Troubleshooting – Common Amplification and CE Errors/Issues

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Overview

- Trouble Shooting Tools
- Common Capillary Electrophoresis Instrument Issues and Observations
- Trouble Shooting 3500 Issues
- Common Amplification Observations
Overview

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Troubleshooting Tools

Data is usually viewed in the following order:

- Raw data
- EPT data/Status view (during run)
- Capillary view
- Instrument logs
- Analyzed data
  - usually seen first by customer
- Service tools and software (Field Service Engineer)
Why Review Raw Data First?

Data has not been manipulated/analyzed by GeneMapper® software
- No baselining/No smoothing
- No peak detection
- No sizing
- No allele detection
- No table construction
What Raw Data Should Look Like!
Poor Raw Data

Data analyzed correctly, but precursor to a problem
Poor Raw Data
Review Raw Data: Internal Size Standard

- If prepared properly, the size standard is incorporated into every sample along with HI-DI™ Formamide

- Scan for pattern, resolution, peak morphology and peak heights of the size standard
  - Can be used to determine if the instrument hardware and consumables (i.e. polymer, buffer, capillary/array) are working properly
  - In other words, can help you determine if the problem is related to capillary electrophoresis

- Just takes a quick review of the raw data for EACH sample
Troubleshooting Tool: GS500 LIZ & GS500 ROX

- All present
- Good morphology
- Clean baseline
- Adequate signal intensity
- Relatively balanced peak heights
- Check migration by reviewing 250 bp peak (analyzed data)
Troubleshooting Tool: GS600 LIZ®
Size Standard
Review Raw Data: Evaluate Ladders

- Ensure size standard and ladder peaks are present and have good morphology
- Ensure ladder peak heights are at least above Peak Amplitude Threshold (PAT)
- Must have at least one passing ladder per plate
  - If there is only one ladder and it fails, entire plate must be rerun
- If you are using GMID 3.X, remove any failing ladders prior to analysis
  - If not removed, they will all get averaged...throws off good ladders
  - GMIDX software will automatically ignore failing ladders
Review Raw Data: Evaluate Ladders
# Use of the Size Standard & Allelic Ladder

<table>
<thead>
<tr>
<th>Good Quality Result from Unknown DNA Sample(s)</th>
<th>Poor Quality Result from Unknown DNA Sample(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Good Quality Size Standard/Ladder Profile</strong></td>
<td><strong>Poor Quality Size Standard/Ladder Profile</strong></td>
</tr>
<tr>
<td>Good Quality Result from Unknown DNA Sample(s)</td>
<td>No Issues</td>
</tr>
<tr>
<td>Poor Quality Result from Unknown DNA Sample(s)</td>
<td>Investigate sample history and all steps prior to electrophoresis (extraction method, quant results, amplification parameters)</td>
</tr>
<tr>
<td>Single Sample or Few Samples:</td>
<td>Single Sample or Few Samples:</td>
</tr>
<tr>
<td>Verify master mix made properly (master mix dispensed in well, insufficient mixing)</td>
<td>Possibly bubbles/poor injections</td>
</tr>
<tr>
<td>All Samples: Investigate size standard (expiration, storage) &amp; ladder</td>
<td>All Samples: Investigate instrument, reagents &amp; consumables</td>
</tr>
</tbody>
</table>
Review Raw Data: Check Peak Heights & Analysis Range

Analysis Start Point
- past primer peak
- before 75 peak

Analysis Stop Point
Anywhere after 450 peak**
EPT Data/Status View

- Compare a known, good EPT file with problem file
- Ambient temperature
- Oven temperature
- Be aware that default run voltage, injection voltage, laser power, dynamic range and injection time vary between CE platforms

EPT = Electrophoresis, Power & Temperature
Default Run Parameters

- **3130 and 3130xl**
  - Run voltage: 15,000
  - Injection voltage: 3,000
  - Laser Power: 15
  - Injection Time: 3130 = 5 sec  3130xl = 10 sec
  - Typical camera saturation = ~7,000+

- **3500 and 3500xl**
  - Run voltage: 15,000  (13,00 for Globalfiler kits)
  - Injection voltage: 12,000
  - Laser Power: 10
  - Injection Time: 3500 = 15 sec  3500xl = 24 sec
  - Typical camera saturation = ~24,000+

- **3730**
  - Run voltage: 15000
  - Injection voltage: 2,000
  - Laser Power: 25
  - Injection Time: 3730 = 10
  - Typical camera saturation = ~24,000 +
Navigating an EPT File

1. Oven & cell heater stabilize at set temperature

2. Pre-run
   - equilibrates ion balance in system

3. Injection

4. Initial separation
   - voltage stepped up gradually

5. Separation
   - data collection
EPT Data
Capillary View: What to Look For

1. Signal intensity
2. Loss of resolution
3. Migration issues
4. Missing capillaries
5. Possible patterns
Instrument Logs

- Can tell you where or what the problem is
- Trust them!
Analyzed Data

- Analyzed data is usually what people often view first and deem as being good or bad.

- This spurs questions and concerns and may develop into a call to AB.

- Flip side is data may seem to analyze correctly, yet still have problems.

- This is why we start by looking at the Raw Data first.
Check the Controls: AmpFlSTR® Kit
Positive Control

- Sample of known origin that is provided in each AmpFlSTR® kit at a certain concentration*
  - Different kits have different positive controls that may be supplied at different concentrations

- When used according to protocol and when system is working normally, positive control should consistently and reliably produce the same results each and every time

- Primary purpose is to serve as a genotyping control to show that amplification progressed normally
  - Can also help diagnose capillary electrophoresis problems and software analysis issues

*Positive controls are genotyping controls only, not quantitation controls
## Use of the Positive Control

<table>
<thead>
<tr>
<th>Good Quality Positive Control Result</th>
<th>Poor Quality Positive Control Result</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Good Quality Result from Unknown DNA Sample(s)</strong></td>
<td><strong>Single Batch:</strong> Failure to add control DNA; See if reproducible</td>
</tr>
<tr>
<td></td>
<td><strong>All Batches:</strong> Investigate control DNA (expiration, storage, contamination, etc.)</td>
</tr>
<tr>
<td><strong>No Issues</strong></td>
<td><strong>Single Batch:</strong> Mistake made during master mix prep; TC failure; See if reproducible</td>
</tr>
<tr>
<td></td>
<td><strong>All Batches:</strong> Investigate kit reagents (expiration, storage, contamination), TC (parameters, malfunction, temp verification)</td>
</tr>
<tr>
<td><strong>Poor Quality Result from Unknown DNA Sample(s)</strong></td>
<td></td>
</tr>
</tbody>
</table>
| Investigate sample history, extraction method, quant results, dilution ratios, TE-4, sample inhibition/degradation | }
Check the Controls: Internal Laboratory QC Controls

- Known standard with a known DNA profile from the customer's lab or purchased from a manufacturer
- Often taken through the entire process from extraction through data analysis
- Used in conjunction with the previously mentioned controls to determine if the entire process is working properly
  - Can help determine which stage of the process had an error
Overview

- Trouble Shooting Tools
- Common Capillary Electrophoresis Instrument Issues and Observations
- Trouble Shooting 3500 Issues
- Common Amplification Observations
Common Capillary Electrophoresis Instrument Issues and Observations

- Front –End Troubleshooting
- Migration Problems
- Avoiding Bubbles
- Shadow Peaks
Common Capillary Electrophoresis Instrument Issues and Observations

- Front –End Troubleshooting
- Migration Problems
- Avoiding Bubbles
- Shadow Peaks
Front-End Troubleshooting

- Common Front-End Problems
  - Fluorescent contamination
  - Low or no signal
  - Failed injections
  - Loss of resolution
  - Inconsistent peak migration
  - Erratic EP current
  - Extraneous peaks (spikes)
What is the Front-End?

The Front-End includes all replaceable hardware, reagents & consumables

- Pump & polymer blocks
- Buffer & water reservoirs/jars
- Ferrules and tubing
- Capillary array
- Polymer, buffer, water
- 96-well plates & septa
Indications of Possible Front-End Problems

- Allelic ladder and size standards exhibit the same problems as the sample data
- Poor data occurs across multiple plates
- The same samples run on another instrument do not exhibit the same problems
Fluorescent Contamination

Possible Causes

- Poor quality water or reagents (buffer, polymer, formamide)
- Incorrect or infrequent cleaning of system components
- Contamination originating from the sample
- Improper use of canned air
Separated Baseline Due to Fluorescent Contaminant
No Data / No Signal

Possible Causes

- **Injection problem**
  - Air bubble in sample tube
  - No current between electrode and capillary (air bubble in electrophoresis system)
  - Sample volume in tubes too low
  - Sample (& size standard) not added
  - Suboptimal autosampler calibration
  - Clogged/poor capillary/array

- **Detection problem**
  - Dead laser (no signal)
  - Poor spatial calibration

Raw Data
Low Signal

Possible Causes

- Poor quality system reagents (polymer, formamide, buffer, water)
- Incorrect sample preparation
- Suboptimal autosampler calibration
- Poor/improperly stored/exhausted array or capillary
- Bubbles/Failure to centrifuge plates
- Contaminant
Loss of Resolution
Loss of Resolution

Possible Causes

- Poor water quality
- Poor quality system reagents
- Insufficient capillary filling
  - Leak in the system fittings
- Air in the system
  - Bubbles
- Impurities
  - Protein, salts
  - Detergents
- Poor/exhausted array
- Poor instrument maintenance
Spikes: Analyzed vs. Raw Data

Analyzed

Raw
Example of Spikes in the Allelic Ladder

AL190704LIZZIE/7700...0.fsa  5 Blue

AL190704LIZZIE/7700...0.fsa  5 Green

AL190704LIZZIE/7700...0.fsa  5 Yellow

AL190704LIZZIE/7700...0.fsa  5 Red
Spikes: Possible Causes

Possible Causes

- Dust or lint from non-lint free tissues
- Dried polymer deposits
- Dried buffer deposits
- Old or poor quality formamide
- Air bubbles
- Electrical surges
- Poor/exhausted capillary/array
- Improper use of canned air
- Use of powdered gloves
Notice a Trend?

- All of the previously mentioned problems share many of the same causes
- The trend will continue...
Steps to Resolve Front-End Problems

- Check array for damage (detection cell and load end)
- Perform water wash using high quality water source
  - At least 18 MΩ
- Install fresh polymer, buffer and water
- Clean the buffer jar and buffer/water/waste reservoirs
- Set up samples with fresh aliquot of HI-DI™ Formamide and new septa
- Inject allelic ladder and size standards for 10 consecutive runs to see if data problems persist
If Problems Persist

- Install array port plug and perform water wash 4X with 40ºC bottled water
  - Run water wash wizard through the step that flushes the PDP with water
  - At this point, cancel the wizard and start it again
  - Do this until the pump system has been flushed 4X

- Install a new array

- Set up instrument with high quality water and different lot of polymer/buffer

- Inject new lot of allelic ladder and size standards for 10 consecutive runs to see if data improves
  - Set up with different lot of HI-DITM
Common Capillary Electrophoresis
Instrument Issues and Observations

- Front –End Troubleshooting
- Migration Problems
- Avoiding Bubbles
- Shadow Peaks
Peak Migration Problems
Peak Migration Problems: Common Causes

- Polymer on instrument for >7 days
- Poor/expired reagents; poor quality water
- Buffer
  - Not changed daily
  - 1X buffer not prepared correctly
  - Incorrect buffer level on anode and/or cathode side
- Ambient room temperature fluctuations
- Bubble in path of EP current
  - Can be in the capillary or in the polymer tubing/block
- Contaminant in front-end
- Non-AB® sample plates (injection abnormalities)
- Incomplete filling of capillary with polymer
  - Check for leak at pump fittings, detection cell or damaged lower block
- Oven gasket (addressed by AB® retrofit)
Steps to Correct Migration Problems

- Check for polymer leak at PDP fittings and at lower block pin valve
- Be sure to use high quality water source for:
  - Cleaning the system
  - Making 1X buffer
  - Filling the water/waste reservoirs
- Perform water wash and install fresh polymer, buffer, water and septa
- Set up samples with fresh aliquot of HI-DI™ formamide
- Maintain consistent lab temperature (20-30°C)
- Use AB® sample plates & always spin down plates
- If problems persist, replace the array
Influence of Environmental Temperature

Comparison of EPT view

<19°C

24.5°C
Influence of Environmental Temperature

Data Migration Rate

19°C

24.5°C
Influence of Environmental Temperature
Low Molecular Weight Peak Morphology

3130xl System: rt = 18 °C

3130xl System: rt = 21 °C

3100 System: rt = 23°C
Common Capillary Electrophoresis Instrument Issues and Observations

- Front –End Troubleshooting
- Migration Problems
- Avoiding Bubbles
- Shadow Peaks
Problems Often Caused by Bubbles

- Failed injections
- Inconsistent peak migration
- Spikes in data
- Damage to interconnect tubing and/or PDP
- Lower block damage
- “Leak detected” errors
- Erratic EP current
Lower Block Damaged by Bubbles: Arcing
More Damaged Lower Blocks
Erratic EP Current Due to Bubbles
Avoiding Bubbles

- Warm & degas polymer prior to installing
  - Allow polymer to sit at room temperature for at least an hour with cap loosened

- Changes in ambient temperature can result in bubble formation
  - Be sure the lab thermostat is set to a consistent temperature 24 hours a day (20-30ºC)
  - Do not place the instrument next to heating or air conditioning vents

- Periodically check that all PDP fittings are finger-tight

- Check for bubbles every day in the pump chamber, tubing, array port

- Use Bubble Remove Wizard to get rid of bubbles

- Stubborn bubbles may require a Water Wash Wizard
Common Capillary Electrophoresis Instrument Issues and Observations

- Front –End Troubleshooting
- Migration Problems
- Avoiding Bubbles
- Shadow Peaks
What are Shadow Peaks?

- The artifact peaks appear as “shadow peaks” to true DNA peaks observed in the electropherograms of amplified samples co-injected with GeneScan™ 500 ROX™ or GeneScan™ 500 LIZ® size standard.
- In most cases, these artifacts are most prevalent in the dye channel corresponding to the size standard and do not affect accurate sizing of the size standard peaks.
- Results of investigations performed at Applied Biosystems to determine the cause of the shadow peaks suggest that the peaks are caused by pre- and/or post-injection hybridization.
Hypothesis

- The extra peak is most likely the non-denatured form (dsDNA) of the fragment (ssDNA)
  - dsDNA could migrate faster than ssDNA for two reasons
    > Primary effect: dsDNA has twice as many negative charge than ssDNA
    > Configuration of dsDNA makes faster migration more favorable
  - The sum of the peak height (dsDNA + ssDNA) within a locus is conserved between a non-denatured and denatured sample

General schematic of CE

Some fragments are present as dsDNA and travel faster than ssDNA during CE injection. It denatures once it hits the oven.
Verification Study
Replacement of Water Wash with Buffer

Experiments have been performed which demonstrate the elimination or significant reduction of the shadow peaks on Applied Biosystems® 3130 Genetic Analyzers when the water in the water reservoir rinse tray (tray 4 in figure below) is replaced with 1X Genetic Analyzer Buffer.

Note: The configuration for the 3100 is slightly different. See manual for configuration.
Verification Study
Replacement of Water Wash with Buffer

- GeneScan™ 500 LIZ® and GeneScan™ 500 ROX™ size standards as well as in samples amplified with the AmpFLSTR® Identifiler® kit and co-injected with GeneScan™ 500 LIZ® size standard.

- Studies were performed to examine the impact of the buffer replacement:
  - genotype concordance, precision, peak resolution, intracolor balance, and overall peak height
  - AmpFLSTR® SGM Plus and AmpFLSTR® Identifiler® kits analyzed on 3100 and 3130x/ capillary electrophoresis instruments.
Results – Shadow Peak Ratio

Note: GS500 LIZ - 20 hours after mixing with HI-DI™; 9947A - 6 ng reaction, 15 hours after mixing with HI-DI™
Verification Study

Replacement of Water Wash with Buffer

- **Shadow Peak Height Ratio**
  - Undetermined because shadow peaks were not observed
  - Buffer wash reduced peak height ratio from 2-20% to 0-2% (ABJ MCB)

- **Peak Height**
  - Calculated relative change in peak height from water to buffer wash
  - Decreased by an average of 18%

- **Genotype Concordance**
  - 100% concordance for samples with complete profile
  - Number of incomplete profiles increased with 0.125ng samples most likely due to decrease in peak height

- **Sizing Precision**
  - No differences due to water or buffer capillary wash
  - Met specification; standard deviation below 0.15bp for all alleles

- **Intracolor Balance**
  - Similar results between water and buffer wash
Shadow Peaks Are Not Restricted to the ILS

- Not all loci showed this problem

- Data generated below followed normal CE procedures except they have omitted the denaturation step
  - The customer solved the problem by rerunning and/or denaturing the samples
Shadow Peaks - Conclusion

- The extra peaks (shadow peaks) observed are non-denatured fragments

- These peaks were present due to incomplete denaturation

- Not all of the dye and loci were affected – indicating that some fragments are harder to denature.

- Shadow Peaks generally appear first in the size standard.

- During testing, it was generally found that arrays with high number of runs (over 200 runs) are more prone to shadow peak formation.
Shadow Peaks - Recommendations

- The following recommendations can help minimize observation of the shadow peaks:
  - Target an appropriate amount of input DNA.
    - Excess DNA loading on the capillary tip is more likely to cause shadow peak formation.
    - Exceeding recommended DNA:formamide ratio results in reduced denaturing.
  - Perform capillary electrophoresis testing on freshly prepared plates (<8-24 hours).
  - Ensure proper aliquoting and storage of HI DI™ formamide to avoid uptake of water in the formamide.

- If shadow peaks are observed in a particular injection, the following steps may reduce or eliminate the peaks:
  - Re-inject the prepared sample.
  - If no improvement in results is seen, re-denature the sample by heating to 95°C for 3 minutes and chilling for at least 3 minutes on ice and re-inject.
  - If again no improvement is seen, re-prepare the amplified product with fresh HiDi™ formamide and size standard, denature and re-inject.
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Instrument Restart Procedure

Intended to resolve miscommunications between Data Collection software and the instrument

Power off the instrument and the PC

1. Power on the instrument and wait for it to fully boot to solid green.

2. Power on the PC to Windows Vista® OS login screen, but do not login at this time

3. Log in to the PC OS

4. Wait for all 3500 services to be fully started (green check mark symbol is in the bottom tray)

5. Launch the 3500 Data Collection software.
Reset the Instrument

When
- Fatal error as indicated by the red status light
- Instrument does not respond to the Data Collection software

Reset with the Reset button
- Shut down the computer
- Close the instrument doors
- Reset the instrument with the Reset button, as shown

Or, Reset by restarting the system (see previous slide)
Hardware:
The Autosampler Fails to Move the Plate

Possible Causes:

- CBC septa separated from CBC
- Plate base or retainer seated improperly

Action:

- Properly secure CBC septa, plate retainer and/or plate base in autosampler
Hardware:
RFID Read Failure

Possible Causes:
1. System needs refresh
2. Malfunctioning RFID label
3. Miscommunication between instrument and DC software

Possible Solutions:
1. Click Refresh on Dashboard
2. Try a previously used consumable with a known working RFID; contact AB if known working RFID fails to read
   (Suggestion - keep empty consumable with known working RFID for troubleshooting purposes)
3. Restart the system
Hardware:

Polymer Delivery Pump (PDP) is Noisy and/or Vibrating

Possible Causes:

- PDP is not pushed to back wall
- Array locking lever is incorrectly positioned

Action:

- Restart the instrument
- Check Buffer Pin Valve Lever movement
- Push PDP against the back wall
Hardware:
Bubble Detect Error Message: Polymer Delivery Pump (PDP)

Possible Causes:

- Air bubbles in PDP

Action:

- Run the Remove Bubble Wizard
**Consumables:**

Electric Discharge Message, Electrical Current Error Message, Arc Detect Errors, Unstable Current, Crackling Noise

**Possible Causes:**

- Insufficient Buffer level in ABC or CBC
- Excess buffer in small overflow chamber of ABC

**Actions:**

- Replace low buffer container with new container
- Tilt or transfer excess buffer to the large chamber of ABC
Consumables:
Debris in PDP or Upon Removal of Polymer Pouch from Polymer Deliver Pump (PDP)

Possible Causes:

- Shipping seal of polymer pouch has become delaminated; polyethylene remains on pouch fitment

Actions:

- Prior to installation, remove the entire seal from the pouch fitment
- Run the Bubble Remove wizard
- Run the Wash the Pump Chamber and Channels
Software:
Miscommunication Between Data Collection and the Instrument

Possible Causes:
- Door is left open for an extended period of time
- RFID read failure
- User launches software then starts the instrument

Actions:
- Click the refresh button on the dashboard screen
- Start up sequence:
  1. Start the instrument
  2. Turn on computer and launch the software
Software: Dialog Box Unreadable

- The Load plate for run message does not display correctly because the window does not refresh properly

- Click **OK** in the error message and follow the prompts
Software: Re-Inject Button Dimmed

Issue: Re-inject button dimmed when injection selected

Possible Causes:
1. Injection contains samples with assays that specify >1 Instrument Protocol, or
2. Injection contains >1 Results Group

Possible Solutions:
1. Select injection with the Instrument Protocol of interest, or
2. Select samples that specify the same Results Group
Software: No Green Checkmark

Issue: Software status icon = X instead of green check mark

Solution: Right-click the status icon, then select Services
- If any item does not display a checkmark, click the item to start the service
Software: Plate Does Not Link

- Possible Cause: Spatial/spectral calibration not performed

- Possible Solution: Run spatial and/or spectral and relink the plate
Misc: “Spatial Calibration Error” Message

- Issue: Instrument cannot perform spatial calibration with fill
- Possible Cause: Conditioning reagent installed instead of polymer
- Possible Solution: Replace conditioning reagent with polymer and fill array
Log Files

- **3500UsageStatistics.txt** – Provides a summary of the number of plates run, as well as number of run types (sequencing, fragment, and HID)
  - Stored in: x:\Applied Biosystems\3500\UsageData

- **3500ConsumableUpdates.txt** – Provides a summary of consumables installation information and dates
  - Stored in: D:\Applied Biosystems\3500\LogFiles

View using a text editor such as Wordpad
Overview

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Common Amplification Observations

- **Artifacts**
  - Incomplete A nucleotide addition (-A)
  - Dye-labeled artifacts

- **Overamplification**

- **Partial profiles/Imbalanced profiles**
  - Stochastic effects
  - Inhibition
  - Degradation
Common Amplification Observations

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What are Artifacts?

- Inherent anomalies in molecular biology systems
  - Artifacts will always exist
  - Can cause interpretation issues for forensic samples

- Two sources of artifacts
  - **PCR-related**
    > E.g. Stutter, -A peaks, Dye-Labelled molecules
    > Usually reproducible (observed when sample is re-injected)
  - **Post PCR and CE Instrument-related**
    > E.g. Spikes
    > Usually non-reproducible (NOT observed when sample is re-injected)
Addition of 3’ Non-templated ‘A’ Nucleotide

- Inherent feature of AmpliTaq® enzyme

- Primers are designed to maximize ‘A’ addition rather than try to prevent it

- Post PCR incubation at 60°C provides more time for ‘A’ addition
Incomplete Addition of 3’ Non-template ‘A’

Possible Causes

- Too much input DNA
  - Not enough time to add ‘A’ to all products
  - ‘A’ nucleotides may become limiting

- Incorrect thermal cycling parameters
  - Failure to program final 60°C extension step

- Use of a non-validated thermal cycler/block

- Thermal cycler failure

- Poor/incorrect storage of enzyme

- Reduced/altered reaction volumes
-A vs. Split Peaks Due to Cold Temperatures

- Which is –A and which is caused by electrophoresis?
- Solution: Re-inject
  - If split peaks disappear upon re-injection on another CE or when room temperature is higher, problem is not related to amplification
- To resolve –A:
  - Discover source of problem (see previous slide)
  - Rectify source of problem
  - Re-amplify under proper conditions
Decreasing Injection Time

5 sec injection

1 sec injection

Decreasing injection time does not remove the –A!
Dye-Labeled Artifacts

- Usually have abnormal peak morphology
- Most artifacts have been characterized by Applied Biosystems and published in kit user manuals
- With proper use of kit, artifacts should remain below 50 RFUs
Dye-Labeled Artifacts

Exaggerated by:

- Excessive exposure to heat or light
  - Do not denature longer than 3-5 minutes
  - Increased 60°C PCR extension time
- Improper storage of reagents
- Shipping/handling issues
- Use of expired or degraded reagents
- Using non-validated PCR system
- Reduced/altered reaction volumes
- Increased injection time
- Using more than the recommended PCR product for electrophoresis
Common Amplification Observations

- Artifacts
  - Incomplete A nucleotide addition (-A)
  - Dye-labeled artifacts

- Overamplification

- Partial profiles/Imbalanced profiles
  - Stochastic effects
  - Inhibition
  - Degradation
Overamplification

- Presence of pull-up peaks, increased stutter ratios, noise and -A peaks
- Poor peak morphology
- Compromised interpretation
- Off-scale data flagged by GeneMapper® ID Software
Overamplification – Possible Causes

- Too much input DNA
  - DNA not quantitated before addition
  - Quantitation method inaccurate
    > Results outside dynamic range
      - Ideal solution is to dilute and re-quantitate prior to amplification
    > Refer to quantitation presentations for troubleshooting info

- Too many PCR cycles

- Reduced/altered reaction volumes

- Poor pipetting

- Thermal cycler not performing properly
Common Amplification Observations

- **Artifacts**
  - Incomplete A nucleotide addition (-A)
  - Dye-labeled artifacts

- **Overamplification**

- **Partial profiles/Imbalanced profiles**
  - Stochastic effects
  - Inhibition
  - Degradation
Partial Profiles & Stochastic Effects

Possible Causes

- Insufficient DNA input/limited sample
- Quantitation
  - DNA not quantitated
  - Inaccurate quantitation method
  - Quantitation method does not provide an effective indication of amplification potential (e.g. Quantiblot)
- Degraded or inhibited DNA
- Improperly stored/expired reagents
- Thermal cycler not performing properly
Imbalanced Profiles: Degradation vs. Inhibition

Both usually represented by typical “ski slope” effect

**Distinction requires close examination of:**

- Sample origin & extraction method utilized
  - Source (whole blood, semen, tissue, etc.)
  - Substrate
  - Environmental element exposure
- Quantifiler results
  - Inhibitors produce IPC with higher than expected $C_T$ values
  - Degraded samples would not produce unexpected IPC results
- DNA profile
  - Off-scale short amplicon peaks usually indicate inhibition
  - Random marker dropout also usually indicates inhibition
Imbalanced Profiles: Degradation or Inhibition?

Electropherogram from a Crime Lab

Sent Chelex extract of blood from victim to Applied Biosystems

Sample re-amplified at AB with 50% DNA concentration

Sample Degraded?

PCR Inhibited

Off scale peaks

Still off scale
Imbalanced Profiles: PCR Inhibition
Possible Causes

- Too much input DNA (Preferential Amplification)
  - DNA not quantitated before addition
  - Quantitation method inaccurate

  > Refer to quantitation presentations for troubleshooting info

- Inhibitors present in the sample
  - Sample may require **dilution** or **clean-up** to reduce level of inhibition and improve profile quality

  > Hematin and Heme

  > Soil

  > Indigo dyes

  > Phenol

  > Etc.
Reaction Volume

- Profiler Plus® & COfiler® kits are *optimized* & validated with a 50 μl reaction volume

- Identifiler®, Yfiler® & MiniFiler® kits are *optimized* & validated with a 25 μl reaction volume

- Performance expectations and interpretation guidelines documented in the AmpFSTR® Kit User Manuals are based on these validated reaction volumes
  - E.g. Inter- and intra-color balance; signal intensity; heterozygote peak balance; stutter percentage; capacity to cope with sample inhibition

- Quality Control evaluations prior to kit release are performed at the validated reaction volumes
  - Therefore, if your lab uses a reduced reaction volume, it is possible that you may see artifacts/anomalies that we did not
General Amplification Recommendations

- Do not use any kit components beyond kit expiration dates
- Do not combine components from different kit lots (kits are QC’ed together as a lot)
- Do not alter reaction volumes or thermal cycling parameters from manufacturer’s recommendations
- Store all kit components appropriately:
  > Primer set, reaction mix and controls stored at 2 to 8 °C (limit primer set exposure to light)
  > Taq Polymerase stored at -15 to -25 °C
Troubleshooting Amplification Summary

- To minimize the number of amplification issues:
  - Ensure the correct amount of DNA is added to the reaction through the use of an effective quantitation technique
  - Follow manufacturer’s recommendations for amplification using the Applied Biosystems Kits
    > Reaction volume
    > Input DNA concentration
    > Use of a validated thermal cycler/block
    > Use of correct thermal cycling parameters
  - Ensure thermal cycler calibrations and proper temperature verifications are performed routinely

- Unless otherwise directed, use colorless tubes/plastics throughout the analytical process as recommended by Applied Biosystems
Thanks!
Legal Statements

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