BrightBox QUANTITATION

EdgeBio
A CALIBRE SCIENTIFIC COMPANY

BrightBox Quantitation FAQ

Usability

Q: How does the kit work?
A: The kit uses a variety of enzymes and oligonucleotides to create a fluorescent signal. The same amount of fluorescence is created per library molecule (irrespective of fragment size).

Q: Why is there temperature cycling? Is it PCR amplification?
A: Temperature cycling produces a linear amplification of fluorescent signal; however, library molecules are NOT amplified.

Q: What temperature should I store the kit at?
A: Store the kit at 4°C.

Q: Why did my kit arrive thawed on ice packs?
A: Components may be shipped frozen or thawed on ice without impacting usability, but all tubes should be stored at 2-8°C upon receipt.

Q: Can I freeze the Assay Mix and Standards more than once?
A: Yes, the Assay Mix and Standards can be frozen and thawed a number of times, but this may lessen effectiveness.

Q: How stable are the reagents at room temperature?
A: The reagents are stable at room temperature for up to 1 week.

Q: What is the dynamic range of the kit?
A: When using 1uL, the Standards have a dynamic range of 2-30nM, the reactions are accurate from 1nM to 50nM. If you are using 2uL, the dynamic range for your libraries will be 1-15nM.

Q: What type of elution buffers are compatible with the Assay?
A: All elution buffers tested did not affect performance. Our team has tested up to 5uL of low TE and 0.1% of Tween and have seen no inhibitory effect.

Set Up

Q: How should I mix the Standards?
A: Vigorously vortex the tubes.

Q: How should I mix the Assay Mix?
A: Gently invert the tube. DO NOT vortex, as there are enzymes in the Assay Mix.

Q: Do I need to assemble the reactions on ice?
A: No, assembly can be performed at temperature.

Q: How long can I leave the kit exposed to light?
A: Avoid prolonged exposure of the Assay Mix to light.

Q: How long can I leave the assembled reactions before running the plate?
A: The assembled reactions are stable up to 24 hours at room temperature.

Q: Why do I need a pre-reaction read?
A: This reduces well-to-well variability and is similar to setting a “baseline” fluorescence for each well in the first few PCR cycles of a qPCR.

Q: What should my first set of experiments be?
A: Our recommended testing method:

- Program the instrument using 10-cycle settings in the protocol.
- Run an old plate to ensure file quality.
- Run only the Standards, to ensure you are familiar with the assay, the instrument is running correctly and you are able to export the correct information.
- Run your own libraries with the Standards again. Run 8 or 16 libraries to familiarize yourself with the kit. Decrease the number of cycles as needed.

Q: My instrument indicates the read step must be more than 10 seconds, what should I do?
A: 10 seconds is not a mandatory time for the read step and can be increased. Some instruments require a minimum read step time of 19 seconds.

Q: Are there templates for my instrument?
A: Yes, please contact customer service.

Q: What type of replicates should I run?
A: Run technical triplicates. There is no need to run biological replicates.

Q: What methods can I use to remove well-to-well variability?
A: Preform a pre-reaction read and remove this value from the post-reaction read. Also, normalize the FAM values to ROX (if available), which is mixed in the Assay Mix.

Kit Feedback

Q: Who is currently running this assay?

A There are many large companies and genome centers. 2 of the biggest genome centers in the US are testing/using and the largest in the UK. Companies which use NGS as a readout (for cancer screening for example) also currently use the kit.

Q: What are their feedbacks if anyone is running it?

A: They love the simple workflow, it allows them to cut out the day that they usually reserve for quantitation allowing them to get the NGS machine loaded on the 2nd day instead of the 3rd.

Kit Information

Q: Will BrightBox work for Ion Torrent libraries?
A: It won't work in the ion torrent libraries. They have different adapters and don't have the P5 and P7.

Q: Do you have any internal data (or from other labs using the kit) on BrightBox's compatibility with Illumina DNA prep kit (formerly Nextera DNA Flex)? I see that Nextera XT is listed in the brochure.
A: Nextera flex has been validated by a user but no no data is available

Q: Currently NGS assays are not validated in my lab. I see that "Number of Reads" was assessed, comparing BrightBox with qPCR. Is there another assay, other than NGS, that may be used to validate BrightBox?
A: People usually compare it to their current method like qPCR. There is an example of this in the brochure at the bottom left of the 2nd page (BrightBox vs qPCR). However, comparing to read numbers is best because other methods can introduce bias that could decrease the correlation to correct values which are obtained using BrightBox

Q: Is the expiration date of your products 1 year from the date of manufacture?
A: Yes, 1 year from date of manufacture.

Q: Would you please let me know how the solution was measured 12 times in the brochure?
A: The solution from 1 kit was measured 12 times and measured by 1 qPCR system.

Q: What are the size of DNA fragments used in the BB standards?
A: Currently the BB standard is 150 bases.

Q: Finally, could you let us know the approximate length of DNA molecules in the standards of the kit
A: The standard has a 50 bp insert. The standard is single stranded, so will perform at half the fluorescence if used in qPCR

Q: Your FAQ document says that the same amount of fluorescence is created per library molecule (irrespective of fragment size). Is there any limitation regarding the fragment size of the library prep? What is it?
A: Testing has been done internally for libraries with an insert of up to 500bp. Users have run libraries up to 700bp.

Kit information continued

Q: The BrighBox Assay detects only the adapter region and should be independent of the length of the library, but is there any back data that shows no variation in quantitative results when, for example, the concentration is the same but the length of the library is different?
A: There is no data comparing libraries with different sizes, however, the figure in the brochure that shows the standards, is using a standard that has a 485bp insert. The current standards have an insert of 50 bp.

Q: Can I quantitate the BBA standards with other methods?
A: Do not run the BBA standards with qPCR. This will result in incorrect value for the standards.

Q: What type of accuracy and reproducibility is expected?
A: Most users see a 7-10% variability in read numbers for equal molar pooled libraries. This is equal to or better than what is seen with qPCR with fragment analysis, and much better than found with other methods.

Q: Can more wavelengths been taken during the capture steps?
A: It is fine if more fluorescent channels are captured. Only the FAM and ROX channels will be used during analysis.

Kit Format

Q: Are other reagents or consumables required that are not included in the kit?
A: The only other reagent required is water. Any type works.

Q: Is there a control included? If not, is there a product that is recommended to use with BrightBox as a control? E.g. we use STD0 for KAPA.
A: Yes the kit. Contains 6 controls. One negative control, 2, 5, 10, 20 and 30nM

Q: For 100-reaction size kit, is that referring to the amount of Assay Mix provided? For each of the standards, how many reactions worth are included? I.e., how many sets of standards can be prepared?
A: The 100 reaction kit has enough for 33 libraries or controls if they are run in triplicate as recommended. It also contains enough standard for 20 runs (20uL) in the 100 reaction kit, and 50 runs (50uL) in the 500 reaction kit.

Q: For 100-reaction size kit, is that referring to the amount of master mix provided?
A: The 100 reaction kit has enough Assay Mix for 33 libraries or controls if they are run in triplicate as recommended.

Kit Mechanism

Q: I would like to understand better how your chemistry works. Your FAQ document states that the kit uses a variety of enzymes and oligonucleotides to create a fluorescent signal and that there is no amplification of the library molecules. What additional details can you provide?

A: Two of the oligonucleotides bind to the P5 and P7 sequences, and the enzymes ensures that these occur on the same molecule.

- During each "round" the same amount of fluorescent dye (regardless of insert size) is released by the action of the enzymes.
- Since the reaction is linear, each "cycle" runs the linear reaction again, ie 10 cycles doubles the fluorescence (excluding background signal) from 5 cycles.
- Unfortunately, the mechanism is proprietary, and we cannot disclose more at this time.

Q: There is 5-10 temperature cycles in BB protocol and you claim that there is no amplification. Why is there these temperature cycles?

A: The BrightBox Assay is a linear reaction and this "cycling" allows the linear reaction to run multiple times.

Q: Does the BrightBox assay detect adapter dimers?

A: The BrightBox Assay detects any fragment with an intact P5 and P7, and unfortunately, an adapter dimer contains both of these. qPCR also detects adapter dimers, and due to the preference for amplification of short fragments (which BrightBox does not), qPCR greatly impacted by their presence. The most accurate way of determining the amount of adapter dimer is to run a fragment analysis, and if there are unacceptable levels of adapter dimer, then to repurify the intact library.

- We are always looking to improve our products at Calibre scientific and would like to know if detecting adapter dimers is something that you would find useful? Would you like to be able to quantitate the amount of adapter dimer and library at the same time, or just remove its participation in the library quantitation?

Q: Is there a particular tendency for BrightBox Assay to be lower in concentration than the qPCR method in libraries without adapter dimers, for example?

A: Yes, because The BrightBox assay will not pick up PCR dimers. However, The BrightBox assay will detect adapter dimers the same as libraries with different insert sizes. The adapter dimers are essentially a library with a 0 bp insert. When talking to users, most ask about adapter dimers, but their library methods are optimized to remove most of these, and in reality, most are fine with not being able to differentiate adapter dimers from full libraries.

Competition

Q: The Kapa kit and other qPCR quant kits give also results from a std curve in pM range. Why do you claim that BB provides molarity information that Kapa do not?

A: The Kapa kit provides Cts. The Ct values of the values of the libraries are compared to Ct values from the standards. However, because the qPCR reaction uses an intercalating dye the Ct values need to be corrected for library size in comparison to the standard. This is done in the excel sheet that is provided and is why you need to know the "average" library size to utilize qPCR quantitation. The standard for Kapa is 452 base pairs, 387 bp for Thermo, 399 bp for NEB etc.

- To correct for library size these companies ask you to do a calculation such as:
  - Adjust library concentration for size using: Adjusted Conc. = Calculated Conc. × 399 / library size (bp)

Q: Is there any data that shows the comparison to competitor products?

A: At the bottom of left of the brochure, you will find a comparison of BrightBox vs qPCR.

Q: The Kapa protocol says that "depending on the library's expected concentration, 1:1,000 – 1:100,000 dilutions may be appropriate. At least one additional 10-fold dilution of each library is recommended". Kapa also recommends two separate dilutions per library (1:100, 1:1.000, 1:10.000, 1:100.000). How does this relate to the number of qPCR reactions I need to run?

A: The qPCR protocols suggest a separate dilution in order to get good accuracy. If you follow the protocol and run qPCR on "1:1,000; 1:10,000; 1:100,000 dilutions may be appropriate. At least one additional 2-fold dilution of each library is recommended" then it could even more than 12 qPCR reactions (each dilution requires triplicate qPCR).

Q: Also, when a user needs 12 rxns per library from KAPA does that take into consideration that they're in triplicate as well?

A: KAPA recommends doing 2 dilutions per library which means 3 qPCR for each 1:10,000 dilution, 3 qPCR for each 1:100,000 dilution (12 qPCR reactions). However, this method is customized per user, some (only run triplicate for 1:10,000 or 1:100,000 dilutions (3 qPCR reactions per library), some run triplicates for 1:1,000 dilution 1:10,000 and 1:100,000 dilutions (9 qPCR reactions per library). Some users run triplicate for 1:1,000 1:10,000 1:100,000 1:1M (12 qPCR reactions per library).

Q: What is the comparison to fragment analysis only?

A: Mass quantitated by the BioAnalyzer is VERY inaccurate for mass resulting in an inacturate estimation of concentration.

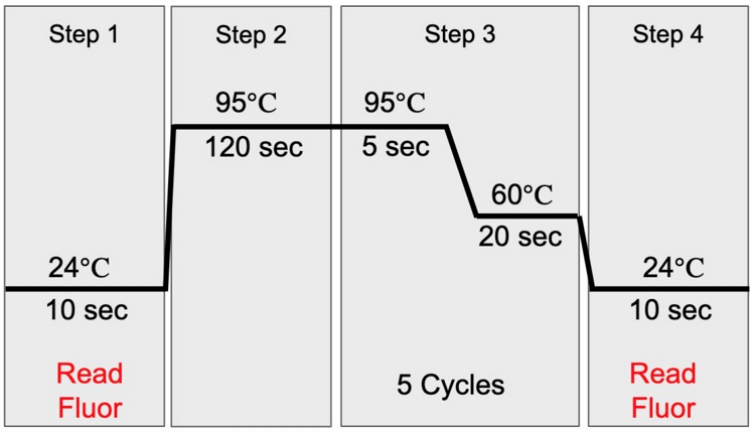
Running the Kit

Q: Will the Applied Biosystems QuantStudio 1 and Applied BioSystems 7500 work as a detection system?
A: Yes it will. The QuantStudio 1 is actually a new instrument, much like the one we use (QuantStudio 3 and 5) at Calibre Scientific

Q: Can Applied Biosystems 7900 and 7900HT be used?
A: Yes

Q: Is an instrument needed for the readout? or is this just a consumable read by (say) fluorimetry?
A: Most people run the assay on a qPCR instrument because it does the temperature incubation and fluorescent reading. You can use a plate reader with a thermocycler (to do the incubation) though.

Q: In your protocol, the workflow on page 4 shows temperature cycling, does this include the 24 degree steps?
A: The figure on page 4 is supposed "summarize" the temperature incubations/cycling etc. The first and last steps are actually just a read, so can be reduced to 1 second.



Q: The protocol states that the library below the 1nM is quantitated in 10 cycles, but it does not mention how much sample should be used. Do you have a correspondence table between the concentration and the required volume of the sample?
A: The limit of the assay is using 1uL of 2nM library (1 x 2). If you have a library that is less than 2nM, then you will need to add more library. As an example:

- 2uL of 1nM
- 3uL of 0.7nM
- 4uL of 0.5nM
- 5uL of 0.4nM

- The worksheet actually corrects for the volume added, which is why you need to add the "volume" of library added to each reaction to the cells.

Q: A Ramp Rate of 0.11 °C/s was set, however, a Ramp Rate of 0.06 °C/s in the video uploaded to Youtube. Is there any problem with the measurements at set Ramp Rate 0.11 °C/s?
A: A faster ramp rate of 0.11 °C/s is fine.

Q: We measured each library with n=3. If there is an outlier among three measurements, is it acceptable to delete it from a cell on the spreadsheet and calculate with n=2.
A: Definitely, this is why it is good to run in triplicate

Running the Kit continued

Q: What would be a good way to test out the kit?
A: Our recommendation for testing would be:
- o Program the instrument using the settings in the protocol - some instruments have a minimum of a 19 second step, so the read steps (which are normally 10 seconds) can be extended to 19 or reduced without a problem.
- o Run with an old plate to make sure the file is good. Some instruments have issues with only one read.
- o For your first run we would recommend just using the standards (1uL of each) in order to make sure that you are familiar with the assay, that the instrument is running correctly and that you are able to export the correct information.
- o Lastly, run your own libraries with the standards (1uL of each), again, we would recommend just running 8 or 16 libraries to familiarize yourself with using a multichannel pipette before going to a high number of libraries. The reagents are very stable at room temperature (more than 1 week) so do all of the assembly at room temperature (no need for ice). The assembled reactions are also very stable (more than 24 hours) so don't worry too much about this.
    - There are also links to YouTube videos for qPCR instrument set up and data analysis in the manual.

Q: FAQ recommends 10 cycles in the first experiment and then considering reducing the number of cycles. But IFU says to program qPCR instruments for 5 cycles. Should users always use 10 cycles at the first time?
A: Some instruments are not as sensitive as others. For this reason, we recommend starting with 10, then decrease as they see fit. 10 cycles is 10 minutes so most users don't mind doing this and decrease when they are comfortable with the kit.

Q: Why in some place does it say to use 10 cycles?
A: We recommend starting with 10 cycles (it is a linear reaction, not amplification) because we are not sure how sensitive your instrument is. We would start with 10, and move down to 5 as you become familiar with the assay and performance on your machine. This will only change it from 5 minutes to 10 minutes of incubation.

Q: The FAQ recommends 10 cycles in Step 2 for the initial assay, but does setting more than 10 cycles have a negative effect on the data? Was it decided from any support data that setting more than 10 cycles is not effective?
A: During development we found that using more than 10 cycles usually does not improve data quality. However, if users are optimizing the protocol, and usually have low concentration libraries, they could try 15 cycles .

Q: What volume of library should I try first?
A: We recommend using 1uL of standard and up to 5uL of library and running the reactions in triplicate. Try 1uL of library first to make sure you are in the 2-30nM linear range

Q: Most users first assay with 1ul and evaluate the results, however, it appears that low concentration samples can give variations.
A: We normally recommend only using 1uL, if values are less than 2nM values then we recommend trying more volume

Q: Is there any other way to increase the lower detection limit except to increase the sample volume to 2 or 3ul?
A: Unfortunately not. The Assay has a lower limit of approximately 2nM (1uL)

Q: Will the amount of library and the amount of fluorescence not be proportional under 5 cycles? Could you let us know what kind of knowledge was used to determine 5 cycles is sufficient?
A: Yes, in general it is proportional, but I would not expect it on all instruments since background is usually additive (10+3 vs 5+3). The sensitivity at different cycle numbers was determined using standard curve at each condition, and looking to see where linearity was not maintained.

Running the Kit continued

Q: After I perform pooling, what is the concentration of the libraries? Is it possible to use BrightBox Assay in this circumstance?
A: The BrightBox Assay can be run on pooled libraries if the final concentration is above 2nM. For example, if each library is 2nM, and 100 are pooled, then each has a concentration of 20pM, however, the overall library concentration remains at 2nM which can be quantified.

Data Analysis

Q: What is the "pre-read" and "post-read"?
A: The important parts are taking a "pre-reaction" read and a "post-reaction" read. By removing the pre-reaction from the post reaction read it will normalize the well-to-well variation. This is similar to what is done during qPCR where the "baseline" is set in the first few cycles for each well. If you are using a Qubit or plate reader you will only be able to take a "post-reaction" read. It is fine to only take a "post- reaction" read using a qPCR instrument as well.

Q: What does ROX do?
A: ROX will also normalize volumes within each well, so can be useful in reducing well-to-well variability.

Q: Why are some of my low concentration values negative?
A: These values are still accurate. Due to the method that fluorescence is generated in the assay and the specific instrument used, occasionally the fluorescence in the pre-reaction read can be higher than the post-reaction read which results in a negative value. This is fine since it is just a subtraction and all reactions will have the same amount removed (relative to volume of each reaction). This is why the plotted data is linear even though some of the values are negative.

Q: Values for libraries are below the 2nM, can I use these?
A: Libraries that have values below the 2nM value should not be used. Try using 2 or 3ul of this library to get an accurate quantitation.

Q: What is the reason that the measurement value of Std 1 (0nM) is eliminated from the calibration curve calculation?
A: Standard 1 is removed from the Standard Curve because its value represents the background with no template in it.

- In general, No template control values are not used for a standard curve. The NTC provides a value for "background" which is why values below this should not be used.
- https://en.wikipedia.org/wiki/Standard_curve

Q: Is there any reason that BrightBox assay does not use compensated ROX/FAM value but uses raw signals? Normally, compensated signals are used for quantification.
A: The protocol does analyze FAM/ROX signal. ROX normalizes for the volume of Assay Mix that is put into each reaction (10.2uL vs 9.5uL), but using raw fluorescence is also acceptable for the BrightBox Assay.

Q: What should I use the graph for in the data analysis spreadsheet?
A: The graph is only for display purposes, and the concentration calculation does not use the graph

Analysis

Q: One of my technical replicates is far off from the other 2. What should I do?
A: Remove the odd one out, this can be caused by wells with a higher background fluorescence.

Q: The 2nM are very low and close to background, what should I do?
A: This can happen for less sensitive instruments. Increase the number of cycles to linearly increase the amount of fluorescence.

Q: How do I perform data analysis?
A: Use the attached spreadsheet and watch the appropriate YouTube videos. A set of videos has been created to guide you through instrument programming and data analysis using a variety of qPCR instruments:

- QuantStudio 3, 5, 6Pro and 7Pro: programming and data analysis:
  https://www.youtube.com/watch?v=pfOfLzf4slc&t=4s
- LC 480: programming and data analysis:
  https://www.youtube.com/watch?v=OinPdue_eyc&t=7s
- StepOnePlus: programming and data analysis:
  https://www.youtube.com/watch?v=hSMqTC-8L_g&t=24s
- StepOnePlus: data analysis using a customized worksheet
  https://www.youtube.com/watch?v=HtpLO-sK-jw
- Agilent AriaMX: programming and data analysis:
  https://www.youtube.com/watch?v=nLro9kQchtI&t=4s
- Bio-Rad CFX: programming and data analysis:
  https://www.youtube.com/watch?v=ryT8JhnwAv0
- QuantStudio 6 and 7 Flex: programming and data analysis:
  https://www.youtube.com/watch?v=aCxRJhrQQHU
- Data analysis only:
  https://www.youtube.com/watch?v=Qd0ylCU_dcw&t=12s

Q: Why are some of my low-concentration values negative?
A: Occasionally the fluorescence in the pre-reaction read can be higher than the post-reaction read, which results in a negative value. This results in a subtraction and all reactions will have the same amount removed (relative to volume of each reaction). This means the plotted data will remain linear even if some values are negative.