designed peak.
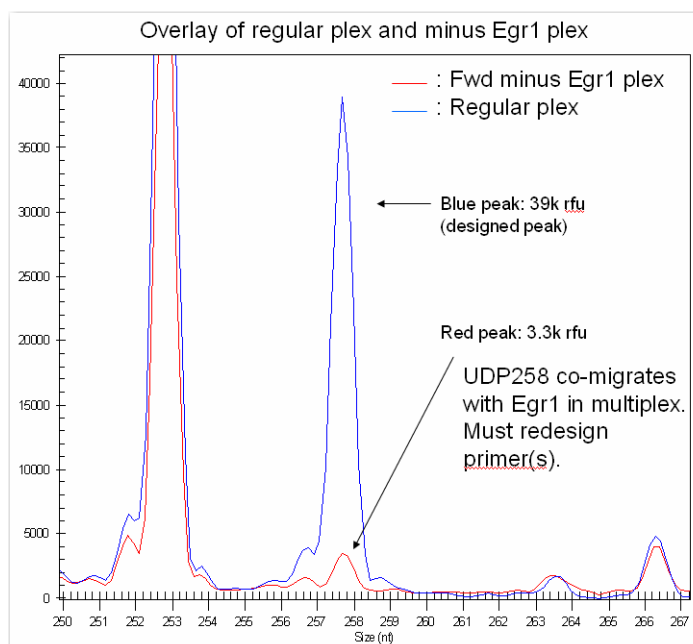
Figure A.1 Co-migrating Undesigned Peak

Repeat Sequences

Repeats in the amplicon sequence can cause DNA polymerase slippage, which results in stutter peaks.

GGCATGGCtttattgttttttttgttttttttgg
tttttttttttttttGGCTTGAC
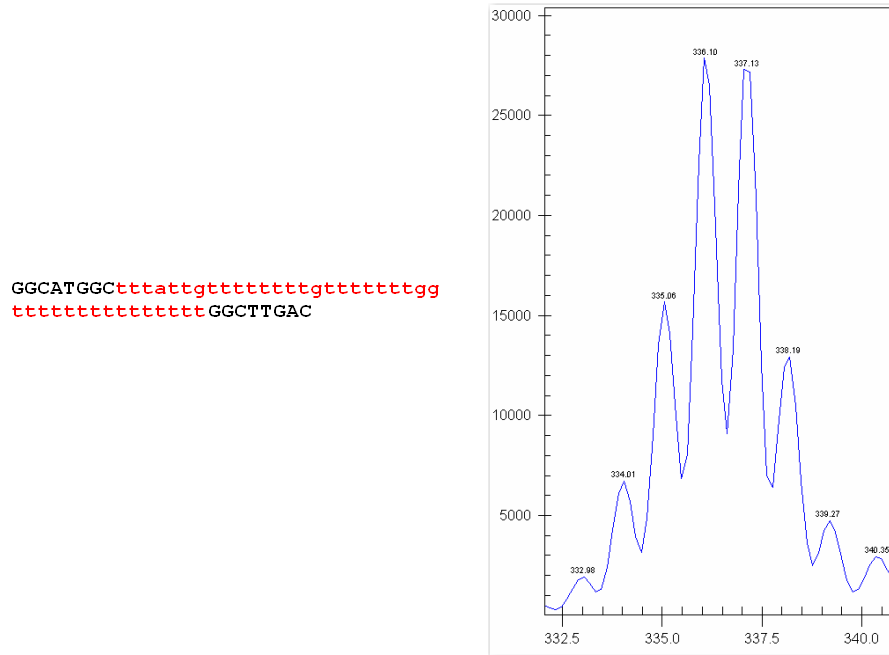
Figure A.2 Repeat Sequence and resulting Stutter Peaks

Over-range Signal in an Unbalanced Profile

A balanced multiplex profile is achieved by attenuating the reverse primer concentration of the high expressers. See **Figure A.4 on page 41** for a Balanced GeXP Profile.
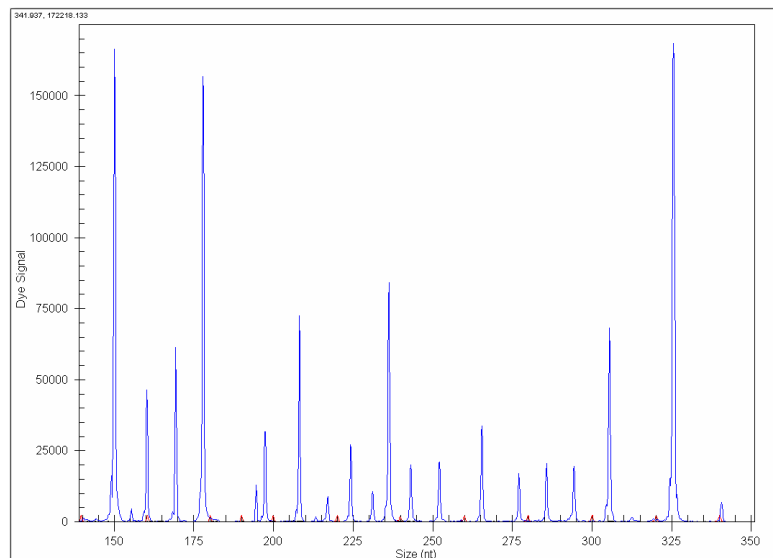
Figure A.3 Unbalanced GeXP Profile - Over-range

Over-range Signal in Balanced Profile

Any signal >120,000 RFU in analyzed data lies outside the linear range of detection, with standard GeXP data analysis. Pre-dilute the PCR product to bring all fragments within the linear range.
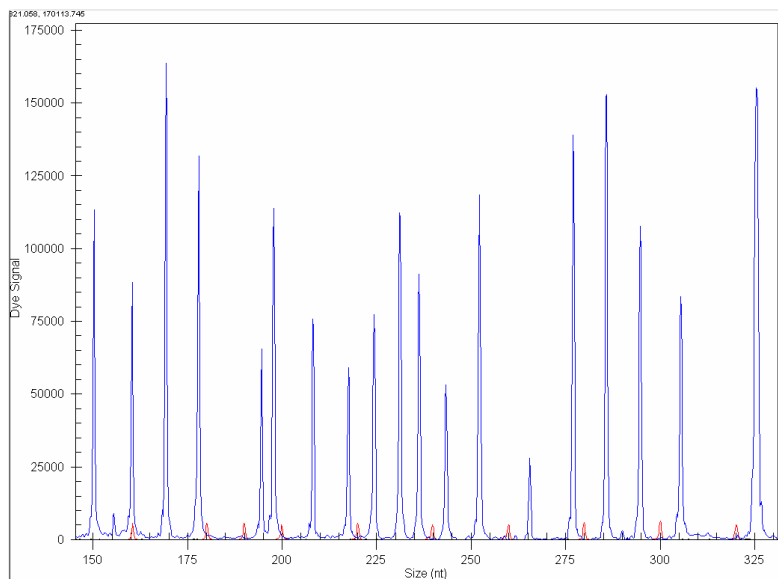
Figure A.4 Balanced GeXP Profile - Over-range

Genomic DNA Contamination

Genomic DNA contamination of RNA samples leads to the production of noise peaks in the RT minus reaction.
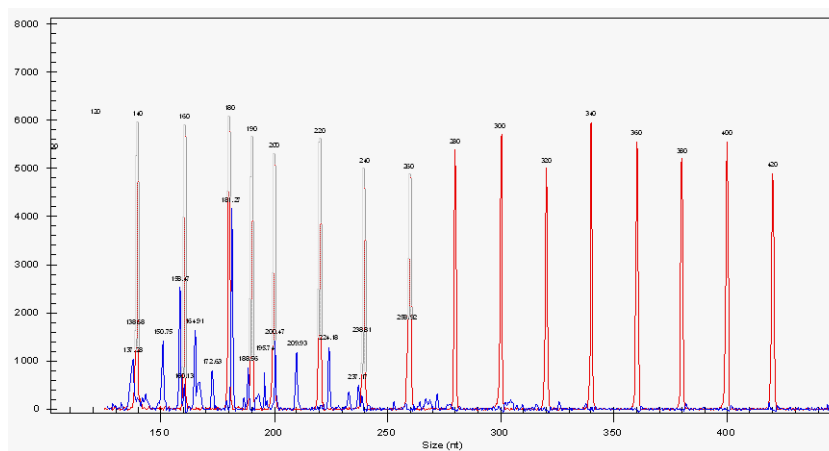
Figure A.5 Noise Peaks in RT minus reaction

Signal Drop-Off

Smaller fragments have a much higher signal than larger fragments, due to thermal cycling temperature fluctuation or incorrect DNA polymerase.
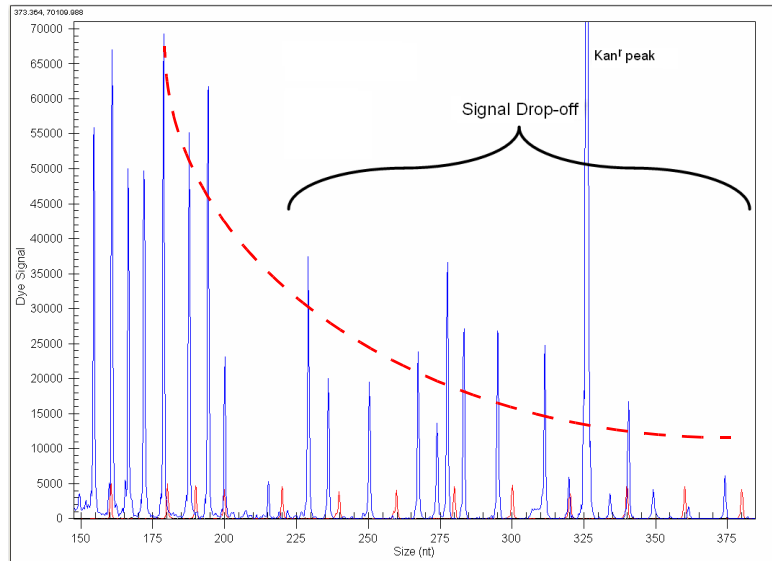
Figure A.6 Electropherogram with Signal Drop-off

Dye-Channel Pull-up

If the D4-dye signal is too strong, a small green D3-dye peak may be observed underneath the over-ranged peak.
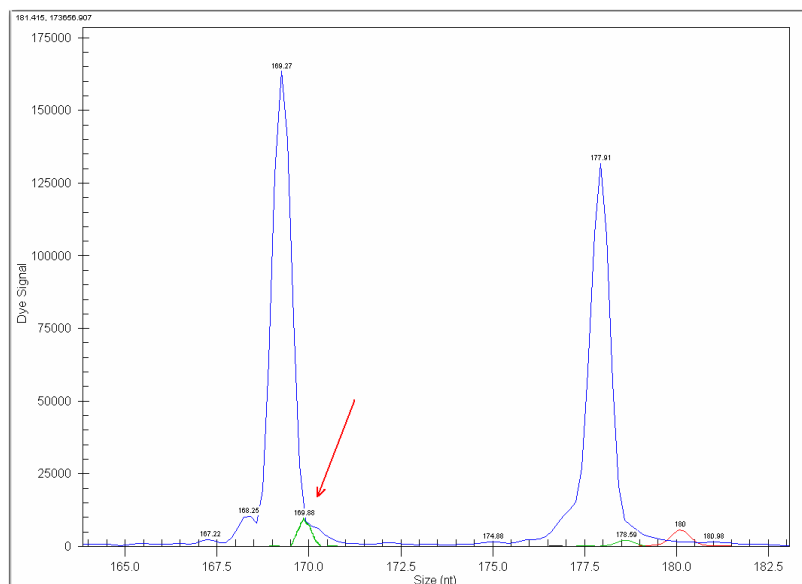
Figure A.7 D3-dye Channel Pull-up

Unresolved Protein-Nucleic Acid Complex

A non-specific protein-nucleic acid complex will consistently migrate in a particular size region, specific to the multiplex.
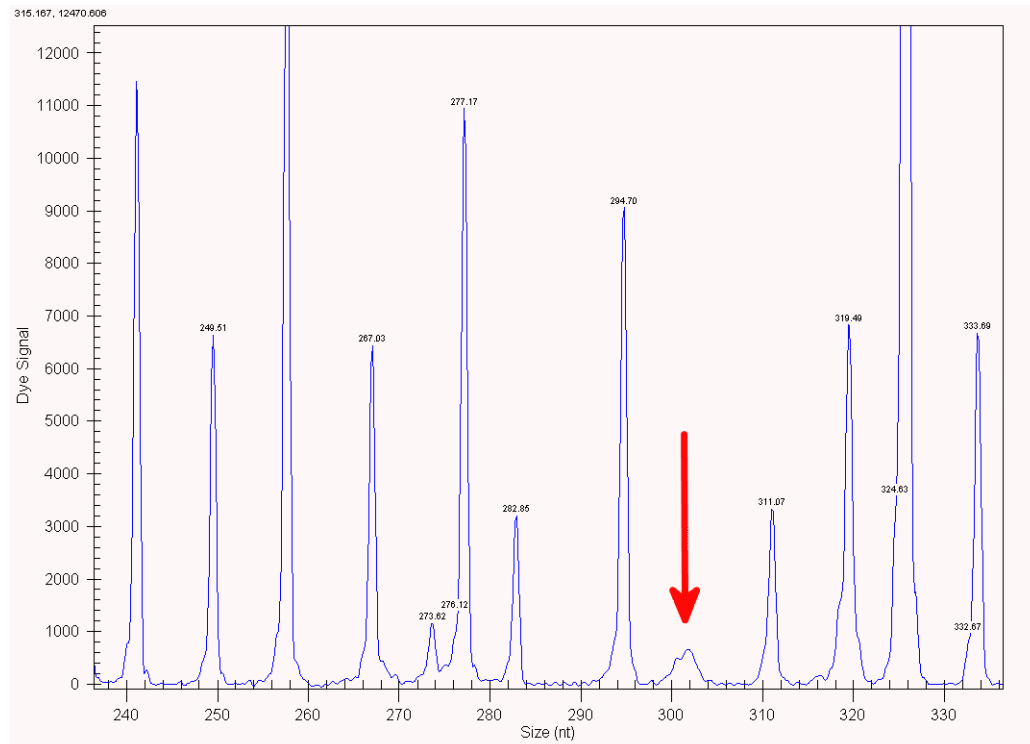
Figure A.8 Unresolved Fragment

Modifying Analysis Parameters

Default Analysis Parameters are too stringent to allow peak call.
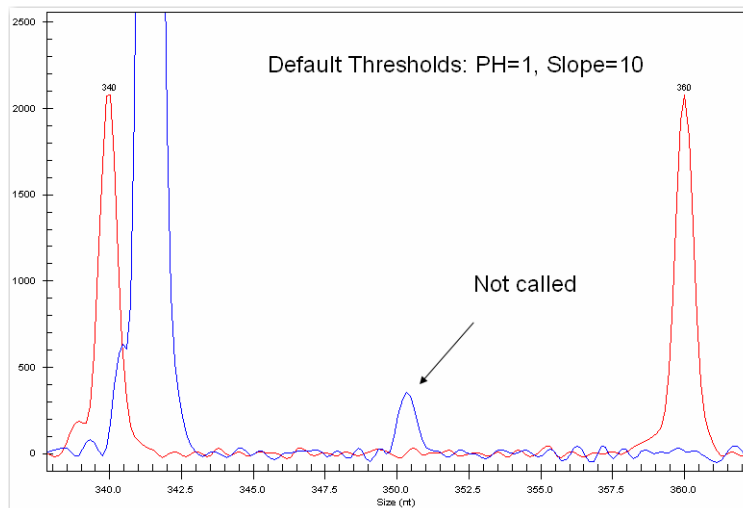
Figure A.9   Default GeXP Analysis Parameters

Modified Analysis Parameters allow peak call.
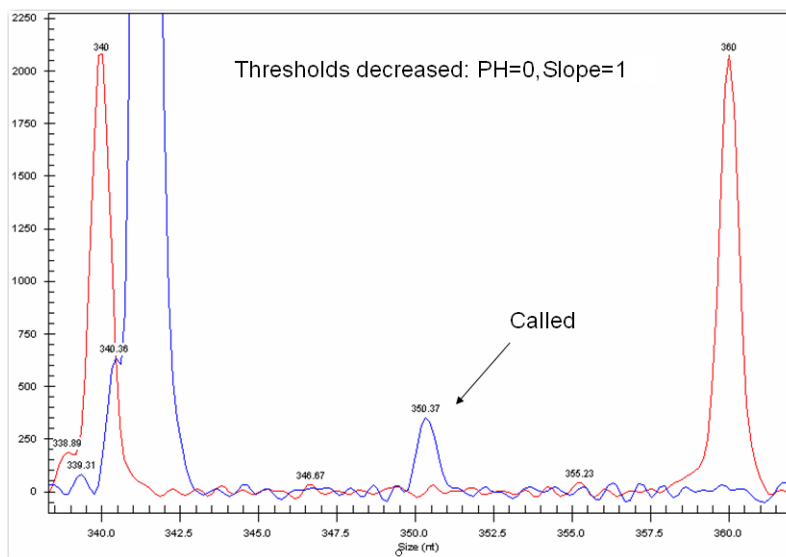
Figure A.10   Modified GeXP Analysis Parameters

Split Peaks

Peaks that are significantly over-range tend to "split" and be called as two peaks in the analyzed data.
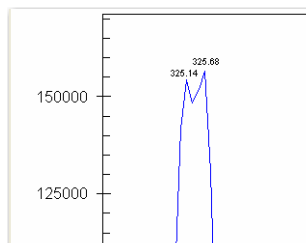
Figure A.11 Split Peak

Appendix B

B.1   Error Messages

This Appendix covers the "Common Error Messages for the GeXP Data Tool" and the "Common Error Messages for GeXP the Quant Tool".

Common Error Messages for the GeXP Data Tool

The File Format Is Incorrect

If there is an error opening the input file, or if the file is not in the expected format (i.e., required column headings are not found), an error message will be given and no further processing of the file will take place.
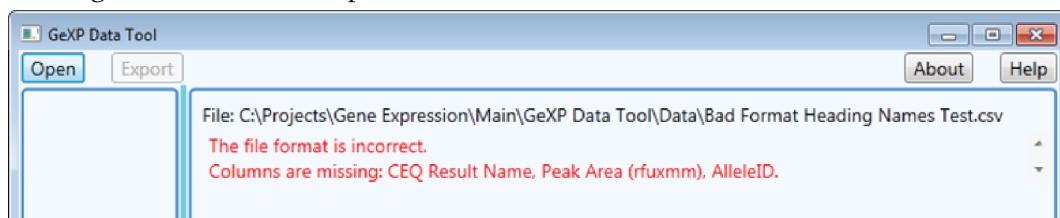
Figure B.1 Input file format is incorrect

If the input file was modified in Microsoft Excel, the double quotes inside the input file will be removed by Excel. The GeXP Data Tool will not be able to accept this modified input file even if it was saved in .csv format.
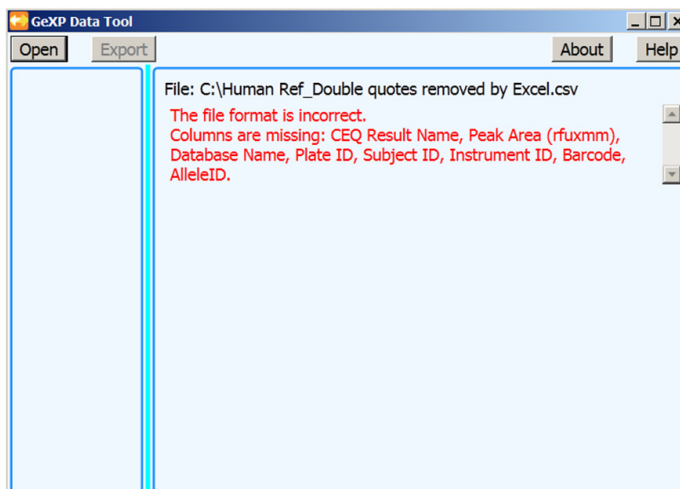
Figure B.2 Error caused by modifying the input file in Excel

The Gene Name Must Not Begin With A Digit

If any gene name begins with a digit, the entry in the Normalization gene list and the table heading for that gene will have an underscore character (_) added as a prefix. The table heading for that gene will be highlighted as a warning with a yellow background. A tool tip indicates the prefix to the gene name.

Figure B.3 Gene Name Must Not Begin With A Digit

This Row Does Not Contain Any Useful Data

Samples with no replicate data will be highlighted as errors with a red background. A tool tip indicates the error and data will be excluded from the export file.

Figure B.4 Row Does Not Contain Any Useful Data

Another Gene has no Data for this Standard Concentration

If a sample **Standard** has no data in one gene, the corresponding standard row for all genes will be highlighted as warnings with a yellow background. A tool tip explains the warning and data will be excluded from the export file.

Figure B.5 Another gene has no data for this standard concentration

Common Error Messages for GeXP the Quant Tool
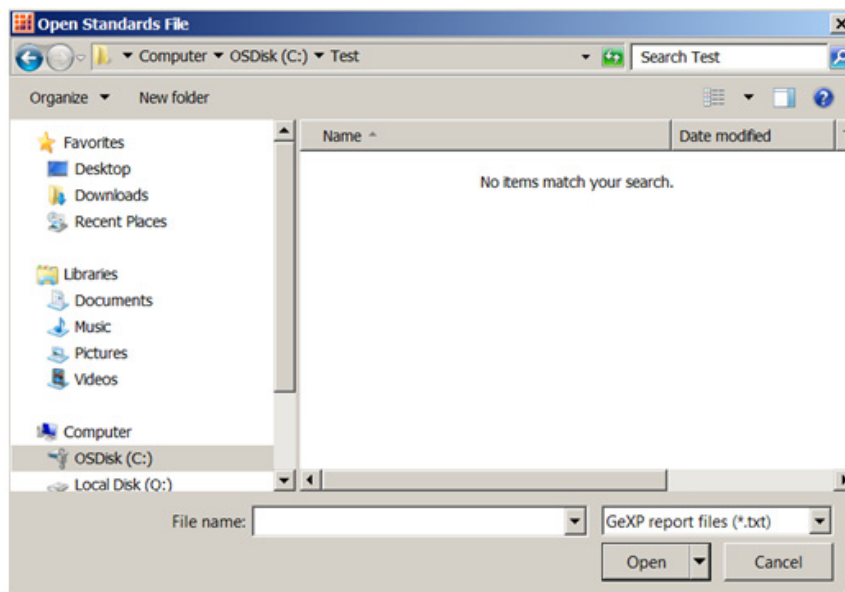
No Items Match Your Search

Figure B.6 Exported data file cannot be found

This error message in Figure B.6 will occur if the exported data file has been renamed, and the file extension ".txt" was removed as shown in Figure B.7 below.
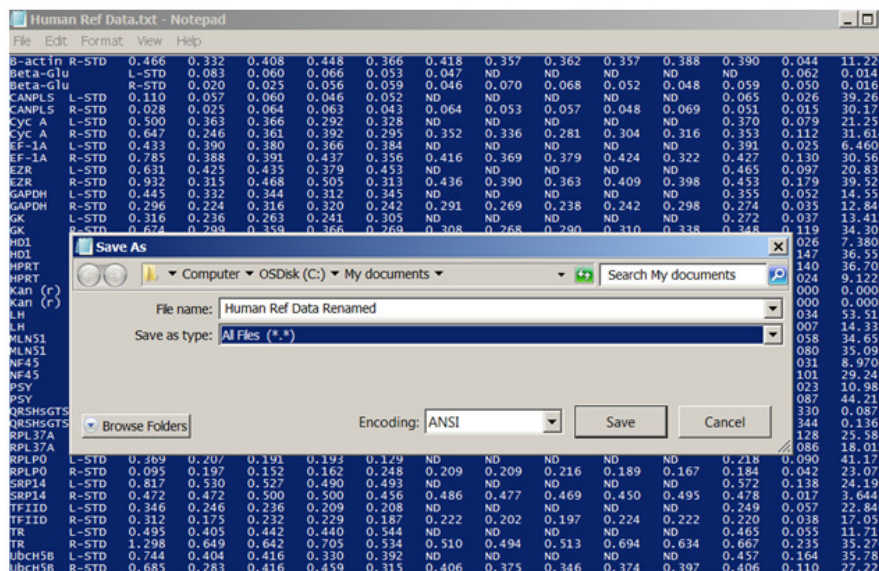

Figure B.7 Exported data file was renamed and the file extension ".txt" was removed

Standard Or Sample Names May Not Be Correctly Formatted

This error message will occur if the loaded file contains standards or samples with invalid names. The number of lines indicates the number of invalid names.
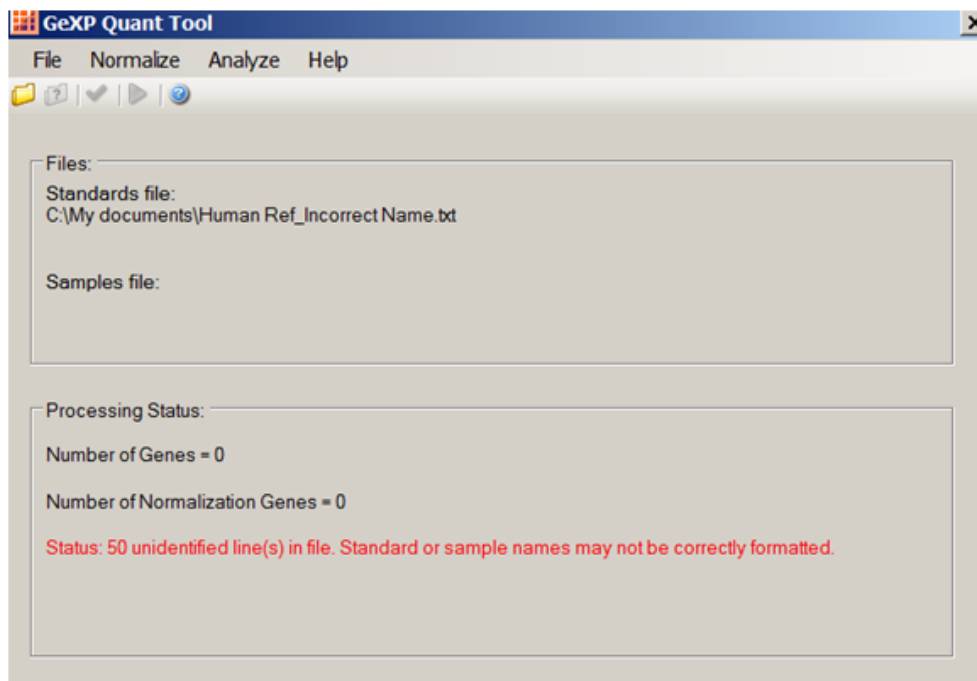
.

Figure B.8 Standard or sample names may not be correctly formatted

Problem Building Samples For Gene …

"Problem building samples for gene NM_012940" (Figure B.9) indicates that there was an internal processing error caused by unexpected contents or formatting in the input files. Click the **Ignore** button to continue analysis. Then, review the **Summary** worksheet in the report file for this particular gene (NM_012940).
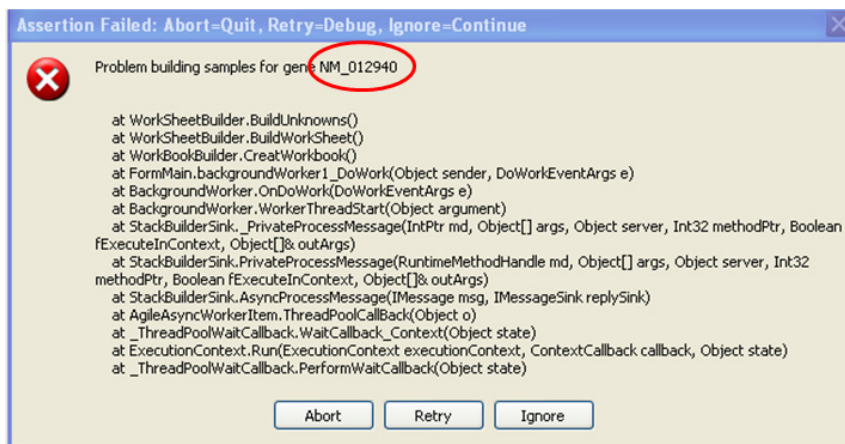
.

Figure B.9 Problem building samples for a particular gene

"#VALUE!" Was Shown In The Cell For "Curve Fit (R^2)" For A Particular Gene

As shown in Figure B.10, the R^2 value for gene NM_012940 could not be returned by the Quant Tool. Figure B.11 shows that there was no data entry for gene NM_012940 at 1 ng and 2 ng standard concentrations due to low signal. As a result, the R^2 value cannot be calculated for the standard curve for this gene. In order to calculate the R^2 value, data points for ALL genes at 1 ng and 2 ng standard concentration need to be removed from the .txt file. Then, analysis in GeXP Quant Tool is repeated. The R^2 value will be provided in the report from Quant Tool (Figure B.12)

.

9	Genes	Curve Fit (R2)
10	J00719	0.9953
11	Kan_r_	
12	NM_001044770	0.9997
13	NM_012540	0.9990
14	NM_012541	0.9986
15	NM_012683	0.9996
16	NM_012692	0.9999
17	NM_012693	0.9999
18	NM_012940	#VALUE!
19	NM_013149	0.9998
20	NM_017008	0.9993
21	NM_019184	0.9991
22	NM_022381	0.9999
23	NM_022536	0.9993

The R^2 value of this gene cannot be returned by the Quant Tool.

Figure B.10 No R^2 value available

.

Gene: NM_012940								
Standards:								
Concentration (ng)	Mean	Std Dev	% CV		Replicate #1	Replicate #2	Replicate #3	Replicate #4
1	#DIV/0!	#DIV/0!	#DIV/0!					
2	#DIV/0!	#DIV/0!	#DIV/0!					
3.9	0.037	0.003	9.2		0.036	0.041	0.039	0.032
7.8	0.120	0.011	9.0		0.133	0.127	0.111	0.107
15.6	0.215	0.021	9.9		0.233	0.222	0.227	0.179
31.3	0.425	0.013	3.0		0.407	0.431	0.441	0.419
62.5	0.620	0.049	7.9		0.642	0.685	0.598	0.554
125	0.972	0.050	5.2		0.954	1.055	0.921	0.957
250	1.519	0.080	5.3		1.644	1.443	1.532	1.456
500	2.772	0.088	3.2		2.812	2.757	2.879	2.639
Concentration minimum	#DIV/0!							
Concentration maximum	#DIV/0!							
Curve Fit(R2)	#VALUE!							

Figure B.11 The R^2 value cannot be calculated due to missing data

.

1	Gene: NM_012940			
2	Standards:			
3	Concentration (ng)	Mean	Std Dev	% CV
4	3.9	0.037	0.003	9.2
5	7.8	0.120	0.011	9.0
6	15.6	0.215	0.021	9.9
7	31.3	0.425	0.013	3.0
8	62.5	0.620	0.049	7.9
9	125	0.972	0.050	5.2
10	250	1.519	0.080	5.3
11	500	2.772	0.088	3.2
12	Concentration minimum	0.037		
13	Concentration maximum	2.772		
14				
15	Curve Fit(R2)	0.9999		

Figure B.12 The R^2 value is calculated after data points for all genes at 1 ng and 2 ng standard concentration were removed

No Value, "#VALUE!" and "#DIV/0!" in GeXP Quant Tool Report

Missing values (as shown by empty cells in the red rectangle in Figure B.13 below) or error "#VALUE!" are due to no data exported from GeXP Fragment Analysis.

Error "#DIV/0!" means the formula used is dividing by zero or empty cells. The most likely cause of this is a missing Geometric Mean (GEOMEAN) value on the Normalization_Values worksheet. In addition, no data points in the replicate wells will also cause this error.

Figure B.13 No Value, "#VALUE!" and "#DIV/0!" in GeXP Quant Tool Report

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