Table of Troubleshooting Symptoms

The table below lists troubleshooting symptoms and a page reference for an example of the symptom and possible causes and actions to take to resolve the problem. If there are two or more possible causes for the symptom, the causes are grouped and listed in the following order: data analysis issues, electrophoresis issues, then sequencing reaction issues.

Table 37 Table of troubleshooting symptoms

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Sample Manager Errors	
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Incorrect Basecalling	
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Too many mixed bases called	205
Irregular Signal	
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Signal starts later than expected: • Signal starts later than expected: no resolution loss • Signal starts later than expected: with resolution loss	 213 214
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Sudden drop in signal: • Sudden drop in signal: corresponds to basecalling stop when sequencing short template • Sudden drop in signal: early sudden drop with sequence termination • Sudden drop in signal: sudden drop with continued basecalling	 219 220 221
Top-heavy data: • Top-heavy data: gradual loss of signal • Top-heavy data: ski slope profile • Top-heavy data: preferential amplification of short sequence • Top-heavy data: split peaks with excessive signal	 222 223 224 225

Table 37 Table of troubleshooting symptoms *(continued)*

Symptom	Example on Page
Abnormal Peak Shapes	
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Improperly spaced peaks, especially peaks in the first 100 to 150 bases	229
Large peaks (blobs) in the first 120 bases	230
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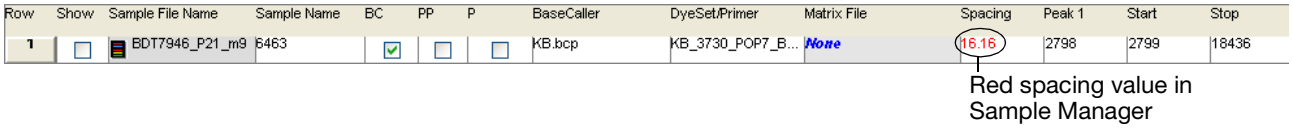
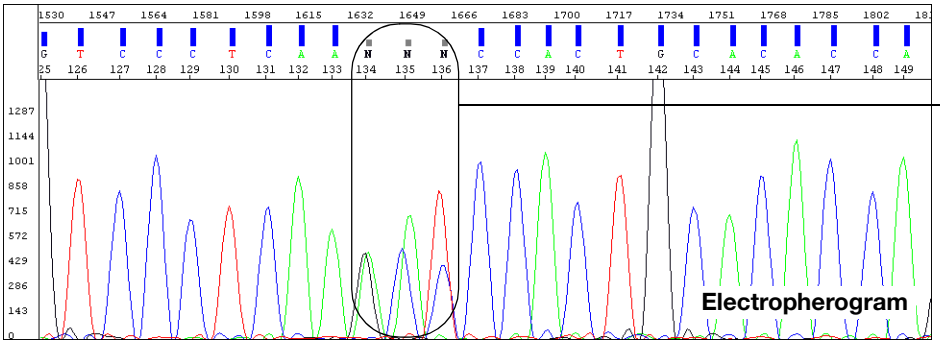
Troubleshooting Examples

Spacing value is red in Sequence Analysis or Sequence Scanner Software



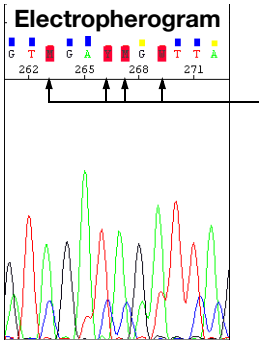
Possible Cause(s)	Recommended Action
Data analysis issue: The red color indicates that the basecaller applied a default value for spacing. The basecaller determined that the sample cannot be analyzed because the spacing estimation algorithm failed. This error may occur if the data has been collected using modified run modules or if data are poor.	Verify that analysis settings are appropriate for the run setup.
	Manually set a spacing value and reanalyze the data. To estimate a spacing value: 1. Refer to the raw data after 1000 scan points. 2. Measure the distance between the crests of two adjacent peaks with the same color. For more information, see the appropriate Sequencing Analysis Software user guide.

Mixed base not called correctly



Possible Cause(s)	Recommended Action
Data analysis issue: The quality threshold setting and the mixed bases settings are not correctly defined in the analysis protocol.	1. Review the quality threshold setting (page 149) and the mixed bases settings (page 149) in the analysis protocol that you used for the analysis. 2. Correct the settings if necessary, then reanalyze the data. **Note:** Significant improvements in mixed basecalling have been made with later versions of Sequencing Analysis Software and the KB basecaller. Please check the Applied Biosystems web site for the latest updates.

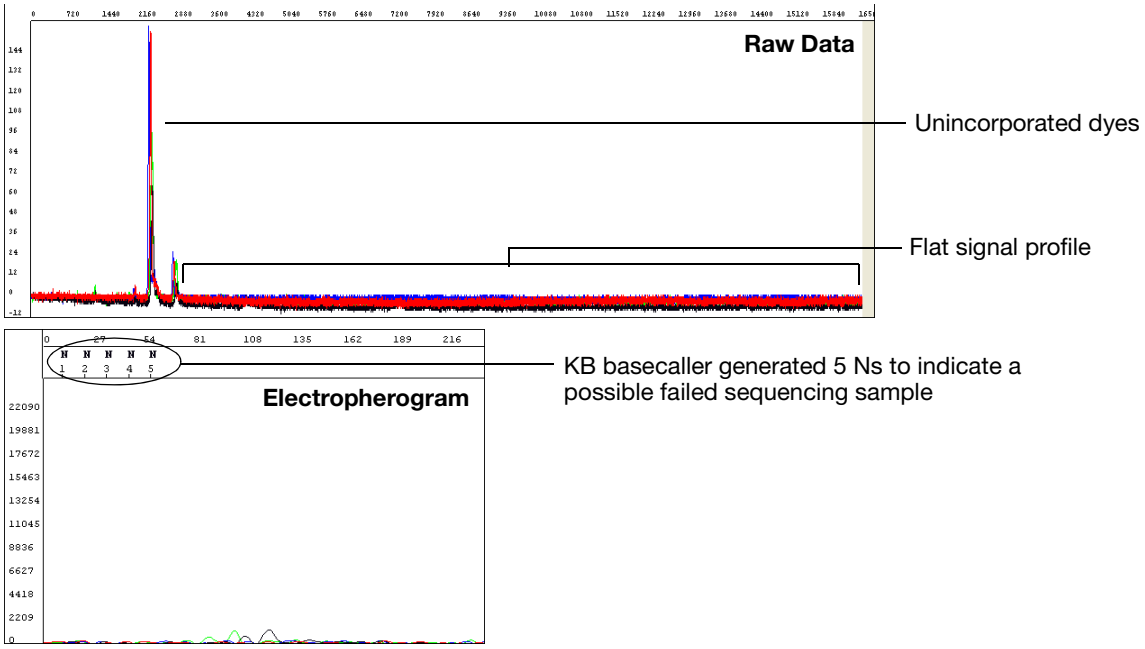
Too many mixed bases called



Possible Cause(s)	Recommended Action
Data analysis issues:	
2nd highest peak threshold for mixed base identification is set too low. The recommended range is 15 to 25%.	Review the Mixed Bases settings in the analysis protocol that you used for the analysis (page 149). Change the settings if necessary, then reanalyze.
Electrophoresis issues (likely in multiple lanes and/or runs):	
Carryover from contaminated septa.	Replace septas and change buffer, water, and waste.
Electrical noise.	Check the uninterruptible power supply (UPS).
Contaminated water or buffer because of dirty containers, microbial growth, or use of tap water for cleaning.	Clean all reservoirs, upper and lower polymer block, and septa with deionized water.
Poor or incorrect spectral calibration (spectral pullup).	Perform the spectral calibration again.
Shifted spatial calibration.	Perform the spatial calibration again.
Poor CCD alignment.	Contact Applied Biosystems to arrange a service engineer visit.
Sequencing reaction issues (in individual samples or multiple samples):	
Secondary primer site in the template was sequenced.	Design a new sequencing primer (page 38).
Secondary amplification product in the PCR product used as a sequencing template.	Use gel purification to isolate the desired product. For more information, see "Purifying PCR Products for Sequencing" on page 41.
	Design new PCR primers or optimize amplification parameters to obtain a single product. For more information, see "Preparing PCR DNA Templates" on page 37.
PCR primers were not completely removed from the PCR product used as a sequencing template.	Remove PCR primers completely before using PCR products as sequencing templates. For more information, see "Purifying PCR Products for Sequencing" on page 41.
Mixed templates.	Review the DNA quality.

Possible Cause(s)	Recommended Action
Pull-up caused by overloading the capillaries with too much product.	Review DNA quantity.
	Use standard run modules
	Click the **Annotation** tab and examine the Ave Signal Intensity. Excessive signal: • 3730/3730*xl* instruments: >10,000 rfus • 310 and 31XX instruments: >1000 rfus Load less labeled sample by performing one of the following: • Remove some of the sample and replace with Hi-Di™ Formamide • Inject sample for less time • Resequence the samples, using less template in the sequencing reaction, especially if you use the BigDye® XTerminator™ Purification Kit (see Table 8, "Recommended DNA template quantities for cycle sequencing," on page 63).
Stutter during either PCR amplification and/or cycle sequencing. Stutter is most common in any homopolymeric region greater than 2 bases. It can also be seen with simple repeated DNA sequences. The results are worse when the stutter occurs during PCR amplification. It is thought that stutter occurs when a partially extended primer and template dissociate, then reanneal improperly before extension continues. Partially extended primers and templates commonly dissociate during the reaction, but if they reanneal with complete fidelity, the reaction produces only one product. Improper annealing results in one or more products that are represented in the sequencing results.	If stutter occurs during PCR amplification, little can be done to correct the problem, except using anchored sequencing primers. If stutter occurs during cycle sequencing: • Try using dRhodamine terminators. They have been shown to be less prone to produce stutters, specifically with poly-T regions. • Some customers have found that they can get past poly(A) regions using a mixture of oligo dT_{18} primers with either a C, A, or G as the 3′ terminal dinucleotide or 2-base anchors.

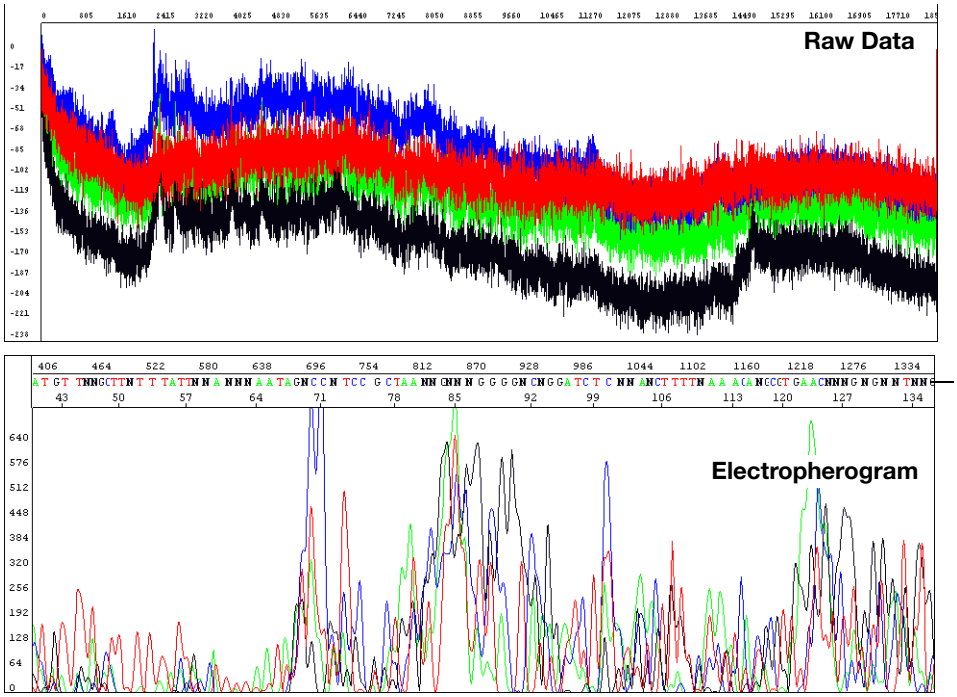
No signal



Possible Cause(s)	Recommended Action
Sequencing reaction issues (likely with multiple or all samples):	
Loss of labeled product during purification of extension products.	See Chapter 5 for suggestions on retaining labeled product during purification.
Thermal cycler malfunction.	Determine with the manufacturer how to test your thermal cycler for proper performance.
One of the components of the sequencing reaction (template, primer, or Ready Reaction Mix) was either omitted, was the wrong material, or was of poor quality.	Review the entire experiment carefully. 1. Check the quantitation and quality of the sequencing reaction components. 2. For each component, replace the component, perform a sequencing run, then evaluate the results until you have identified the problem or replaced all of the reaction components. 3. Run a DNA template control to determine whether the sequencing reaction failed or the template quality is low (page 64).
Insufficient template added to sequencing reactions, leading to too few sequencing products generated during PCR.	Check DNA quantitation and quality (page 44 and 45).
Template contains sequencing inhibitors such as phenol (page 44).	Follow recommended procedures to prepare templates. Check DNA quality (page 44). If necessary, clean up dirty templates.

Possible Cause(s)	Recommended Action
No enzyme activity because Ready Reaction Mix was stored improperly or it separated upon storage.	Check the color of the Ready Reaction Mix. If the color is not uniform, the Ready Reaction Mix separated upon storage. Mix the Ready Reaction Mix gently before using it.
	Run a DNA template control to test enzyme function (page 64).
Weak priming because of poor primer design.	Review primer design (page 38). Make new primers, then repeat the sequencing experiment.

Low signal

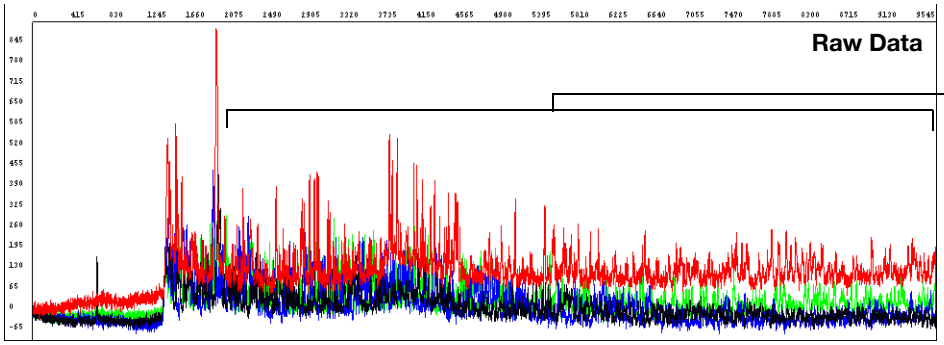


Electropherogram shows Ns (with ABI or KB basecaller) or low quality bases (with KB basecaller)

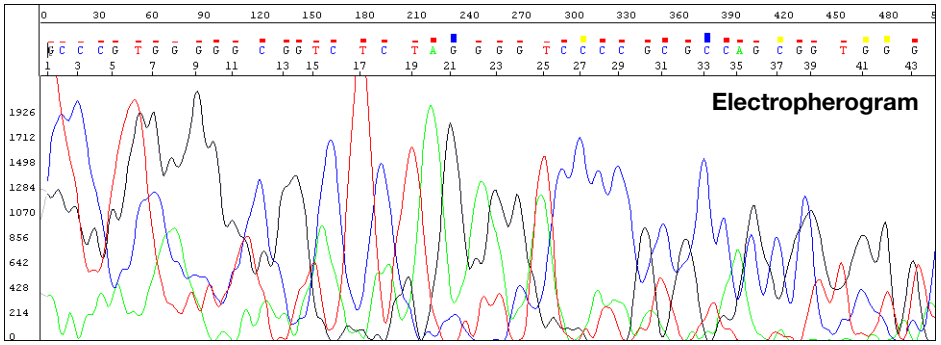
Possible Cause(s)	Recommended Action
Electrophoresis issues:	
One or more broken or blocked capillaries.	Visually check the capillaries. If any are broken or blocked, replace the entire array. If subsequent runs show failure in the same capillary, replace the entire array.
	Check the results using the long read sequencing standard.
Optical path is obstructed (3100/3100-*Avant* instruments only).	Check the laser power, using the EPT in Data Collection Software. Perform the spatial calibration again.
	Check whether you can hear the shutter clicking during data collection. If you cannot hear it click, contact Applied Biosystems for a service engineer visit.
	If all capillaries show no signal or low signal, contact Applied Biosystems for a service engineer visit.

Possible Cause(s)	Recommended Action
Sample evaporated because water was used as the injection solution.	Use Hi-Di™ Formamide to resuspend your samples (see page 122). For future experiments, consider using the BigDye® XTerminator™ Purification Kit to purify samples (see page 88).
	Use a heat sealer to seal the plates (3730/3730*x*/ instruments only).
	Add more resuspension solution to the samples before loading them.
Sample volume is too low.	Resuspend samples using sufficient volumes (at least 10 µL) (see page 122).
Autosampler alignment is off and the tips did not enter the sample.	1. Verify the correct run module was used. 2. If you are using samples purified with BigDye® XTerminator™ Purification Kit and your autosampler was recently calibrated, run the BDX Update utility. Select **Start ▸ All Programs ▸ Applied Biosystems ▸ BDX Updater**. (The utility is installed with the BigDye XTerminator run modules.) 3. Contact Applied Biosystems to arrange a service engineer visit.
Slightly unstable current and voltage during electrophoresis.	Check the current and voltage.
Buffer is old.	Replace the buffer according to the procedures in your instrument user guide.
Too much template or sample temporarily clogging the capillary.	Reinject the sample.
Injection failed.	• Verify correct run module was used. • Verify correct volume in well. • Verify capillaries are not broken or blocked.

Low signal throughout



Low signal throughout the entire sequence



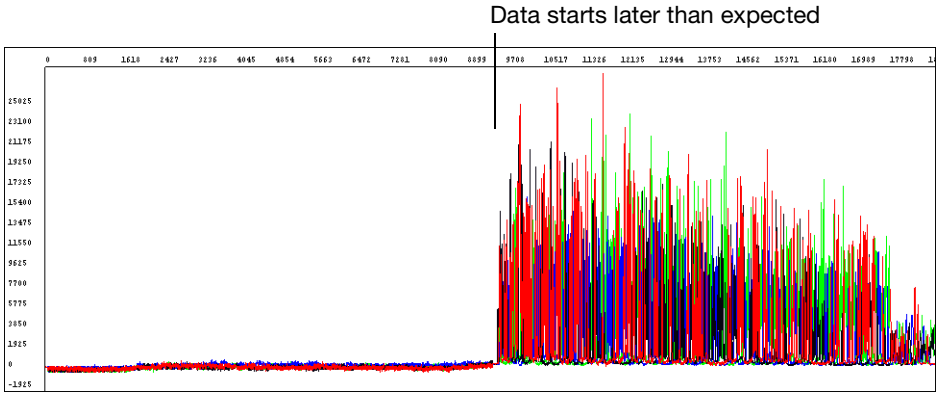
Ave Signal Intensity:	G (93), A (120), T (143), C (127)
Noise:	G (9), A (10), T (12), C (10)
Signal:Noise:	G (10), A (12), T (12), C (12)

Annotation tab shows low average signal intensity values for data from 3730 instrument

Possible Cause(s)	Recommended Action
Sequencing reaction issues:	
Sequencing reaction failed.	Check the control template and primer.
Partial loss of labeled products during purification of extension products.	See Chapter 5 for suggestions on retaining labeled product during purification.
Sample contains salts from insufficient purification of templates, PCR products, or sequencing reactions with ethanol precipitation. Salts in the sample interfere with proper electrokinetic injection.	Review DNA quality, PCR purification, and sequencing reaction purification steps.
The amount of Ready Reaction Mix in the reactions was insufficient, usually because the sequencing chemistry was diluted.	Follow recommended procedures to prepare sequencing reactions with Ready Reaction Mixes. See page 66 for recommended procedures. Applied Biosystems does not support diluted reactions or guarantee the performance of diluted BigDye chemistry.
Not enough primer or template in the cycle sequencing reaction.	Review DNA quantity (page 199). Use the amounts recommended on page 63. Run a DNA template control to check sequencing reaction quality (page 64).

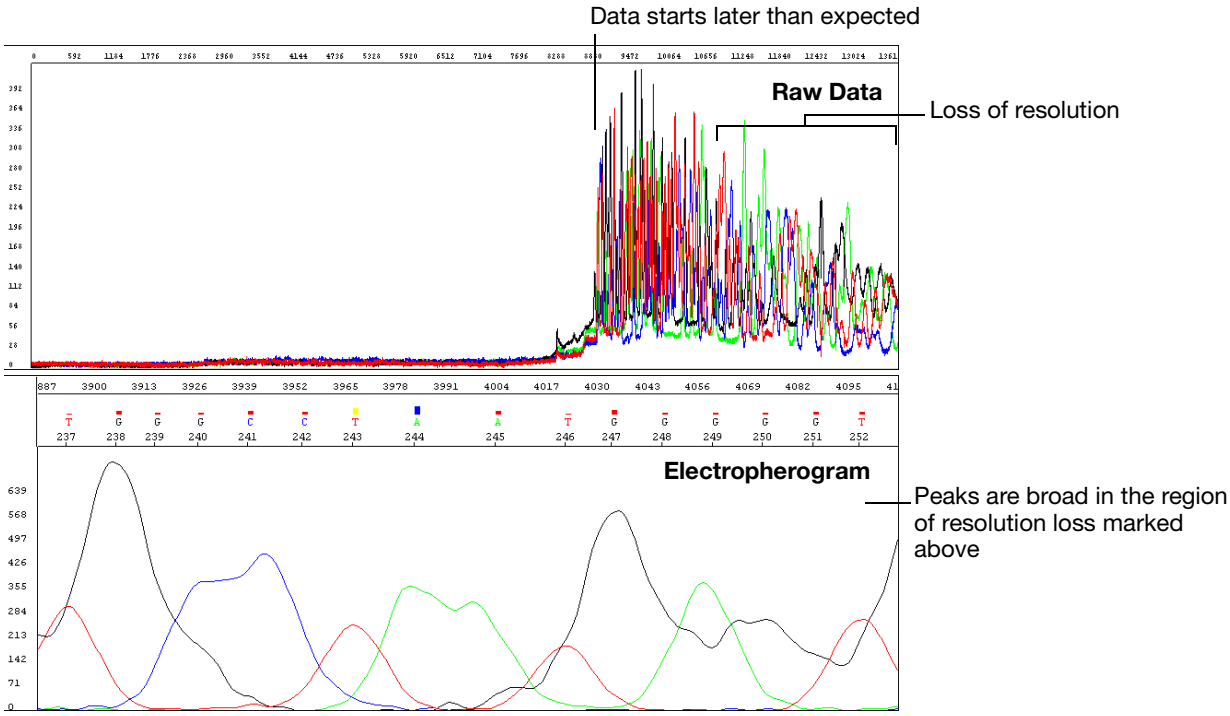
Possible Cause(s)	Recommended Action
Poor template quality.	Follow recommended procedures to prepare templates. Check DNA quality (page 44). If necessary, clean up dirty templates. Run a DNA template control to check sequencing reaction quality (page 64).
Failure caused by difficult template sequence.	Use Table 7 on page 56 to select a chemistry kit for certain difficult templates.

Signal starts later than expected: no resolution loss



Possible Cause(s)	Recommended Action
Electrophoresis issues:	
Incorrect capillary length (Length to Detector) or run module was selected.	1. Review run information in the Annotation tab using Sequencing Analysis Software (see page 197): – Length to Detector – Run module 2. If an incorrect selection was made, run the samples again using the correct settings.
Variation in lab temperature leads to faster or slower runs.	Stabilize the lab temperature.
Sample heated during vortexing step of BigDye® XTerminator™ purification.	1. Repeat the sequencing reactions. 2. Perform BigDye XTerminator purification using recommended vortexer and plate adapter. 3. Run the samples again.
Too much template used.	Run the samples again, using less template.

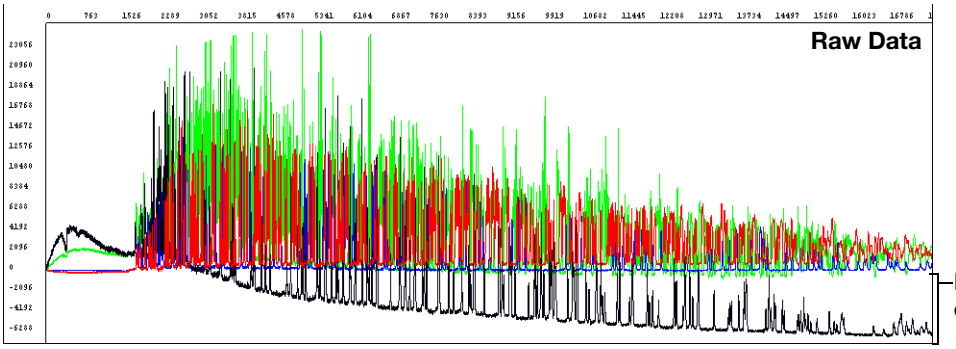
Signal starts later than expected: with resolution loss



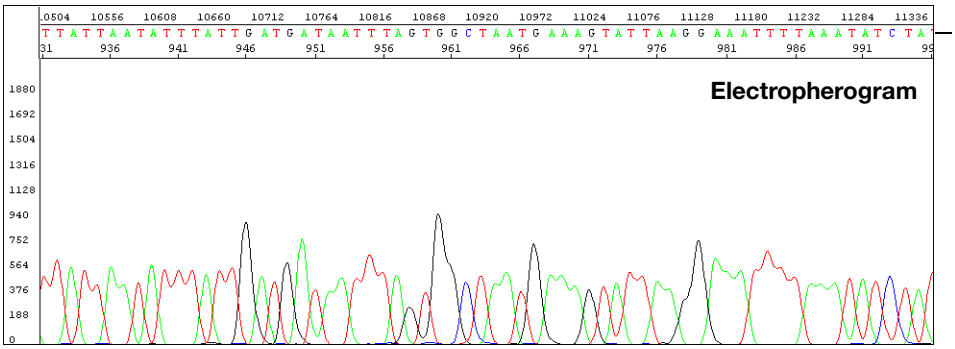
Possible Cause(s)	Recommended Action
Electrophoresis issues:	
Capillaries overloaded with sequencing product, possibly unlabeled DNA or RNA.	Click the **Annotation** tab and examine the Ave Signal Intensity. Excessive signal: • 3730/3730*xl* instruments: >10,000 rfus • 310 and 31XX instruments: >1000 rfus Re-inject the samples using decreased injection time and/or lower voltage. Load less labeled sample by using less template in the sequencing reaction (see Table 8, "Recommended DNA template quantities for cycle sequencing," on page 63).
Temperature in room and/or oven fluctuating.	Review the EPT tab using Sequencing Analysis Software (see page 198). If the oven temperature is fluctuating, the oven may be leaking because of a poor seal. Contact Applied Biosystems to arrange a service engineer visit.
Contaminant migrated through the capillary during electrophoresis.	Run the sample again.
Capillary not filling.	Check the pin valve in the polymer block, amount of polymer in the bottle, leaks in the check valves, and polymer pump function. Contact Applied Biosystems to arrange a service engineer visit.
Temperature in the array heater fluctuating more than ±0.5 °C (3730/3730*xl* and 3130/3130*xl* instruments and POP-7 only).	Using Data Collection Software, check the array heater temperature. If it fluctuates more than ±0.5 °C, contact Applied Biosystems to arrange a service engineer visit.

Possible Cause(s)	Recommended Action
Water in polymer system caused by insufficient flushing after water wash maintenance.	Flush the polymer, using the wizard if possible.
Extension products purified using bead-based kits were injected without removing the magnetic beads. The beads may interfere with the extension products during injection and cause overloading or other injection anomalies.	Remove magnetic beads before loading the sample.
Variables that affect current set incorrectly.	• Replace buffer in system with fresh 1× running buffer. • Inspect system for leaks (wet or dry polymer around fitting indicates a leak) and tighten fittings as needed. • Look for discoloration in the block channels or tubing. If present, perform a water wash on the system using the wizard in Data Collection Software.

Negative baseline: one color



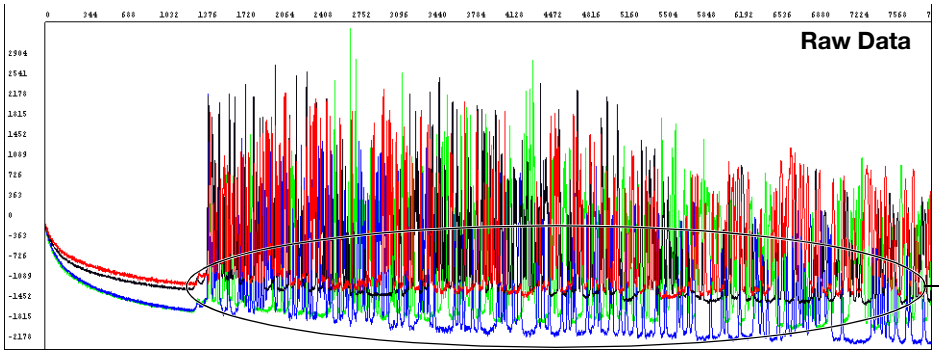
Baseline fluorescence for one color is below 0 rfus



Software corrects the baseline fluorescence during data analysis

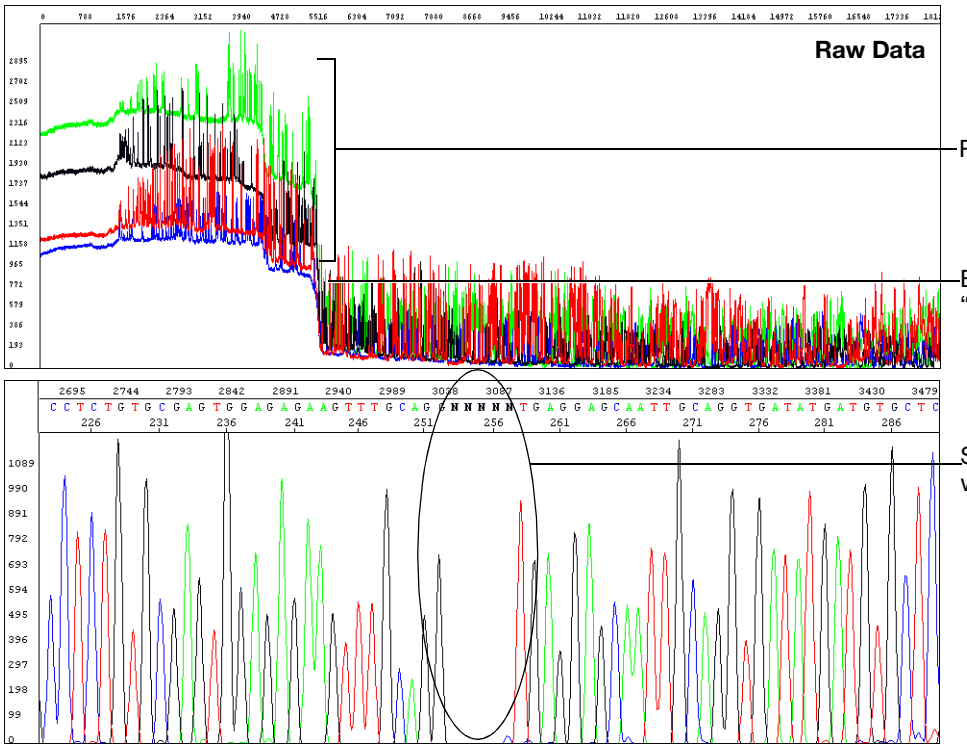
Possible Cause(s)	Recommended Action
You are using an early version of Sequencing Analysis Software. This error, found in versions earlier that v5.2, was corrected in Basecaller updater v2.0.	Upgrade Sequencing Analysis Software v5.2 or later. To obtain the latest software updates and patches, go to **www.appliedbiosystems.com**, then click the link for **Support**.

Negative baseline: all four bases



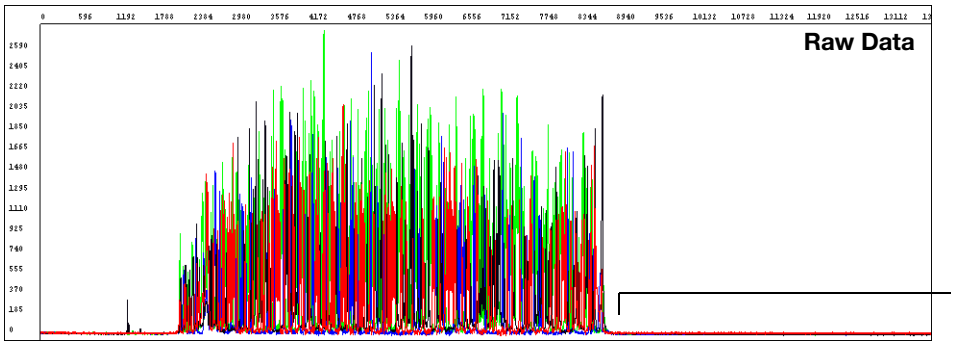
Possible Cause(s)	Recommended Action
Electrophoresis issue: Excessive fluorescent contamination in the detection area that bleaches out over the duration of the run (3730/3730*xl* instruments only).	Use manual control to turn on the laser before starting the run to negate the effects of excessive fluorescent contaminant. Contact Applied Biosystems technical support or a field applications specialist.
	Perform a water wash on all components of the system using the wizard in Data Collection Software, then replace the capillary array.

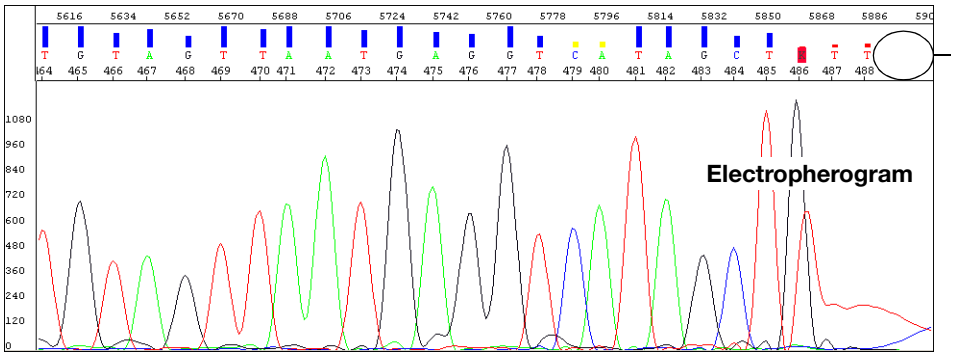
Waterfall baseline



Possible Cause(s)	Recommended Action
Residue from cleansers used on glassware. **Note:** Primarily observed in syringe-based instruments.	Rinse all components with deionized water. Check gasket on syringe(s), replace if necessary. Replace syringe(s).

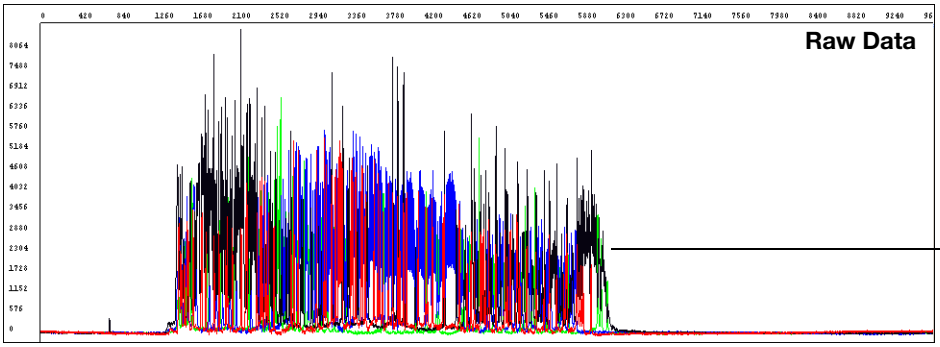
Sudden drop in signal: corresponds to basecalling stop when sequencing short template

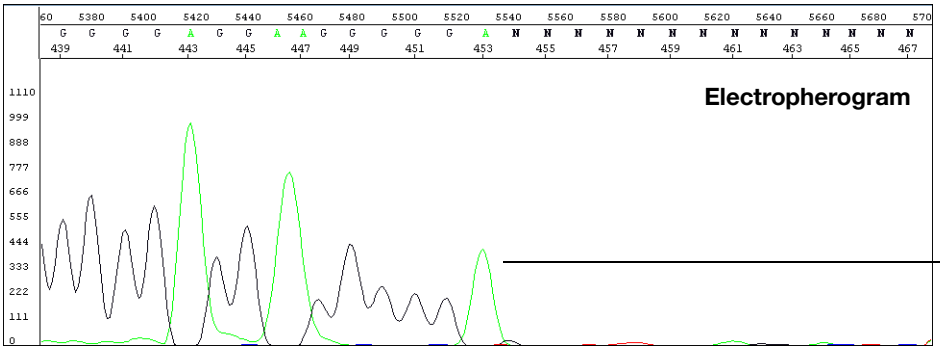




Possible Cause(s)	Recommended Action
Data analysis issue: The drop in signal identifies a PCR stop point and the basecaller stops calling bases beyond this point. With the ABI basecaller, you observe Ns beyond the PCR stop. With the KB basecaller, the analyzed trace is displayed until the last basecall.	Select the **At PCR Stop** check box in the analysis protocol using Sequencing Analysis Software (see page 148).

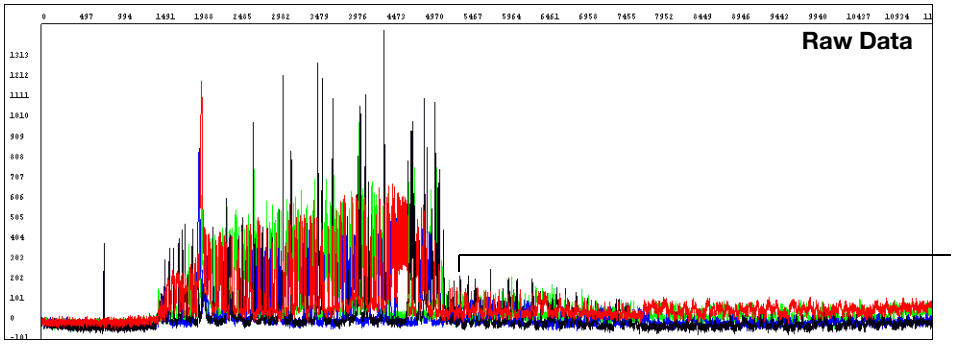
Sudden drop in signal: early sudden drop with sequence termination

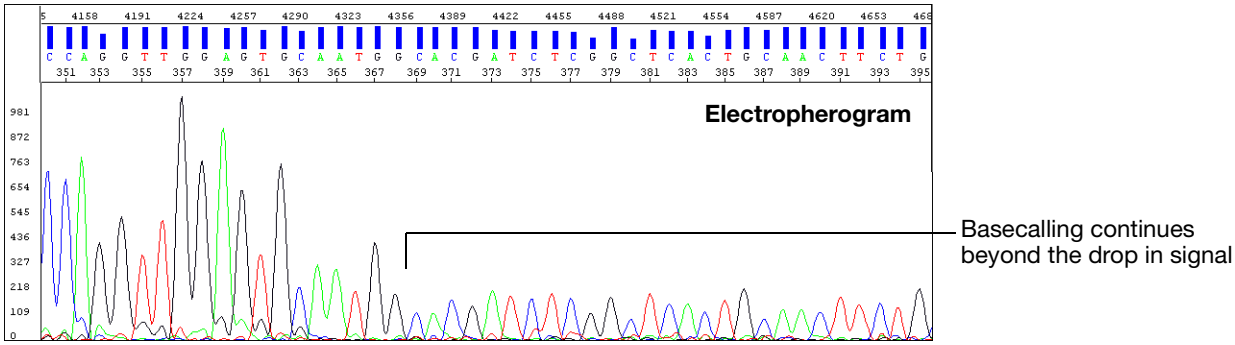




Possible Cause(s)	Recommended Action
Sequencing reaction issue:	
DNA polymerase enzyme stopped at a region of the template that was difficult to sequence.	• Depending on sequence contexts, you can try sequencing some template with dGTP kits (if the problem sequence is G-rich), dRhodamine kits, or BigDye primer kits. • If termination of sequencing was caused by hairpins or secondary structure, redesign primers around the problem region. Some customers report that certain additives can help, but Applied Biosystems cannot recommend any specific protocols.
Not enough Ready Reaction Mix was used in the sequencing reaction.	Follow recommended procedures to prepare sequencing reactions with Ready Reaction Mixes. See page 66 for recommended procedures. Applied Biosystems does not support diluted reactions or guarantee the performance of diluted BigDye chemistry.

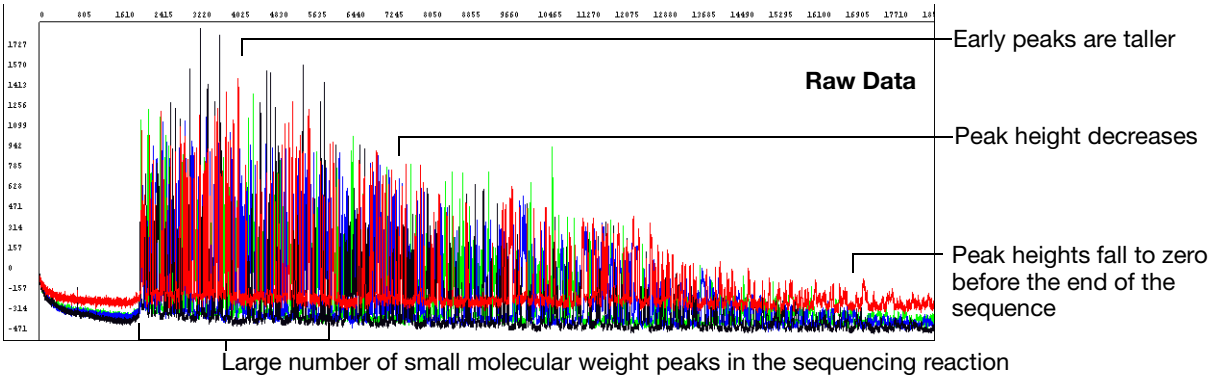
Sudden drop in signal: sudden drop with continued basecalling





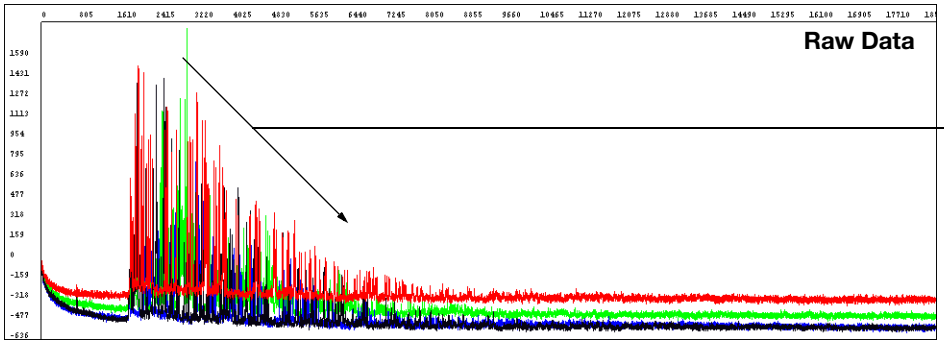
Possible Cause(s)	Recommended Action
Sequencing reaction issues:	
DNA polymerase had difficulty processing through a particular sequence context.	Depending on sequence contexts, you can try sequencing some template with dGTP kits, dRhodamine kits, or BigDye® primer kits.
Not enough Ready Reaction Mix was used in the sequencing reaction.	Follow recommended procedures to prepare sequencing reactions with Ready Reaction Mixes. See page 66 for recommended procedures. Applied Biosystems does not support diluted reactions or guarantee the performance of diluted BigDye chemistry. If the problem persists, try sequencing using the dGTP kits.

Top-heavy data: gradual loss of signal



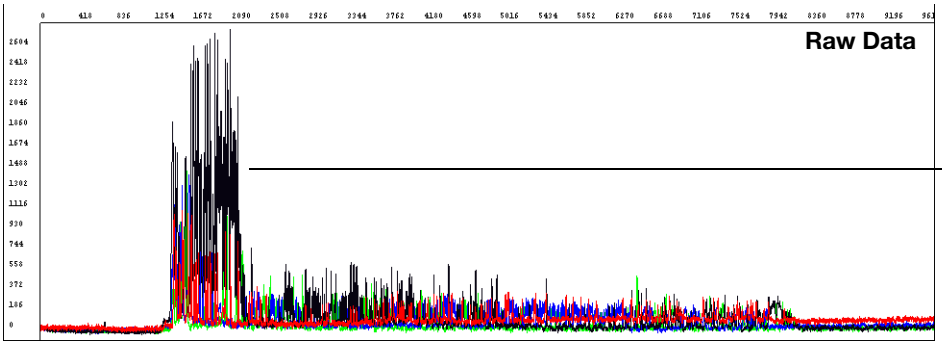
Possible Cause(s)	Recommended Action
Sequencing reaction issues:	
Improper cycling conditions for extension. The extension time is too short or the extension temperature is too high.	Increase the extension time or decrease the extension temperature.
Improper ratio of primer to template in the sequencing reaction.	Set up a matrix of reactions with varying ratios of primer:template to determine which ratio produces the best peak profile.
Sequencing template contains a contaminant that inhibits DNA polymerase activity.	Review how templates are prepared. Try a different method or clean up dirty templates (page 44).
Not enough Ready Reaction Mix was used in the sequencing reaction.	Follow recommended procedures to prepare sequencing reactions with Ready Reaction Mixes. See page 66 for recommended procedures. Applied Biosystems does not support diluted reactions or guarantee the performance of diluted BigDye chemistry.
Template or extension products are degraded. With degraded extension products, the data are noisy, with a higher baseline at the start of peaks.	Review how templates are prepared and stored. Try a different method (Chapter 3) and store at – 20 °C.

Top-heavy data: ski slope profile



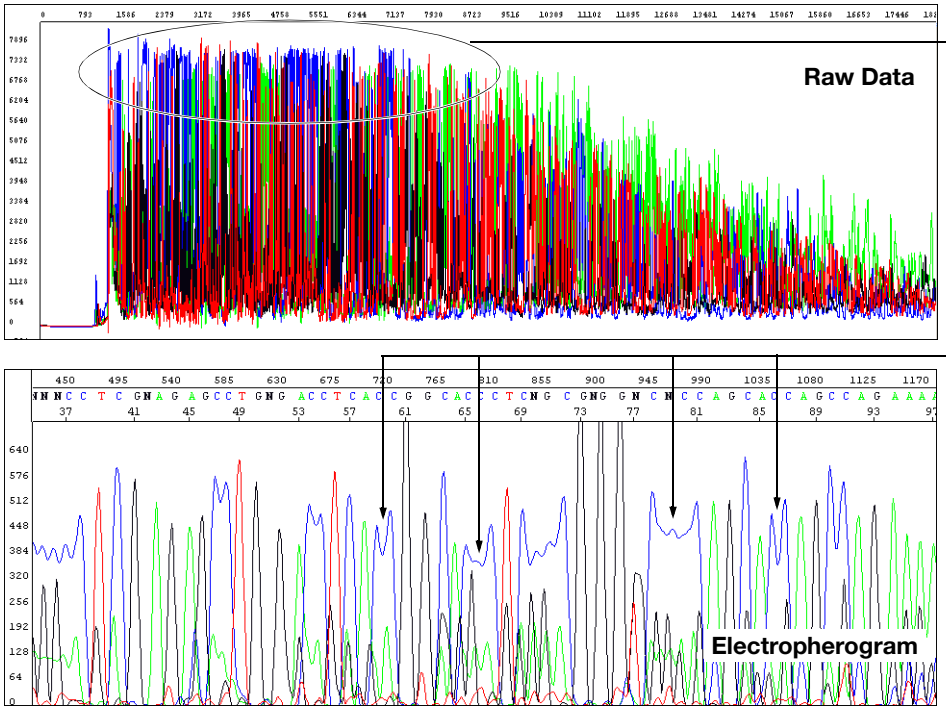
Possible Cause(s)	Recommended Action
Sequencing reaction issues:	
Not enough or too much template was used in the sequencing reaction.	Review the DNA quantity (page 199).
Not enough or too much primer was used in the sequencing reaction.	
Not enough Ready Reaction Mix was used in the sequencing reaction.	Follow recommended procedures to prepare sequencing reactions with Ready Reaction Mixes. See page 66 for recommended procedures. Applied Biosystems does not support diluted reactions or guarantee the performance of diluted BigDye chemistry.
Template is degraded.	Review how templates are prepared and stored. Try a different method (Chapter 3) and store at – 20 °C.

Top-heavy data: preferential amplification of short sequence



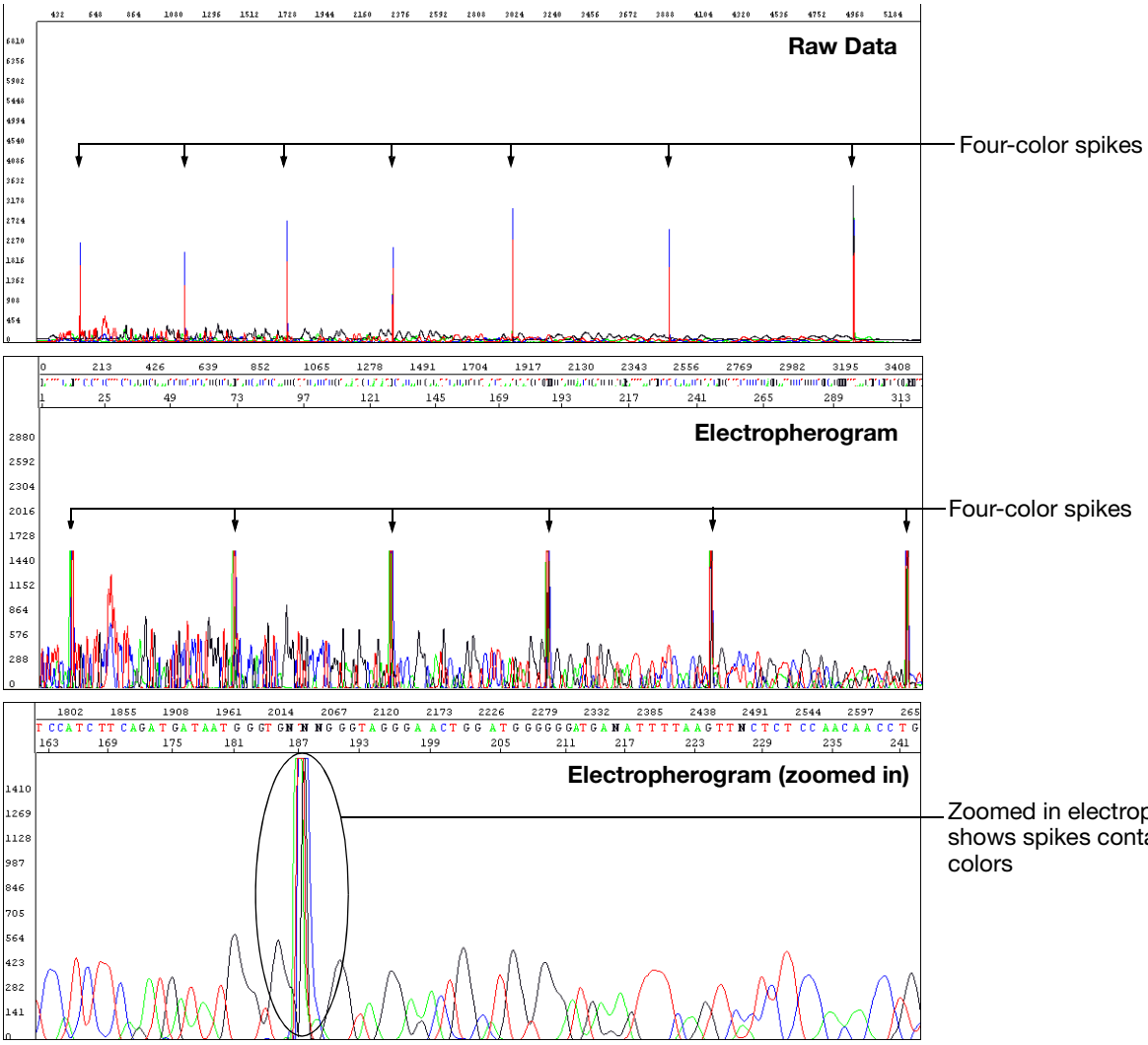
Possible Cause(s)	Recommended Action
Sequencing reaction issue: Primer-dimer formation during the PCR reaction.	Redesign the PCR primers to eliminate the sequences that allow primer-dimer formation.
	Use a “hot start” PCR enzyme to inhibit primer-dimer formation.

Top-heavy data: split peaks with excessive signal



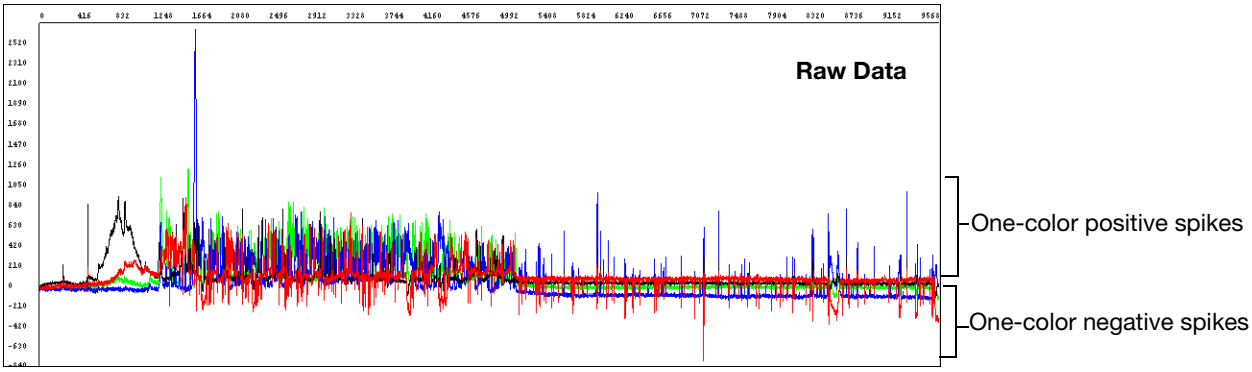
Possible Cause(s)	Recommended Action
Sequencing reaction issues:	
Signal is too high because too much template was used in the sequencing reaction and too much sequencing product was created.	You do not have to repeat the reaction. Click the **Annotation** tab and check the Ave Signal Intensity. Excessive signal: • 3730/3730*xl* instruments: >10,000 rfus • 310 and 31XX instruments: >1000 rfus Especially if your samples were purified using the BigDye® XTerminator™ Purification Kit, load less labeled sample by performing one of the following: • Remove some of the sample and replace with Hi-Di™ Formamide • Inject sample for less time • Resequence the samples, using less template in the sequencing reaction, especially if you use the BigDye® XTerminator™ Purification Kit (see Table 8, "Recommended DNA template quantities for cycle sequencing," on page 63).
	For future reactions, reduce the amount of template in the sequencing reaction.
Injection height incorrect due to incorrect run module.	Use correct run module, especially for samples purified with the BigDye XTerminator Purification Kit (see "Selecting a Run Module" on page 137).

Four-color spikes



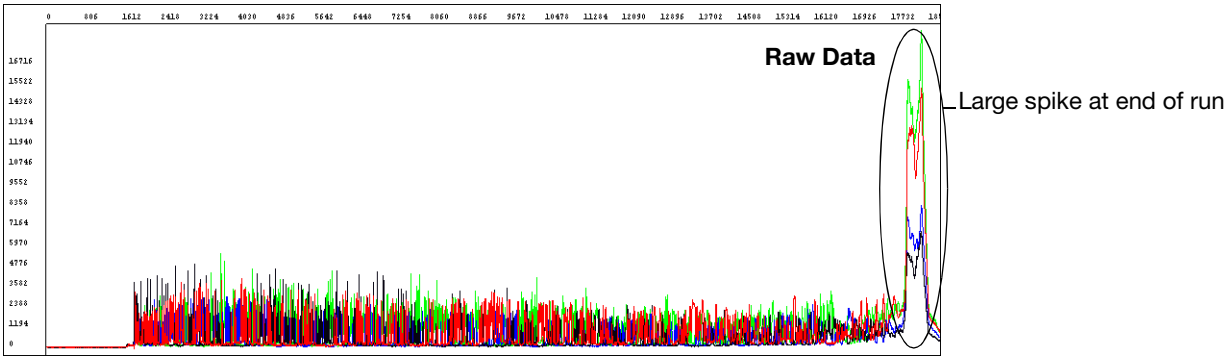
Possible Cause(s)	Recommended Action
Electrophoresis issues:	
Dust, bubbles, or crystals in polymer passed through the path of the laser beam.	1. Eliminate large amounts of dust in the environment. 2. Inspect the upper gel block for bubbles. If present, flush all bubbles out of the system and out of the array manually. 3. Check the polymer bottle for crystals. If present, warm the polymer gently to 30 °C with gentle mixing, then refill the syringes and array with the polymer. 4. Replace polymer if the condition persists.
Polymer is expired or was stored at room temperature for more than 7 days.	Replace the polymer.

One-color spikes



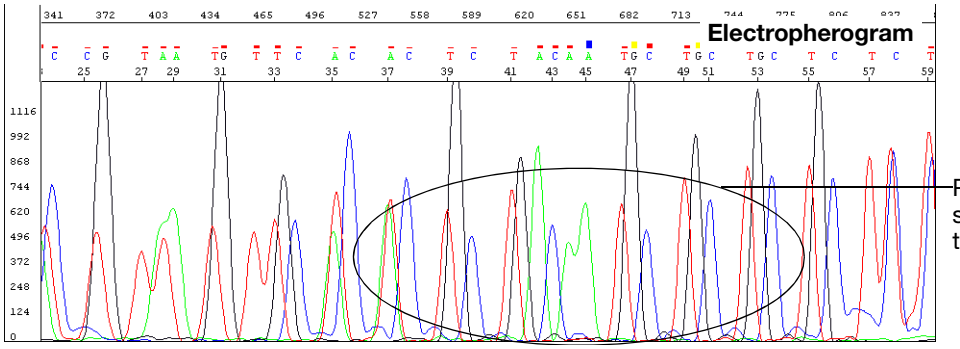
Possible Cause(s)	Recommended Action
Electrical noise or power fluctuations.	Verity the power source, use uninterruptible power supply.
Polymer temperature is too high.	• Verify the shipping temperature of the polymer. • Verify lab temperature is below 26 °C.
Well volume is too low.	• Verify volume is ≥10µL for 96-well plates and ≥15µL for 384-well plates. • If using septa, verify septa are fresh to minimize evaporation.

Large spike at the end of the run



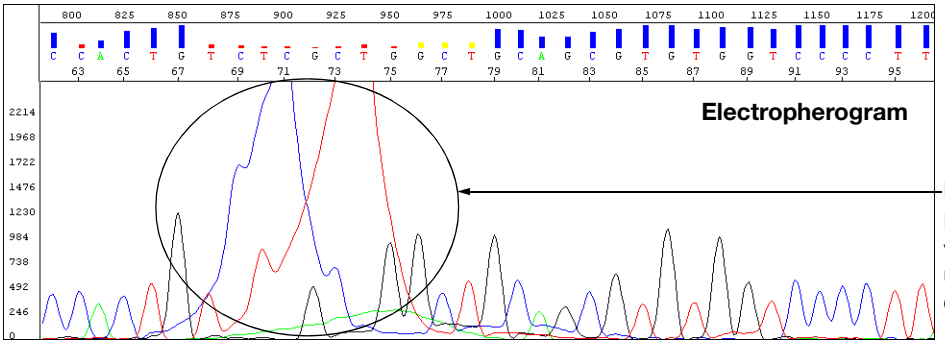
Possible Cause(s)	Recommended Action
The large spike at the end of the run, called a reptation peak, occurs with almost all electrophoretic separations of DNA on capillary instruments. With typical run conditions, data collection stops well before the spike occurs. There is no useful sequencing information in the spike or just before the spike. Because some run modules are designed for the longest possible read lengths, data collection stops just before the spike occurs. Normal run variation within a lab may result in the spike appearing in some electropherograms.	None needed. Shorten the data collection time a few minutes to remove a persistent spike from your data.

Improperly spaced peaks, especially peaks in the first 100 to 150 bases



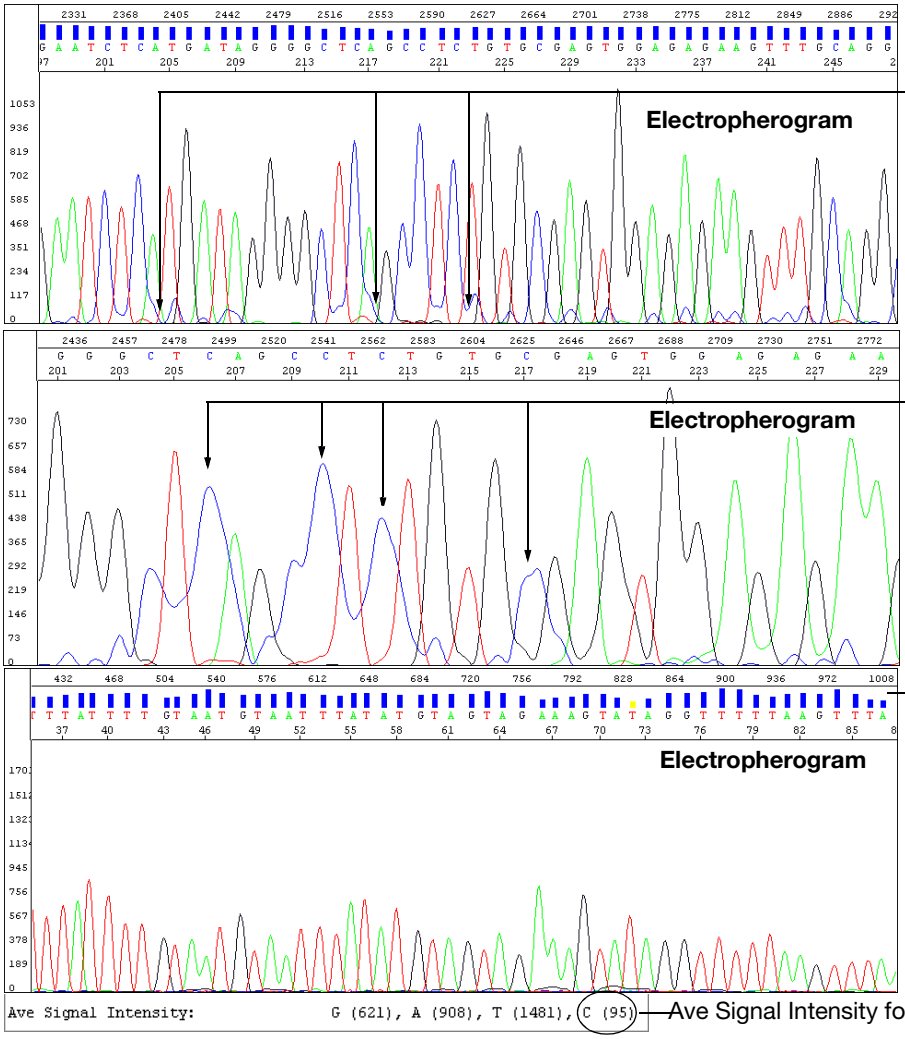
Possible Cause(s)	Recommended Action
Data analysis issue: Wrong mobility file applied to the sequence data.	Reanalyze the data using the correct mobility file to observe proper spacing of all peaks. For more information about mobility files, see page 145.

Large peaks (blobs) in the first 120 bases



Possible Cause(s)	Recommended Action
Sequencing reaction issues:	
Incomplete removal of dye-labeled terminators after the cycle sequencing reaction.	Review the methods described in Chapter 5, "Purification of Extension Products." If you are using a third-party product for purifying extension products, contact the manufacturer for troubleshooting help. For future experiments, consider using the BigDye® XTerminator™ Purification Kit to purify samples (see page 88).
Poor incorporation of terminators, leaving excess unincorporated terminators.	Review the entire experiment carefully. • Check the quantitation (page 45). • Check the quality of the sequencing components. Replace each component, one at a time. • Run a DNA template control to determine whether the sequencing reaction failed or the template quality is low (page 64). • Check expiration dates on all reagents and replace any that have expired.
If using BigDye XTerminator Purification Kit, insufficient mixing during vortexing step.	• Verify plate is firmly attached to vortexer. • Follow protocol for vortexing.
If using BigDye XTerminator Purification Kit, incorrect ratio of BigDye XTerminator reagents.	• Vortex the XTerminator Solution bulk container at maximum speed for at least 10 seconds before dispensing. • Use wide-bore pipette tips to dispense the XTerminator Solution.

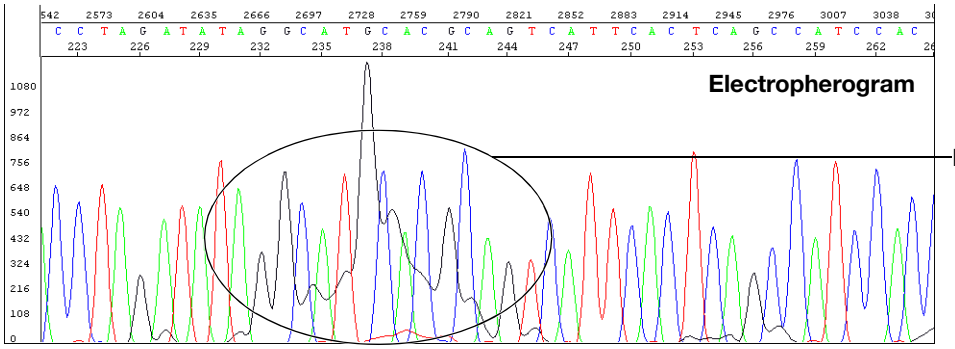
Irregular C peaks using BigDye® Terminators v3.1



Possible Cause(s)	Recommended Action
Sequencing reaction issues:	
The dye labels attached to the ddC terminators are degraded. Initial degradation results in shoulders on all C peaks. With further degradation, the C peaks appear very small or rough or disappear completely.	Protect the fluorescently labeled DNA from light, heat, acidic conditions, and oxygen (see "Storing Sequencing Reactions" on page 121). If no C peaks are visible, repeat the sequencing reactions with fresh reagents.
The Hi-Di™ Formamide is degraded.	Resuspend the samples using a newer lot of Hi-Di Formamide.
Sequencing reactions were exposed to light, heat, acidic conditions, and/or oxygen before they were loaded onto the instrument.	Use tube septa or a heat seal to prevent exposure to air and evaporation of samples, especially if you place the samples in the autosampler more than 6 hours before starting electrophoresis.
	Verify that the primer and template pHs are not acidic.

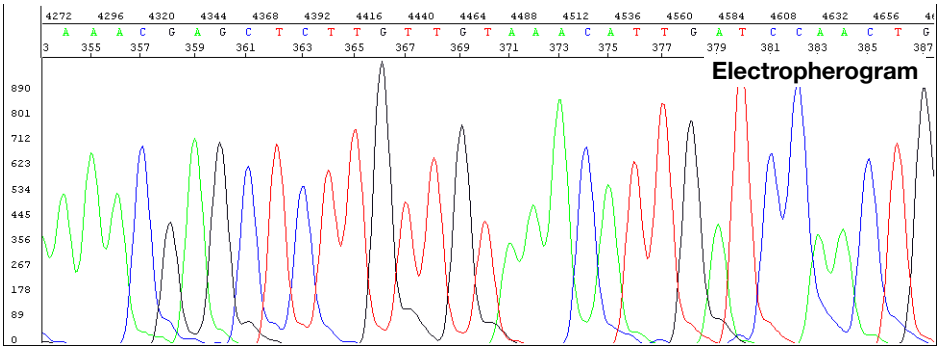
Possible Cause(s)	Recommended Action
Electrophoresis issue:	
The buffer heater is powered on (3730/3730*x*/ instruments only).	Verify that the buffer heater is not powered on.
Severe arcing events can mask the C signal.	• Perform several water washes using the wizard in Data Collection Software. • Disassemble the system and clean out all components with warm water (<42 °C).

Irregular G peaks using BigDye® Terminators v1.1 and 3.1



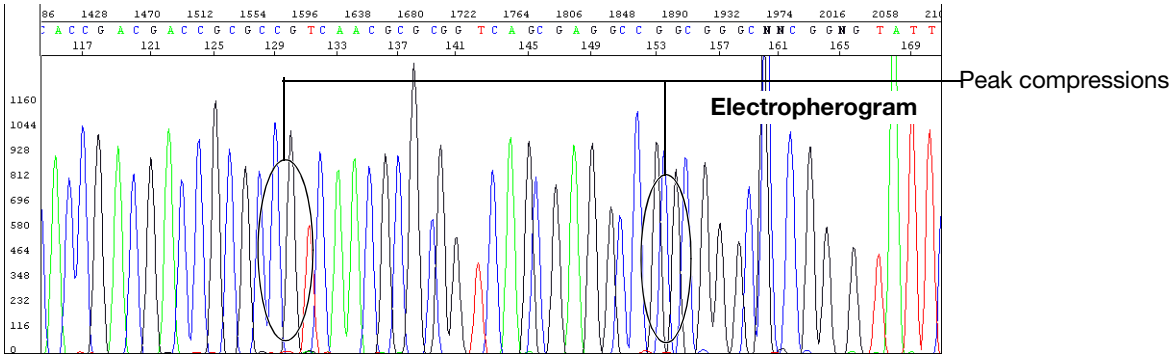
Possible Cause(s)	Recommended Action
Electrophoresis issue: The buffer heater is powered on (3730/3730*xl* instruments only).	If you are using the 3730 instrument, confirm that the buffer heater is not powered on.
Sequencing reaction issues:	
The Hi-Di™ Formamide is degraded.	Resuspend the samples using a newer lot of Hi-Di Formamide.
Sequencing reactions were exposed to light, heat, acidic conditions, and/or oxygen before they were loaded onto the instrument.	Use tube septa or a heat seal to prevent exposure to air and evaporation of samples, especially if you place the samples in the autosampler more than 6 hours before starting electrophoresis.
	Verify that the primer pH and the template pH are not acidic.
The dye labels attached to the ddG terminators are degraded. As shown in the figure above, the pattern for degradation of dye labels on ddG terminators is different than for ddC terminators. The G peak patterns are very irregular, and the complexity increases as degradation progresses. This problem can occur with BigDye Terminators v1.1 and less frequently with BigDye Terminators v3.1.	Protect the fluorescently labeled DNA from light, heat, acidic conditions, and oxygen (see "Storing Sequencing Reactions" on page 121).
Water used as Injection solution. **Note:** Resuspending samples in water leads to breakdown of C and/or G-labeled fragments.	Degradation of the dye labels attached to the ddG terminators is less likely to occur in Hi-Di Formamide or 0.1 mM EDTA.

Shoulders on all peaks



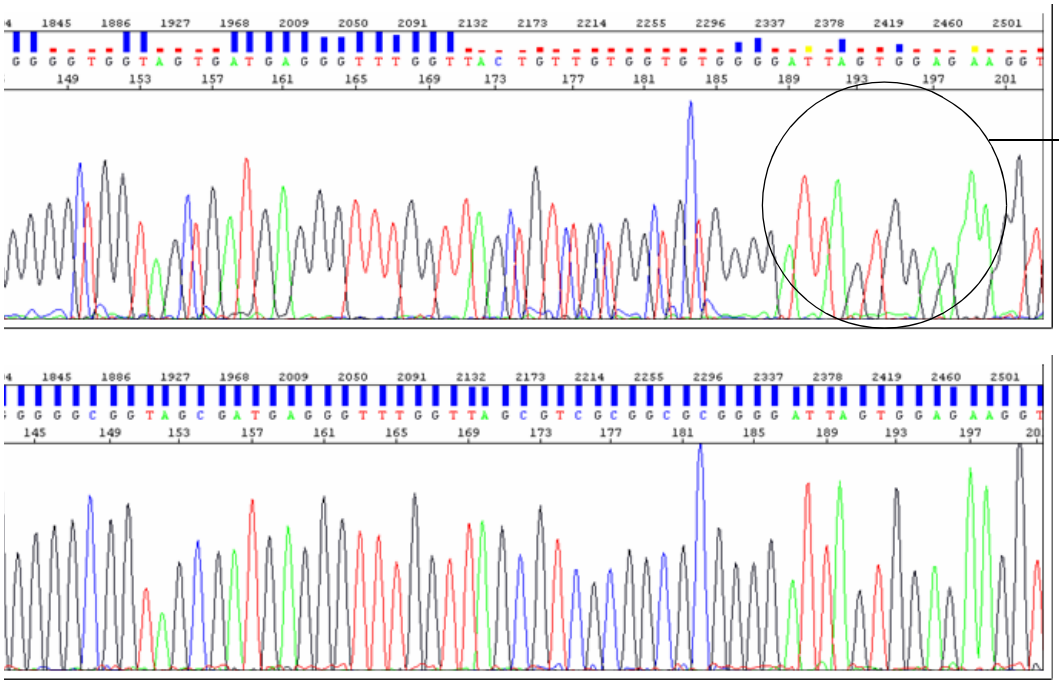
Possible Cause(s)	Recommended Action
Electrophoresis issues:	
Capillary array needs to be replaced.	Replace the capillary array.
Overloaded sample.	Shorten the injection time.
	Amplify less DNA.
Sequencing reaction issues:	
Contamination of the sequencing primer with n+1 or n-1 sequencing primer.	Use the primers with a different template. If the problem persists, resynthesize the primers before repeating the experiment.
Stutter during either PCR amplification and/or cycle sequencing. Stutter is most common in any homopolymeric region greater than 2 bases. It can also be seen with simple repeated DNA sequences. The results are worse when the stutter occurs during PCR amplification. It is thought that stutter occurs when a partially extended primer and template dissociate, then reanneal improperly before extension continues. Partially extended primers and templates commonly dissociate during the reaction, but if they reanneal with complete fidelity, the reaction produces only one product. Improper annealing results in one or more products that are represented in the sequencing results.	If stutter occurs during PCR amplification, little can be done to correct the problem, except using anchored sequencing primers. If stutter occurs during cycle sequencing: • Try using dRhodamine terminators. They have been shown to be less prone to produce stutters, specifically with poly-T regions. • Some customers have found that they can get past poly(A) regions using a mixture of oligo dT_{18} primers with either a C, A, or G as the 3′ terminal dinucleotide or 2-base anchors.
Blending Ready Reaction Mixes from dGTP BigDye terminator kits with BigDye terminator vx.1 kits.	Do not use blended Ready Reaction Mixes of dGTP BigDye terminator kits and BigDye Terminator vx.1 kits.

Peak compressions



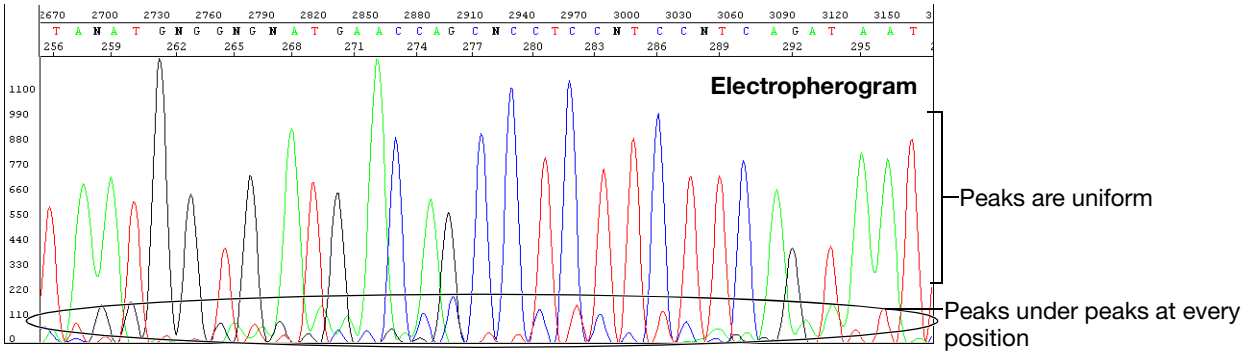
Possible Cause(s)	Recommended Action
Sequencing reaction issue: Observed when sequencing GC-rich regions using dGTP sequencing chemistry. Thought to result from incomplete denaturation of the synthesized DNA.	No corrective action is known at this time. Some customers report that using BigDye primers corrects this problem.

Broad peaks for bisulfite-converted sequences



Possible Cause(s)	Recommended Action
Sequencing reaction issue: Mobility of fragments is uneven because the sample contains both Cs (from methylated samples) and no Cs (from unmethlyated samples).	• Repeat bisulfite conversion • Ensure amplicon is 250 to 400 bp for cloning and 100 to 250 bp for direct sequencing. • Include and extra incubation at the end of thermal cycling run for non-templated A addition.

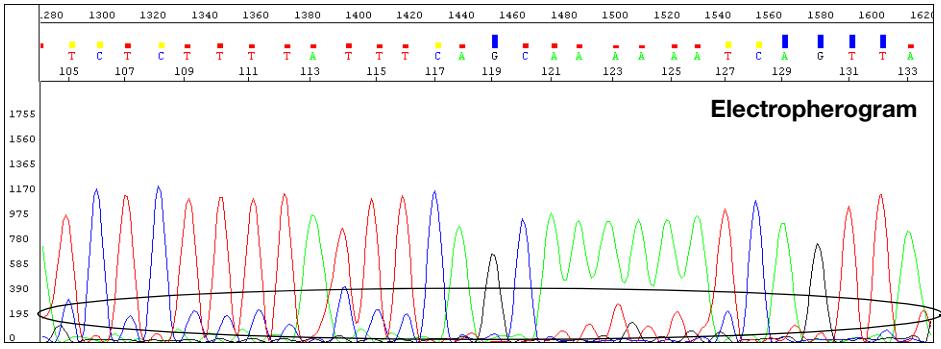
Double peaks: peaks under peaks throughout

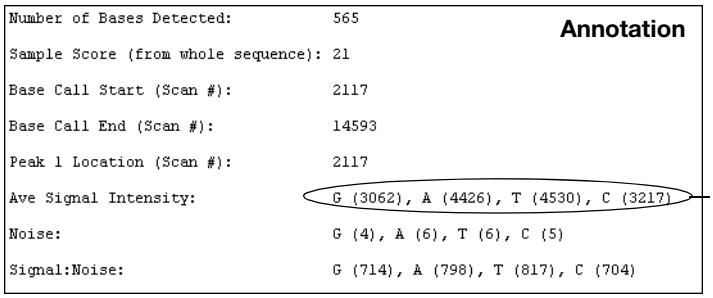


Possible Cause(s)	Recommended Action
Electrophoresis issues:	
Carryover from contaminated septa.	Replace septas, then change buffer, water, and waste.
Electrical noise.	Check the uninterruptible power supply (UPS).
Dirty containers and/or tap water were used to clean instrument components, resulting in contaminated water or buffer.	Clean the containers to be used for cleaning instrument components, then rinse the containers thoroughly with deionized water. It is preferable to use deionized water to clean the instrument components.
Shifted spatial calibration.	Redo the spatial calibration.
Poor CCD alignment.	Contact Applied Biosystems to arrange a service engineer visit.
Poor or incorrect spectral calibration (spectral pull-up).	Redo the spectral calibration.
Sequencing reaction issues:	
Secondary primer site in the template was sequenced.	Design a new sequencing primer (page 38).
Secondary amplification product in the PCR product used as a sequencing template.	Use gel purification to isolate the desired product or design new PCR primers to obtain a single product. For more information, see "Preparing PCR DNA Templates" on page 37.
PCR primers were not completely removed from the PCR product used as a sequencing template.	Remove PCR primers completely before using PCR products as sequencing templates. For more information, see "Preparing PCR DNA Templates" on page 37.
Mixed or contaminated templates or primers.	Review the DNA quality.

Possible Cause(s)	Recommended Action
Stutter during either PCR amplification and/or cycle sequencing. Stutter is most common in any homopolymeric region greater than 2 bases. It can also be seen with simple repeated DNA sequences. The results are worse when the stutter occurs during PCR amplification. It is thought that stutter occurs when a partially extended primer and template dissociate, then reanneal improperly before extension continues. Partially extended primers and templates commonly dissociate during the reaction, but if they reanneal with complete fidelity, the reaction produces only one product. Improper annealing results in one or more products that are represented in the sequencing results.	If stutter occurs during PCR amplification, little can be done to correct the problem, except using anchored sequencing primers. If stutter occurs during cycle sequencing: • Try using dRhodamine terminators. They have been shown to be less prone to produce stutters, specifically with poly-T regions. • Some customers have found that they can get past poly(A) regions using a mixture of oligo dT_{18} primers with either a C, A, or G as the 3′ terminal dinucleotide or 2-base anchors.
Very strong or offscale data	Reduce the signal: • Adjust the injection time and/or lower the voltage • Reduce the template concentration or use less sample

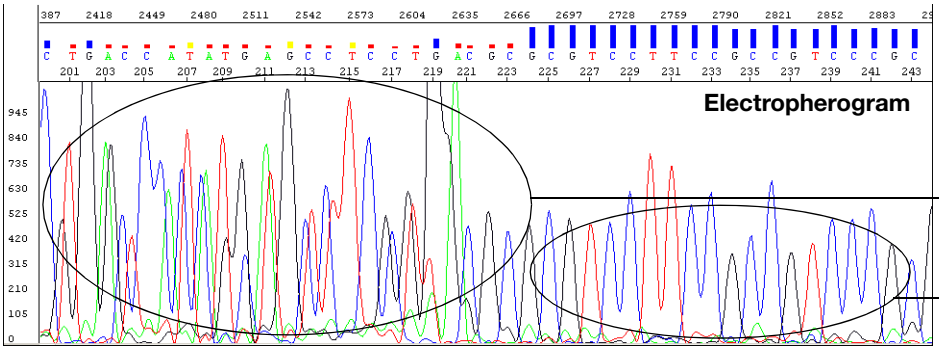
Double peaks: with high average signal intensity values





Possible Cause(s)	Recommended Action
Sequencing reaction issue: Signal is too high for data from the instrument. See page 197 for valid ranges.	Click the **Annotation** tab and examine the Ave Signal Intensity. Excessive signal: • 3730/3730*xl* instruments: >10,000 rfus • 310 and 31XX instruments: >1000 rfus Load less labeled sample by performing one of the following: • Dilute the resuspended product with Hi-Di™ Formamide before loading onto the instrument • Inject sample for less time • Resequence the samples, using less template in the sequencing reaction, especially if you use the BigDye® XTerminator™ Purification Kit (see Table 8, "Recommended DNA template quantities for cycle sequencing," on page 63).
Electrophoresis issue: Modified run module with increased injection time was used.	Use an unmodified standard run module.

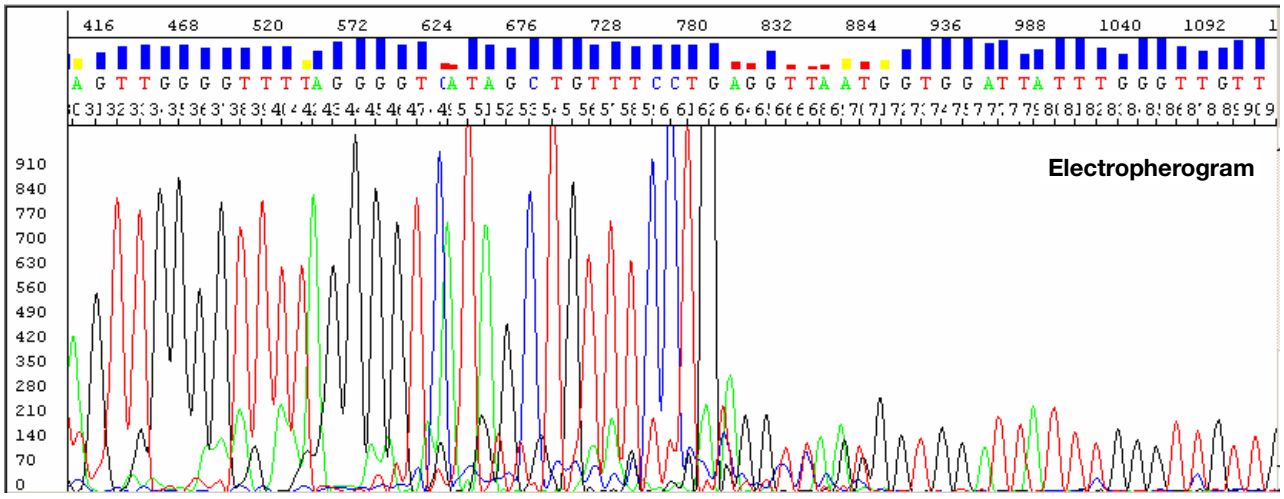
Double peaks: at the beginning of the sequence



See also page 224 for the raw data view

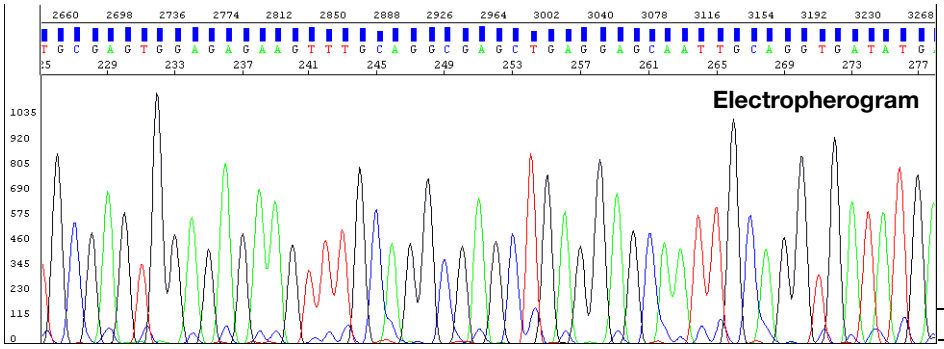
Possible Cause(s)	Recommended Action
Sequencing reaction issue: Observed when a PCR product is used as a sequencing template. Caused by the formation of primer-dimers during the PCR reaction. The primer-dimers anneal and are filled in to create short, non-template PCR products.	If the sequence within the region affected by the primer-dimer sequence is important, either: • Redesign the PCR primers to eliminate the sequences that allow primer-dimer formation or • Use a “hot start” PCR enzyme to inhibit primer-dimer formation
More than 1 PCR product is present in the PCR reaction.	Re-examine the sequence for primer site homology. Redesign as necessary
More than 1 priming site (either upstream or downstream) on the sequencing template.	

Double peaks: at the beginning of the sequence (bisulfite conversion)



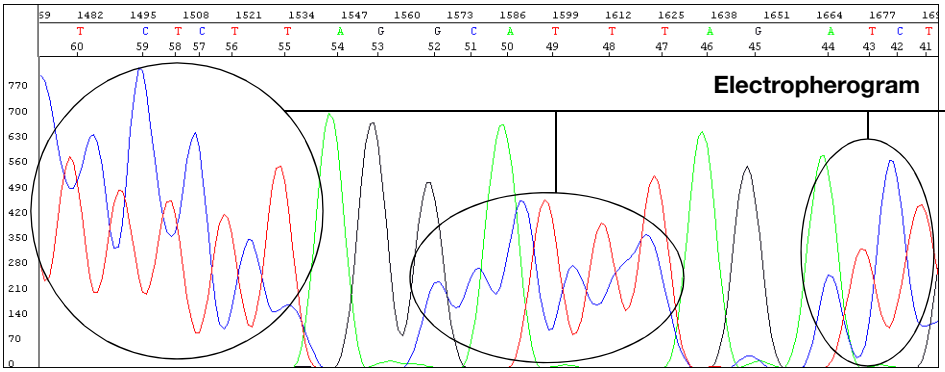
Possible Cause(s)	Recommended Action
Sequencing reaction issue: Observed when a PCR product is used as a sequencing template. Caused by the formation of primer-dimers during the PCR reaction. The primer-dimers anneal and are filled in to create short, non-template PCR products.	If the sequence within the region affected by the primer-dimer sequence is important, use M13 tails with both forward and reverse primers and either: • Redesign the PCR primers to eliminate the sequences that allow primer-dimer formation or • Use a “hot start” PCR enzyme to inhibit primer-dimer formation

Double peaks: specific peaks under specific bases

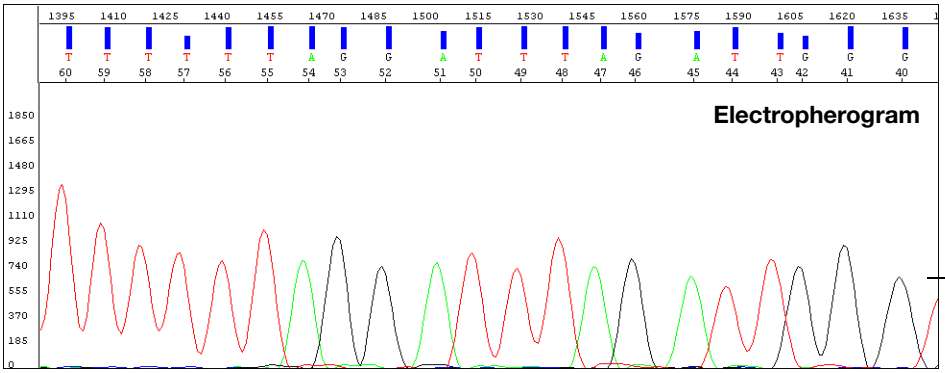


Possible Cause(s)	Recommended Action
Electrophoresis issues:	
Poor or incorrect instrument spectral calibration. Inspection of the raw data shows all secondary peaks directly under primary peaks.	Perform a new spectral calibration run. Follow the procedures in your instrument user guide. Then run your samples again.
Poor quality matrix (310 instruments only).	Create a new matrix file.
Sequencing reaction issue: Signals of the sample exceed the range used for spectral calibration because too much template was used.	Click the **Annotation** tab and examine the Ave Signal Intensity. Excessive signal: • 3730/3730*xl* instruments: >10,000 rfus • 310 and 31XX instruments: >1000 rfus Load less labeled sample by performing one of the following: • Dilute the resuspended product with Hi-Di™ Formamide before loading onto the instrument • Inject sample for less time • Resequence the samples, using less template in the sequencing reaction, especially if you use the BigDye® XTerminator™ Purification Kit (see Table 8, "Recommended DNA template quantities for cycle sequencing," on page 63).

Double peaks: specific peaks under specific bases



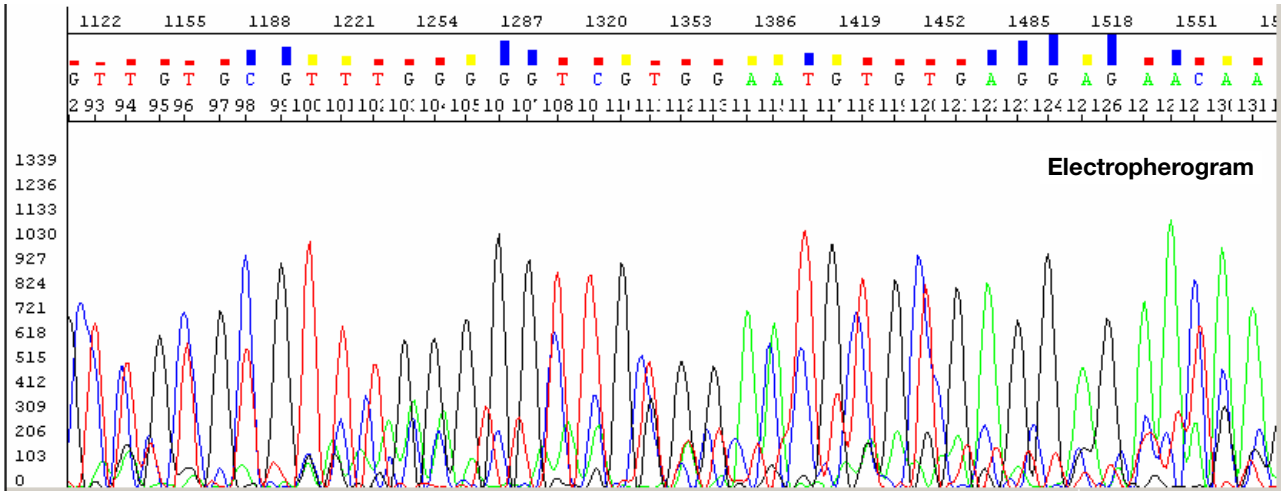
C peaks under many peaks using the ABI basecaller



Clean sequence using the KB basecaller

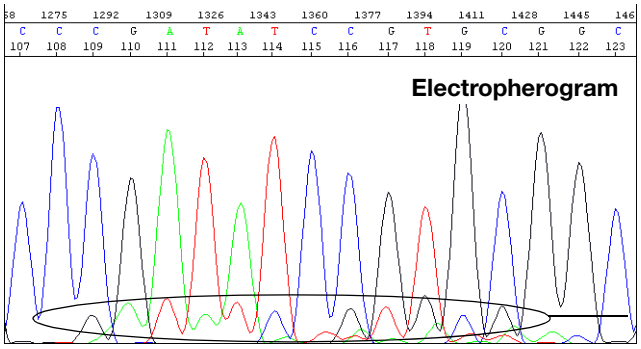
Possible Cause(s)	Recommended Action
Data analysis issue: Using the ABI basecaller when analyzing sequences for bisulfite-treated DNA. Bisulfite treatment of DNA for methylation studies should convert all unmethylated Cs to uracil, so the sequence should contain very few C peaks. However, during sequence analysis, the analysis software overcalibrates for the absence of C peaks.	Use the KB basecaller to analyze sequences for bisulfite-treated DNA.

Double peaks: peaks under peaks throughout (bisulfite conversion)



Possible Cause(s)	Recommended Action
Sequencing reaction issue: Incomplete bisulfite conversion, indicated by the presence of Cs (blue) not adjacent to Gs (black). A C at a non-CpG position serves as an internal control for complete bisulfite conversion. Incomplete bisulfite conversion may be due to: • Impure gDNA • Too much gDNA • Inadequate denaturation of gDNA prior to bisulfite conversion	1. Check DNA quantitation and quality (page 44 and 45). 2. Repeat the bisulfite conversion 3. Repeat the sequencing.

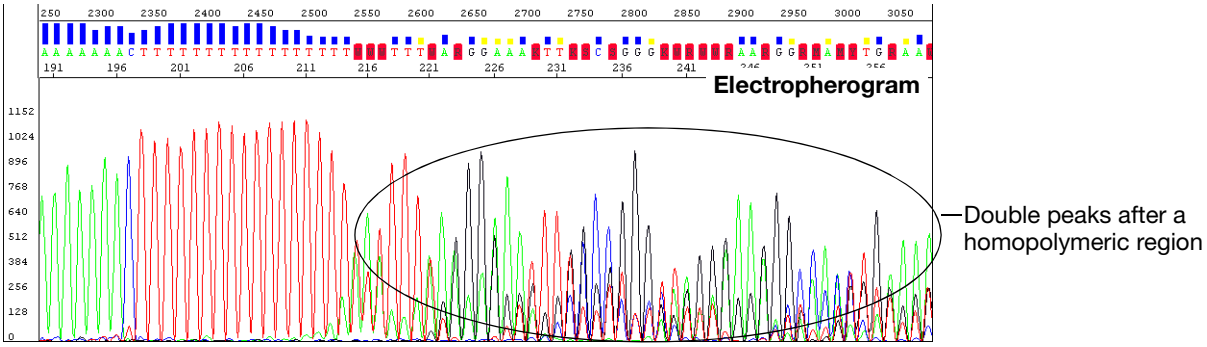
Double peaks: double sequence (n+1 or n-1) throughout



n– 1 sequence

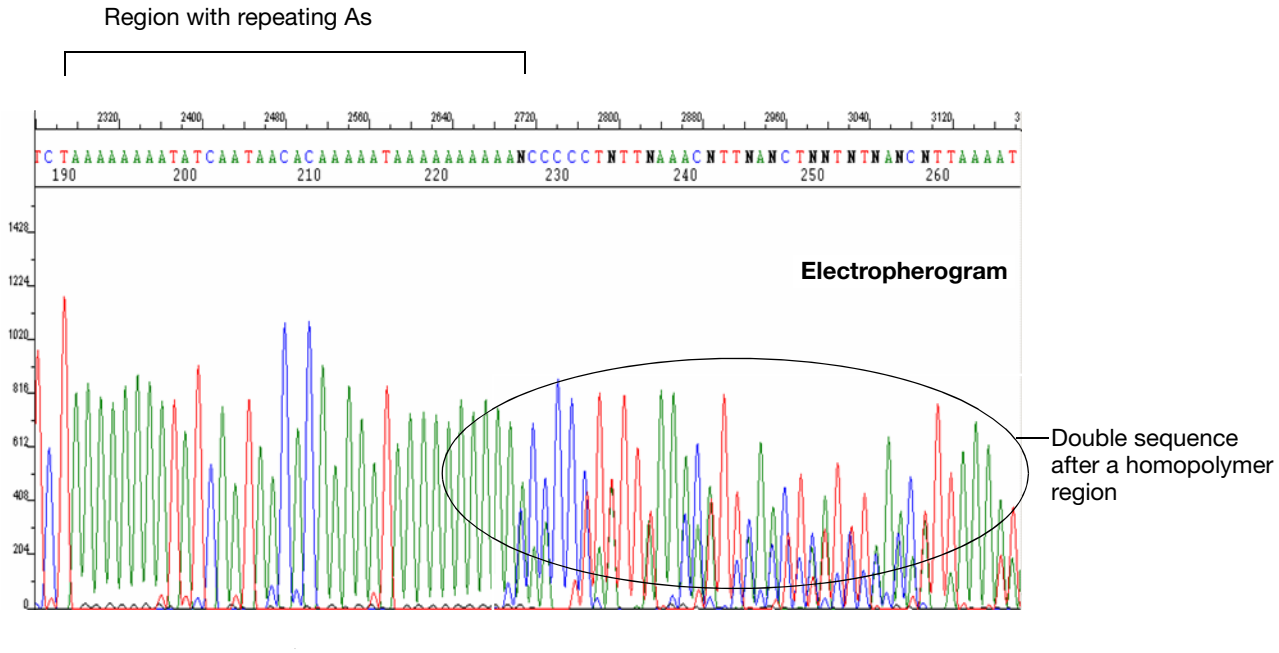
Possible Cause(s)	Recommended Action
Sequencing reaction issues:	
Contamination of the PCR primer with n+1 or n-1 primer.	Use the primers with a different template. If the problem persists, resynthesize the primers before repeating the experiment.
Contamination of the sequencing primer with n+1 or n-1 sequencing primer.	
Sequencing primer contains a run of identical nucleotides, especially 4 or more Gs.	Design new sequencing primers, avoiding runs of identical nucleotides, especially 4 or more Gs.
Homopolymer at the beginning of the sequence.	See page 246.

Double peaks: after a homopolymer or repeated sequence



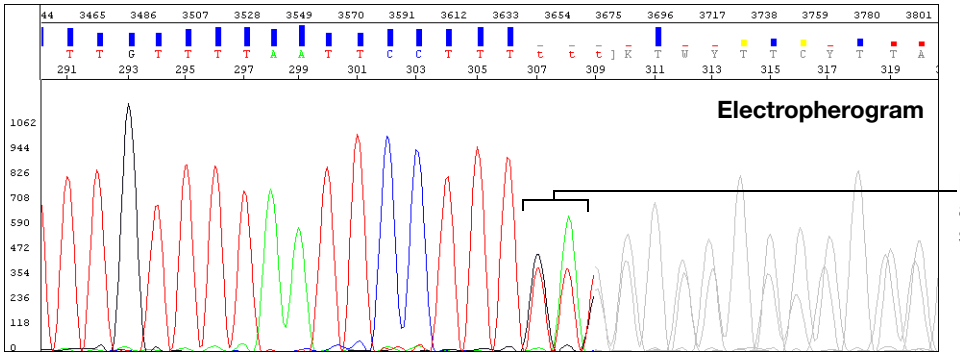
Possible Cause(s)	Recommended Action
Sequencing reaction issue: Stutter during either PCR amplification and/or cycle sequencing. Stutter is most common in any homopolymeric region greater than 2 bases. It can also be seen with simple repeated DNA sequences. The results are worse when the stutter occurs during PCR amplification. It is thought that stutter occurs when a partially extended primer and template dissociate, then reanneal improperly before extension continues. Partially extended primers and templates commonly dissociate during the reaction, but if they reanneal with complete fidelity, the reaction produces only one product. Improper annealing results in one or more products that are represented in the sequencing results.	If stutter occurs during PCR amplification, little can be done to correct the problem, except using anchored sequencing primers. If stutter occurs during cycle sequencing: • Try using dRhodamine terminators. They have been shown to be less prone to produce stutters, specifically with poly-T regions. • Some customers have found that they can get past poly(A) regions using a mixture of oligo dT_{18} primers with either a C, A, or G as the 3′ terminal dinucleotide or 2-base anchors.

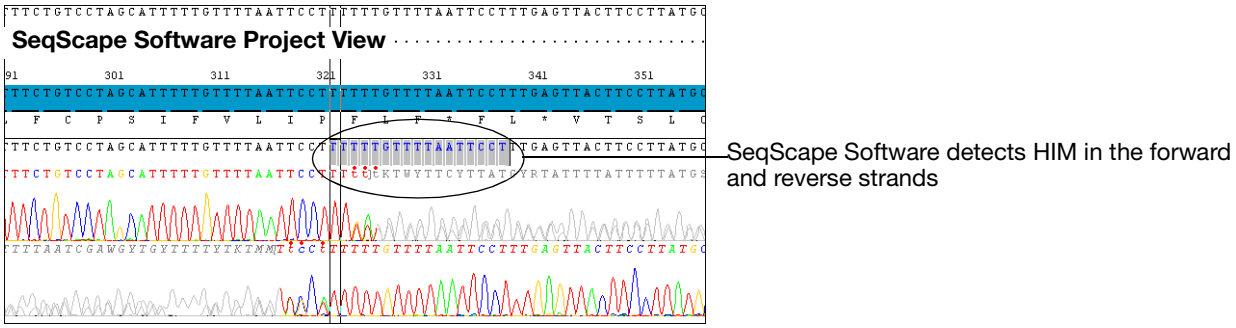
Double peaks: after a homopolymer or repeated sequence (bisulfite sequencing)



Possible Cause(s)	Recommended Action
Sequencing reaction issue: Stutter during either PCR amplification and/or cycle sequencing. Stutter is most common in any homopolymeric region greater than 2 bases. It can also be seen with simple repeated DNA sequences. The results are worse when the stutter occurs during PCR amplification. It is thought that stutter occurs when a partially extended primer and template dissociate, then reanneal improperly before extension continues. Partially extended primers and templates commonly dissociate during the reaction, but if they reanneal with complete fidelity, the reaction produces only one product. Improper annealing results in one or more products that are each represented in the sequencing results.	If stutter occurs during PCR amplification, little can be done to correct the problem, except using anchored sequencing primers. If stutter occurs during cycle sequencing: • Try using dRhodamine terminators. They have been shown to be less prone to produce stutters, specifically with poly(T) regions. • Some customers have found that they can get past poly(A) regions using a mixture of oligo dT_{18} primers with either a C, A, or G as the 3′ terminal dinucleotide or 2-base anchors. • Avoid stretches with > 8 As or Ts. • Use BigDye® Terminator Ready Reaction Mix at full strength. • Use AmpliTaq Gold polymerase.

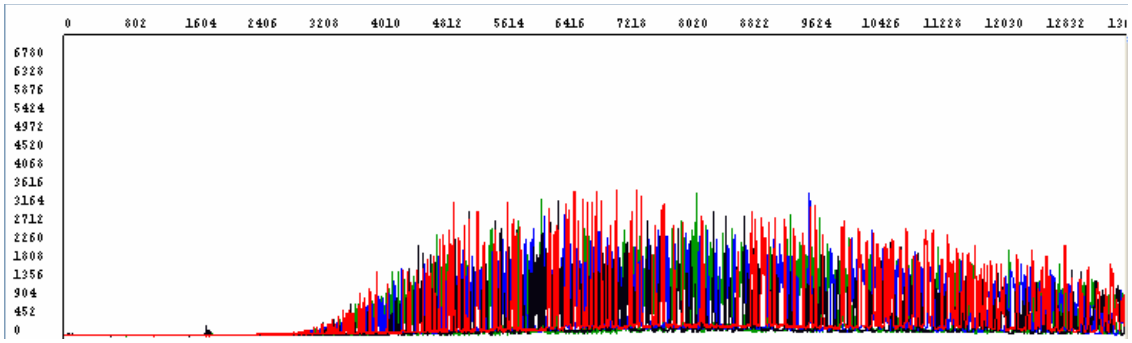
Double peaks: double sequence after clean sequence





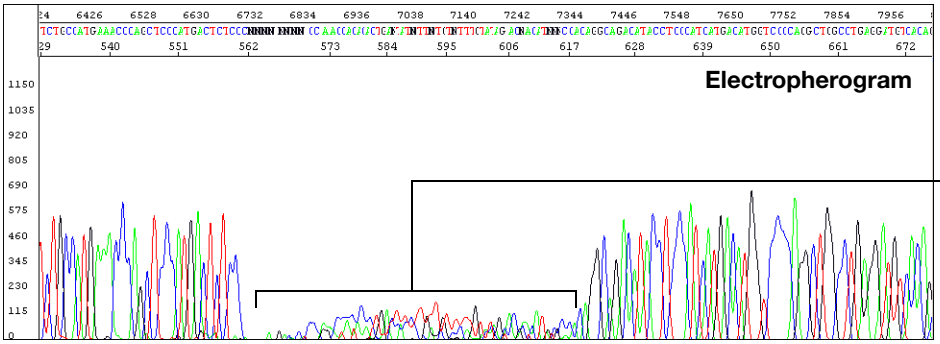
Possible Cause(s)	Recommended Action
Heterozygous indel mutation (HIM).	Obtain forward and reverse sequence data and assemble using SeqScape or Variant Reporter™ Software. • SeqScape Software lists HIMs in the Mutations Report. Review the mutation by clicking the Base Change in the Mutations Report to view the mutation in the Project view. • Variant Reporter Software lists HIMs in the Project Summary Report.

Resolution loss: at beginning of run



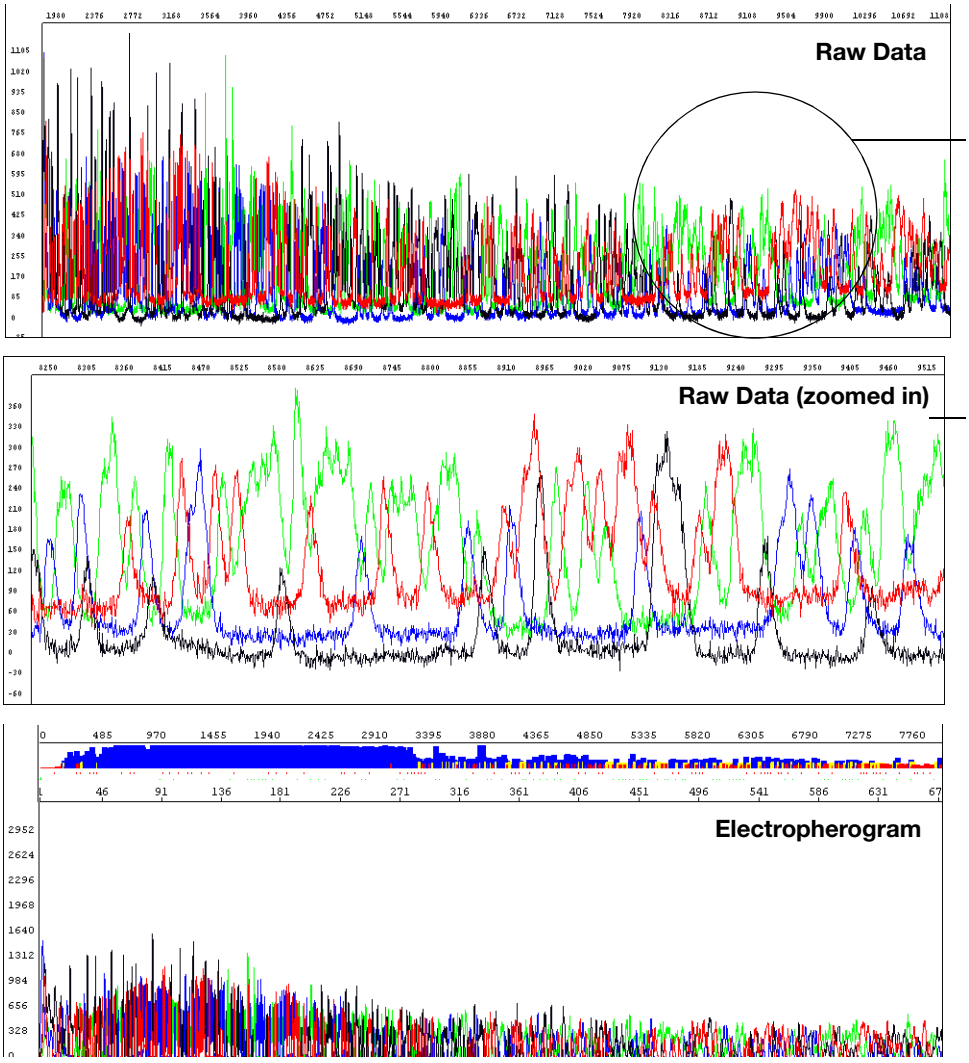
Possible Cause(s)	Recommended Action
Sequencing reaction issues:	
XTerminator™ Solution or premix exposed to temperature over 25 °C.	• Use appropriate adapter for vortexer • Make sure plate does not heat up during vortexing step
BigDye XTerminator Purification Kit reagents past their expiration date.	• Verify expiration dates on reagents and discard if expired • Store XTerminator Solution at 4 °C • Store SAM Solution at room temperature

Resolution loss: in the middle of the run



Possible Cause(s)	Recommended Action
Electrophoresis issues:	
Migration of a contaminant or microbubbles through the capillary during electrophoresis.	Run the sample again.
Syringes, polymer block, or septa contaminated with chemicals during cleaning.	1. Perform a water wash through the polymer delivery system, using the Data Collection Software wizard. 2. Replace polymer, buffer, and water/waste with fresh materials. 3. Run the sample again.
Incomplete replacement of polymer between runs.	Check the polymer delivery system for leaks, looking for residue in and around the polymer block area; check the pin valve for signs of arcing on the tip; check for polymer in the anode buffer jar. If you see evidence of a leak, retighten, then run the sample again. If the leaking persists, contact Applied Biosystems to arrange a service engineer visit.

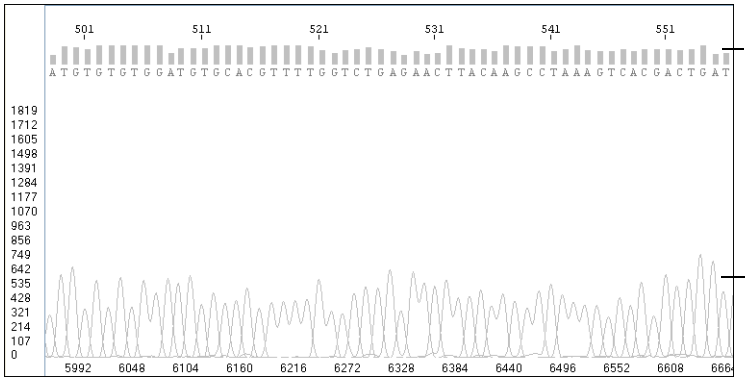
Resolution loss: gradual early loss



Possible Cause(s)	Recommended Action
Electrophoresis issues:	
Capillary array degrading.	1. Perform a water wash through the polymer delivery system, using the Data Collection Software wizard. 2. Replace the capillary/array. 3. Run a sequencing standard. 4. If the problem persists, replace reagents, then run your samples again.
Samples degraded because they sat in the instrument too long, >48 hours.	Prepare additional sample for electrophoresis, referring to "Minimum Sample Volume" on page 122. Then, run the samples again.
Expired or old reagents: polymer, Hi-Di™ Formamide, buffer, or water.	Replace the reagent, then run your samples again.

Possible Cause(s)	Recommended Action
Electrophoresis source is faulty, resulting in unstable current.	Contact Applied Biosystems to arrange a service engineer visit.
Extension products purified using bead-based kits were injected without removing the magnetic beads. The beads may interfere with the extension products during injection and cause overloading or other injection anomalies.	Remove magnetic beads before loading the sample.
Capillaries overloaded with sequencing product.	Click the **Annotation** tab and examine the Ave Signal Intensity. Excessive signal: • 3730/3730*xl* instruments: >10,000 rfus • 310 and 31XX instruments: >1000 rfus Load less labeled sample by performing one of the following: • Dilute the resuspended product with Hi-Di Formamide before loading onto the instrument • Inject sample for less time • Resequence the samples, using less template in the sequencing reaction (especially if you use the BigDye® XTerminator™ Purification Kit) (see Table 8, "Recommended DNA template quantities for cycle sequencing," on page 63).
Blending Ready Reaction Mixes from dGTP BigDye terminator kits with BigDye terminator vx.1 kits.	Do not use blended Ready Reaction Mixes of dGTP BigDye Terminator kits and BigDye Terminator vx.1 kits in these cases.
Use of non-Applied Biosystems reagents.	1. Perform a water wash on all components of the system using the wizard in Data Collection Software. 2. Replace reagents with Applied Biosystems products. **Note:** the performance of non-Applied Biosystems reagents cannot be guaranteed.

High quality sequence in unassembled category in SeqScape Software



Possible Cause(s)	Recommended Action
SeqScape or Variant Reporter™ Software detects no similarity between the sample sequence and the reference sequence. The gray sequence indicates that the trimming of the data to the reference sequence failed.	Make sure that the sample is included in the right project.
Incorrect sample identification when a sample belonging to another project was imported.	