FAQs
Frequently asked questions

The KB™ Basecaller v1.1.1
From Applied Biosystems
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Table of Contents

Executive Summary ...................................................................................................................................... 3
Key Benefits of the KB Basecaller ............................................................................................................... 3
Frequently Asked Questions ........................................................................................................................ 7

Section A: Questions about the KB basecaller v1.1.1 .................................................................................... 7
1. How to obtain KB basecaller v1.1.1? .............................................................................................. 7
2. What applications are affected by the defects found in KB basecaller v1.1?................................. 7
3. What are the effects of these defects? ............................................................................................... 7
4. What does the software update fix? .................................................................................................. 7
5. What is the scope of testing of the software update? ....................................................................... 7

Section B: Questions about the KB basecaller and the Applied Biosystems basecaller ....................... 8
7. What are the general differences among the Applied Biosystems basecaller and KB basecaller? .... 8
8. Can the KB basecaller be used to basecall short PCR products? .................................................... 8
9. Why does the baseline look less smooth when the data is analyzed with the KB basecaller? ........ 8
10. What is the Signal to Noise value found with data analyzed with the KB basecaller? .................. 8
12. Will I get more “good” sample files using the KB basecaller? ...................................................... 9
13. Can the KB basecaller analyze data generated on 373, 377 or 3700 instruments? ....................... 9
14. How can I tell which basecaller was used to analyze each sample file? ......................................... 9
15. Will the Applied Biosystems basecaller be supported in the future? ............................................. 9

Section C: Questions about processing data with Phred software and .phd.1 files. .............................. 9
16. Can I analyze sample files with the KB basecaller and then reprocess it with Phred software? .... 9
17. What software generates .PHD.1 files? ........................................................................................... 10

Section D: Questions about quality values ............................................................................................... 10
18. How should I use quality values to review data? .......................................................................... 10
19. What are the differences between quality values of mixed bases and pure bases? ....................... 10
20. Can I trim my data using quality values? ..................................................................................... 10
21. Is there a table mapping each quality value and the corresponding probability of error? ............. 10
22. Where can I see quality value bars and numbers? ....................................................................... 11
23. Why are the quality value bars displayed in gray color? ............................................................... 11
24. Can quality values bars be printed for the electropherogram or .seq views? ................................. 11
25. Which Applied Biosystems software can display the quality values? ........................................ 11
26. Will I be able to view quality values provided by KB basecaller on other software? ................... 11

Section E: Ns, red spacing values and providing feedback ......................................................................... 11
27. When will I see Ns in samples analyzed by the KB basecaller? .................................................... 11
28. Why does the spacing value sometimes appear in red or have a negative value? ......................... 11
29. How do I provide feedback to the KB basecaller product team? .................................................. 12

References.................................................................................................................................................... 12
Executive Summary

Applied Biosystems introduces the KB™ basecaller designed to reduce manual data review time, elongate the read length of high-quality bases in sequences and thereby substantially reducing sequencing costs. This new algorithm is designed to accurately exact more bases out of the sequencing data generated on current instrument and chemistry platforms provided by Applied Biosystems. KB basecaller v1.1.1 supports all chemistries and run modules available on the ABI PRISM® 310, 3100-Avant and 3100 Genetic Analyzers and the Applied Biosystems 3730/xl DNA Analyzers.

Products integrated with KB basecaller v1.1:
- ABI PRISM® 3100-Avant Data Collection v2.0
- ABI PRISM® 3100 Data Collection v2.0
- Applied Biosystems 3730/xl Data Collection v2.0
- Sequencing Analysis software v5.1
- SeqScape® software v2.1
- MicroSeq®ID software v1.0

Products integrated with KB basecaller v1.1.1:
- Sequencing Analysis software v5.1 Rev B
- SeqScape® software v2.1 Rev B

The KB basecaller v1.1.1 Updater can be applied to:
- ABI PRISM® 3100-Avant Data Collection v2.0
- ABI PRISM® 3100 Data Collection v2

When using Sequencing Analysis software v5.1 Rev B and SeqScape® software v2.1 Rev B you may also choose update the Applied Biosystems Data Collection Software v2.0 using the KB basecaller v1.1.1 Updater from the web. When you analyze your data, you can see the basecaller version on the annotation page or the electropherogram.

To ensure the accuracy of this new basecaller, extensive testing has been conducted on thousands of sequencing samples generated by Applied Biosystems and Applied Biosystems customers. The results show that this new algorithm offers many advantages--the most significant is longer accurate read length. Details of the test and validation process will be published subsequently and posted on our website. Please check at www.appliedbiosystems.com, search for Products, Sequencing Software, documentation.

Key Benefits of the KB Basecaller

A. Increased length of read
B. Increased accuracy in regions of low signal to noise or anomalous signal artifacts
C. Provide per base quality value predictions using equation standardized by Phred software
D. Optional detection of mixed-base with quality values
E. Analyze Short PCR products
F. Detection of failed samples
G. Provide the option to trim data using per base quality value
H. Provide per sample quality value that facilitates determining the quality of each read
I. Optional detection of PCR stop
J. Optional assignment of Ns
K. Optional generation of .PHD.1 files

A. Increased length of read: The KB™ basecaller uses advanced algorithms to accurately extract more bases from the 3' and 5' ends of the sequence.

• Our tests on genomic BAC samples indicate a measurable improvement of roughly 100 bases in length-of-read as compared to the same data analyzed by the Applied Biosystems basecaller and Phred software (v0.020425.c). The tests were performed on a dataset generated by AB and several customer sites using 3730xl instruments. The number of increased bases will vary depending on the run modules used to collect the data.

• The accuracy of start point estimation and the first 50 bases of called sequence are substantially increased. Typically, ~10 more correct calls on average are identified at the 5' end, as compared to the Applied Biosystems basecaller.

B. Increased accuracy in regions of low signal-to-noise or anomalous signal artifacts:

• The KB Basecaller software algorithm increases the accuracy of sequence reads extracted from low signal regions or in data partially contaminated by secondary sequence or by other sources of “chemistry noise.”

• Basecalling errors caused by anomalous chemistry and/or instrument signals (e.g., dye blobs, fluorescent spikes) are substantially reduced. These artifacts are often found in otherwise high-quality “clear-range” data, resulting in the loss of high quality bases down-stream from the noise region. Our tests indicate that KB basecaller software can better distinguish between target DNA peaks and the most common arti-facts, thus allowing the basecaller to better “read through” the noise.

C. Provide per base quality value predictions using equation standardized by Phred software:

• The KB basecaller assigns quality values to every basecall. The quality prediction algorithm is calibrated to return Q values that conform to the industry-standard relation established by the Phred software. The KB basecaller and its output are therefore interchangeable in pipelines requiring Phred software or output.

• Quality value calibration was performed using a controlled set of correct-sequence annotated sample files, representative of production sequencing data generated on capillary electrophoresis platforms. Over 20 million basecalls were used to calibrate the KB basecaller v1.1.1 software. Over 10 million distinct basecalls were used to test the calibration.

D. Optional detection of mixed-base with quality values: The KB basecaller provides the option to detect mixed bases positions and assign IUB codes and quality values to those positions. Quality values are assigned to mixed basecalls using an algorithm similar to that for pure bases. The definition conforms to the Phred relation. Quality values for mixed bases are inherently lower than those of pure bases due to the higher error risk associated with interpreting more complex signals. Note that when using the Applied Biosystems basecaller or Applied Biosystems basecall + Phred software, a second stage analysis is required to determine mixed bases.

E. Analyze Short PCR products: The KB basecaller has been tested for accuracy in basecalling and quality value estimation on PCR products as short as 100 bases. It is possible to basecall products with less than 100 bases; however these types of sample files are not tested.

F. Detection of failed samples:
The KB basecaller indicates gross sample quality via a pass/fail mechanism. A common failure mode is no signal—i.e., insufficient detection of DNA peaks. For the failed samples, the KB basecaller will write a string ‘NNNNN’ as the sequence, signaling that the sample quality is very low and may need to be omitted from further analysis. Failed samples are flagged in reports provided in analysis software. Note that this behavior is different than the Applied Biosystems basecaller that will always attempt to call bases, resulting with sequences of many Ns.

G. **Provide the option to trim data using per base quality value:** Software with KB™ basecaller integrated can be used to automatically determine the clear range region by trimming off the ends using the per-base **quality values** provided by the KB basecaller. The parameters used for trimming are similar to those offered in tools used by genome community.

H. **Provide per sample quality value that facilitates determining quality of reads:** Software with KB basecaller integrated uses the QV provided by the KB basecaller to trim and also determine a sample score. The sample score is the average QV in the clear range region. This single number is a useful metric to determine the quality of the data. The sample score appears in reports generated Sequencing Analysis Software, SeqScape® software and MicroSeq® ID software.

I. **Optional detection of PCR stop:** The KB basecaller can be set to terminate basecalling after a PCR stop. Note that samples with enzymatic failure may have signal properties mirroring those in PCR stop conditions. The algorithm may not be able to distinguish between these two similar signal properties.

J. **Optional assignment of Ns:** In default mode, the KB basecaller will not generate Ns; however, there is an option to reassign Ns to bases with QV below a user settable threshold.

K. **Optional generation of .PHD.1 files:** .PHD.1 files can be generated via auto-analysis or in analysis software. The .PHD.1 files may be used for further analysis by down-stream software such as phrap software. [2]

### General comparison of basecallers

<table>
<thead>
<tr>
<th></th>
<th><strong>Applied Biosystems basecaller</strong></th>
<th><strong>KB basecaller</strong></th>
</tr>
</thead>
</table>
| **What does the software do?** | Process raw traces  
Provides processed traces  
Provides AGCTN calls | Process raw traces  
Provides processed traces  
Provides pure only OR  
Provides pure & mixed calls  
Provides **quality values**  
Provide .PHD.1 files  
Provides a sample score |
| **What are the resulting basecalls?** | Single option available  
Assigns AGCT or N to each peak  
Mixed bases are assigned as Ns. Further process (manual or additional software) is required to reassign IUB codes to the Ns or pure bases | Four options available  
1. Assigns ACG or T and Q value to each peak or  
2. Assigns ACGT and Q value to each peak, any peak with Q value below a defined threshold will be reassigned an N or  
3. Assigns ACG T or a mixed base and Q value to each peak or  
4. Assigns ACG T or a mixed base and Q value to each peak, any peak with Q value below a defined threshold will be reassigned an N |
<table>
<thead>
<tr>
<th>How are failed samples handled? (no signals, chemistry failure)</th>
<th>Will attempt to call all bases, so sample will result with many Ns</th>
<th>Will assign 5 Ns to the entire sample to signal that sample has failed Analysis report will flag files</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline in the processed data</td>
<td>appears smooth</td>
<td>appears less smooth. See FAQ section</td>
</tr>
<tr>
<td>Steps to process data</td>
<td>1. Use Applied Biosystems basecaller to call bases on Windows OS</td>
<td>1. Use KB basecaller to call bases and estimate Q value on Windows OS</td>
</tr>
</tbody>
</table>
Frequently Asked Questions

Section A: Questions about the KB™ basecaller v1.1.1

1. **How to obtain KB basecaller v1.1.1?**
   Sequencing Analysis v5.1 Rev B and SeqScape Software v2.1 Rev B is integrated with KB basecaller v 1.1.1.
   - You may choose to update 3100/Avant Data Collection v2.0 and/or 3730/xl Data Collection v2.0 with the KB Basecaller v1.1.1 Updater, available via the web.

2. **What applications are affected by the defects found in KB basecaller v1.1?**
   KB basecaller v1.1 was integrated in the following products:
   - ABI PRISM® 3100/Avant Data Collection v2.0
   - Applied Biosystems 3730/xl Data Collection v2.0
   - Sequencing Analysis v5.1
   - SeqScape Software v2.1
   - MicroSeq® ID Software v1.0

3. **What are the effects of these defects?**
   The first defect sometimes causes early truncation of the basecalling analysis, which can result in slightly shorter than expected read lengths. This effect can be seen when KB basecaller v1.1 is used to basecall sequencing samples that migrate more slowly through the capillary than indicated by the basecaller's calibration data. This situation usually arises when users modify the run parameters of the electrophoresis system (e.g., temperature).
   The second defect leads to a high rate of deletion errors in data with uncommonly low resolution.

4. **What does the software update fix?**
   This software update addresses only the defects described above. No other changes have been made since KB™ Basecaller v1.1. The update will have very little or no impact on the analysis of data unaffected by the defects.

5. **What is the scope of testing of the software update?**
   The KB basecaller v1.1.1 software update has been tested on data generated with the standard run modules, as shipped with the Data Collection software (v2.0). This software update will not guarantee accurate basecalling analysis on any data collected using modified run modules.

6. **What applications are compatible with KB Basecaller v1.1.1 Updater?**
   This update should be applied to products that include the KB™ Basecaller v1.1 (see list above), except for MicroSeqID Software v1.0. It is not compatible with products that contain KB™ Basecaller v1.0.
   True profile scaling option—Using this method, the processed traces are scaled uniformly so that the average height of peaks in the region of strongest signal is about equal to a fixed value (e.g., 1000). The profile of the processed traces will be very similar to that of the raw traces.
   Do not run the KB Basecaller Updater on MicroSeqID Software v1.0 because it has no known impact on basecalling of short PCR fragments. This software update was not tested with MicroSeqID software v1.0.
Section B: Questions about the KB™ basecaller and the Applied Biosystems basecaller.

7. What are the general differences among the Applied Biosystems basecaller and KB basecaller? Refer to the matrix above.

8. Can the KB basecaller be used to basecall short PCR products?
The KB basecaller has been tested for accuracy in basecalling and quality value estimation on PCR products as short as 100 bases. It may be possible to basecall products with less than 100 bases; however this type of sample files is not tested. Samples significantly shorter than 100 bases may not contain enough signal information needed by the basecaller to process the sample file.

9. Why does the baseline look less smooth when the data is analyzed with the KB basecaller?
Processed signals or traces provided by the Applied Biosystems basecaller will appear smoother than those provided by the KB basecaller because each algorithm uses distinct code that processes the signals somewhat differently.

With the Applied Biosystems basecaller, only AGCT and Ns are assigned to each peak, therefore the user must manually search for mixed bases or use a secondary software to complete the task. To facilitate this secondary process, the Applied Biosystems basecaller subtracts a rather aggressive baseline estimate to present a cleaner baseline in the processed signals.

The KB basecaller can determine pure and mixed bases and therefore there is no need for second stage processing which allows less aggressive baseline subtraction. The processed traces will have a higher baseline. If you have mixed bases, turn on the mixed-base detection option and allow KB basecaller to call bases. Use the mixed base calls and the associated QVs to review mixed bases-- do not simply look at the baseline.

10. What is the Signal to Noise value found with data analyzed with the KB basecaller?
KB basecaller calculates this information and presents the data in the annotation view and analysis report. The Applied Biosystems basecaller will calculate only the signal intensity. The signal to noise value is more informative of data quality than the signal intensity value alone both properties are important in determining quality.

11. What are the two scaling options True and flat profiles available with the KB basecaller?
With the KB basecaller, you have two options for scaling data while options are not provided with the Applied Biosystems basecaller. The user should decide which option is better suited to their particular circumstances. The sequence and QVs called by the KB basecaller are independent of this choice of scaling alternatives The Applied Biosystems basecaller employs a scaling method closer to the “True profile” option than the “Flat profile” option.

True profile scaling option—Using this method, the processed traces are scaled uniformly so that the average height of peaks in the region of strongest signal is about equal to a fixed value (e.g., 1000). The profile of the processed traces will be very similar to that of the raw traces.

Flat profile scaling option—The processed traces are scaled semi-locally so that the average height of peaks in any region is about equal to a fixed value (e.g., 1000). The profile of the processed traces will be flat on an intermediate scale (> about 40 bases).
12. Will I get more “good” sample files using the KB basecaller?
Our tests show that medium and high quality data will yield even more useable bases (longer read length) when analyzed by the KB™ basecaller as compared to results produced by the Applied Biosystems basecaller.

For extremely poor quality data, the KB basecaller will not provide more bases but instead will fail these samples—i.e. no signal, extremely low signals or extremely noisy signals. By calling a string of “NNNNN” for the failed samples (instead a sequence all containing low QVs), the KB basecaller is signaling that the sample is unuseable.

13. Can the KB basecaller analyze data generated on 373, 377 or 3700 instruments?
No, the KB basecaller is not calibrated for this task. It is calibrated to basecall and estimate the basecall quality for 28 specific combinations of instrument/polymer/chemistry/run condition which are currently supported on ABI PRISM® 310, 3100-Avant and 3100 Genetic Analyzers and the Applied Biosystems 3730/xl DNA Analyzers. There are no plans to include support for analysis of data from the ABI PRISM 373, 377 or 3700 instruments.

14. How can I tell which basecaller was used to analyze each sample file?
The Annotation view of each sample file and the print header contains the basecaller name and version number. If no QV value bars are displayed when you select to display those means that the sample file was not analyzed by the KB basecaller.

15. Will the Applied Biosystems basecaller be supported in the future?
AB will continue to provide technical support for the Applied Biosystems basecaller; however, further development and bug fixes will only be done on the KB basecaller. If you encounter a bug in the Applied Biosystems basecaller, please use the KB basecaller as a work around. In 2003, software products will have both the Applied Biosystems basecaller and the KB basecaller support files. Analyzing data collected on new run modules will only be supported on the KB basecaller. In future releases, we will remove Applied Biosystems basecaller support files from the software wherever there is duplicate support in the KB basecaller.

Section C: Questions about processing data with Phred software and .phd.1 files.

16. Can I analyze sample files with the KB basecaller and then reprocess it with Phred software?
In principle, yes, but this is not recommended. The resulting quality values from Phred software will not be calibrated—i.e., it is possible that Phred will over or under-predict quality in certain circumstances because it has not been trained on the type of processed electropherogram produced by the KB algorithms. (Instead, Phred has been trained using the Applied Biosystems basecaller to produce the processed traces.)

In addition, since Phred replaces (and ignores) the initial called sequence, re-processing KB-analyzed samples with Phred will degrade the accuracy of the analysis, on average, in terms of actual sequence error. Analysis improvements provided by KB algorithm outlined above will be essentially lost.

Our tests show that for the same set of sample files, the accurate read length is shortest with the Applied Biosystems basecaller.
Our studies indicate that running Phred software on sample files that were processed by the KB basecaller significantly degrades the quality of the results.
Analysis with KB can output PHD files, which are interchangeable with any pipeline that currently depends on *Phred*.

**17. What software generates .PHD.1 files?**

All of the following software products have KB basecaller integrated and can generate .PHD.1 files

- ABI PRISM® 3100-Avant Data Collection v2.0
- ABI PRISM® 3100 Data Collection v2.0
- Applied Biosystems 3730/xl Data Collection v2.0
- Sequencing Analysis software v5.1
- SeqScape® Software v2.1
- MicroSeq®ID software v1.0

**Section D: Questions about quality values**

**18. How should I use quality values to review data?**

When analyzing data with pure bases: set Low QV = <15, Medium QV= 15 to 19 and High QV= 20+ (default)

When reviewing pure base data use the quality values to

- briefly look at bases with high QV >20
- pay close attention to the medium QV
- quickly review low QV bases but most likely you will discard these bases from further analysis

When reviewing mixed bases, your quality values will be lower than pure bases. For mixed bases, you may want to review and accept basecalls with quality values as low as 10.

**19. What are the differences between quality values of mixed bases and pure bases?**

High quality pure bases will be assigned QVs of 20 or higher. Values between 51 and 99 are rare.

High quality mixed bases will be assigned QVs of 10 or higher. The reason that a high quality mixed base may receive such low QVs is that the probability of error with more complex signals are higher. Do not discard mixed bases with QV between 10 and 20. It is a good idea to review them. Values between 51 and 99 are rare.

**20. Can I trim my data using quality values?**

Yes, when using Data Collection, you can set trimming using QVs in the analysis protocols.

When using Sequencing Analysis, SeqScape or MicroseqID software, you can set trimming using QVs in the Analysis settings.

**21. Is there a table mapping each quality value and the corresponding probability of error?**

Below is a condensed quality value table. To see the full version of this table, look in the help menu or the user manual of Sequencing Analysis and SeqScape software.

<table>
<thead>
<tr>
<th>QV</th>
<th>Pe</th>
<th>QV</th>
<th>Pe</th>
<th>QV</th>
<th>Pe</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>79%</td>
<td>21</td>
<td>0.790%</td>
<td>41</td>
<td>0.0079%</td>
</tr>
<tr>
<td>5</td>
<td>31%</td>
<td>25</td>
<td>0.310%</td>
<td>45</td>
<td>0.0031%</td>
</tr>
<tr>
<td>10</td>
<td>10%</td>
<td>30</td>
<td>0.100%</td>
<td>50</td>
<td>0.0010%</td>
</tr>
<tr>
<td>15</td>
<td>3.2%</td>
<td>35</td>
<td>0.032%</td>
<td>60</td>
<td>0.0001%</td>
</tr>
<tr>
<td>20</td>
<td>1.0%</td>
<td>40</td>
<td>0.010%</td>
<td>99</td>
<td>0.00000012%</td>
</tr>
</tbody>
</table>
22. Where can I see quality value bars and numbers?
Sequencing Analysis, SeqScape® and MicroSeq®ID software provide the option to display/hide quality value bars in displays and printouts. Users can customize the color and range for low, medium and high quality values. For QV $\leq 50$, the length of a bar is proportional to the corresponding quality value. Quality values above 50 will be have the same color and QV bar length as that defined for QV 50. To see the actual quality value for a particular base, hover the computer mouse over the QV bar.
In SeqScape® software and MicroSeq®ID software, the per-base quality values also appear in the reports corresponding to bases identified as mutations.

23. Why are the quality value bars displayed in gray color?
A quality value is assigned to a specific basecall. When you alter the basecall then the quality value is no longer applicable to the new base, therefore it will be displayed as a gray bar. Also when you choose to reassign Ns to bases below a certain QV, the QV bar is not applicable to the N basecall, therefore it will be displayed as a gray bar.

24. Can quality values bars be printed for the electropherogram or .seq views?
There is an option to print/hide QV bars when printing the electropherogram or .seq view of the sample file. QV bars may not be printed, due to space availability, if you choose to print more than 7 panels per page. The actual quality value numbers can not be printed.

25. Which Applied Biosystems software can display the quality values?
Sequencing Analysis v5.X, SeqScape software v2.X and MicroSeqID software v1.X can all display quality values.

26. Will I be able to view quality values provided by KB™ basecaller on other software?
Quality value graphic views were customized for software provided by Applied Biosystems. The design allows for additional functionality such as clear range trimming and more streamlined editing. Most third party do not have graphic views for quality values provided by the KB basecaller.

Section E: Ns, red spacing values and providing feedback

27. When will I see Ns in samples analyzed by the KB basecaller?
When using the KB basecaller, you will see the sequence “NNNNN” when the sample failed analysis. Omit this file from further analysis.

In addition to pure and mixed bases with QV bars, you may also see Ns and gray QV bars when you choose to reassign Ns to all bases below a settable QV threshold. This option was implemented to assist those who analyze data using the KB basecaller and share data with those who do not have software that can display quality values. This option allows you to take advantage of the longer read length, more accurate basecalling provided by the KB basecaller while still viewing data with software that does not display QVs.

28. Why does the spacing value sometimes appear in red or have a negative value?
When the Applied Biosystems basecaller fails to determine a spacing value a sample file, it uses a default value of 12.00 for all run conditions. This number will appear as in red in the sample manager and the annotation view will display a negative 12.00.
When the KB basecaller fails to determine a spacing value for a sample file, it uses a default value specific to the particular instrument/polymer/chemistry/run condition used to generate the sample file. This number will appear in the sample manager and the annotation view will display the value as a negative number.

29. How do I provide feedback to the KB basecaller product team?
Please send detail information and sample files that illustrate your feedback to your local Applied Biosystems applications support representative. You may also send an email to US technical support using this address GAlab@appliedbiosystems.com

References

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