

BrightBox Quantitation Assay Kit Manual



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In the Box

Components may be shipped frozen. Upon receipt, store all tubes at 2-8°C.

BrightBox Assay Mix & Controls (100 rxns) – SKU # 50100

Tube	Concentration	Volume	Tube Label
Assay Master Mix	6X	170µL	Assay Master Mix
Standard 1	0nM Control Library	20µL	Std 1
Standard 2	2nM Control Library	20µL	Std 2
Standard 3	5nM Control Library	20µL	Std 3
Standard 4	10nM Control Library	20µL	Std 4
Standard 5	20nM Control Library	20µL	Std 5
Standard 6	30nM Control Library	20µL	Std 6

BrightBox Assay Mix & Controls (500 rxns) – SKU # 50500

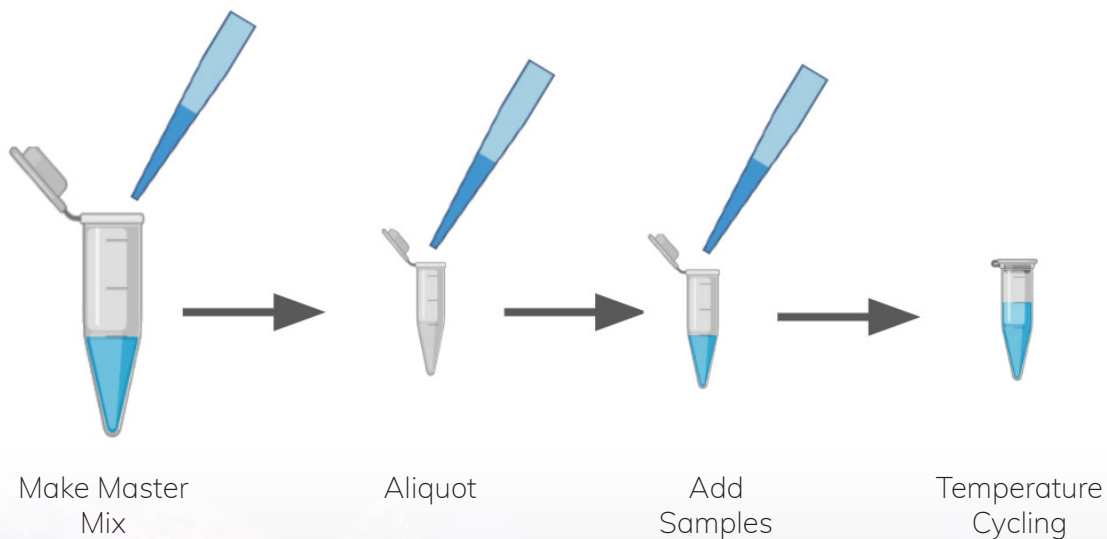
Tube	Concentration	Volume	Tube Label
Assay Master Mix	6X	850µL	Assay Master Mix
Standard 1	0nM Control Library	50µL	Std 1
Standard 2	2nM Control Library	50µL	Std 2
Standard 3	5nM Control Library	50µL	Std 3
Standard 4	10nM Control Library	50µL	Std 4
Standard 5	20nM Control Library	50µL	Std 5
Standard 6	30nM Control Library	50µL	Std 6

For research use only. Not for use in diagnostic procedures.

Notes

- Components may be shipped frozen. Upon receipt, store all tubes at 2-8°.
- Temperature cycling produces a linear amplification of signal; however, library molecules are NOT amplified.
- To increase sensitivity, the number of cycles may be increased. Libraries less than 1nM can be accurately quantitated with 10 cycles.
- On Real Time PCR machines a “Pre-Reaction” fluorescent reading is taken for each well and this value is removed from subsequent readings to remove well-to-well variability. This process is identical to obtaining fluorescent values during the first few cycles of qPCR, which are removed during subsequent readings.

Workflow



Reaction Assembly

- The Assay Master Mix and Standards can be kept at room temperature while reactions are assembled.
- DO NOT VORTEX the Assay Mix.
- DO NOT leave Assay Mix exposed to light for extended periods of time.
- Ensure Standards are vortexed before use.
- Up to 5µL of low TE and 0.1% Tween per 10µL reaction has been tested with no inhibitory effect.
- Triplicate reactions are recommended for each sample or standard.

1. Sample Reactions

Component	Stock Concentration	Volume
Assay Master Mix	6X	1.7µL
Sample		1 - 5µL
dH ₂ O		Volume to make up
Final reaction		10uL

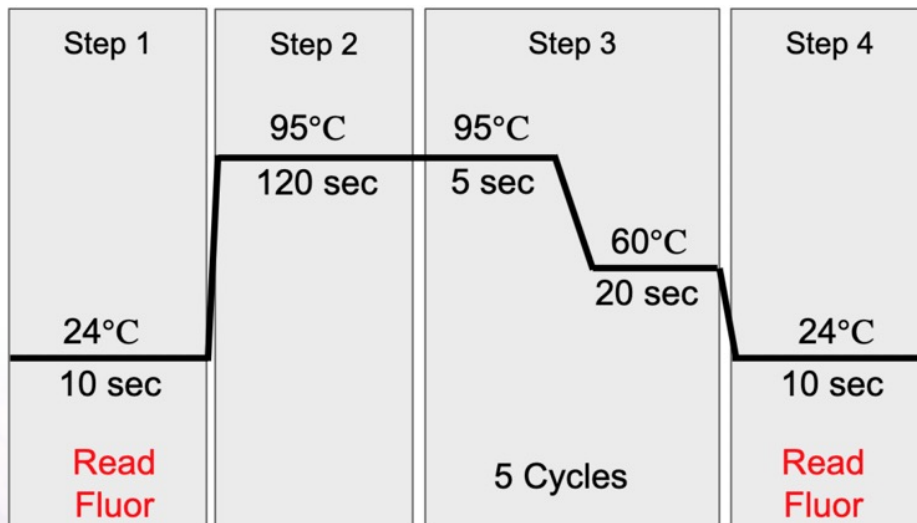
2. Control Reactions

Component	Stock Concentration	Volume
Master Mix	6X	1.7µL
Standard 1, 2, 3, 4, 5, or 6	0nM, 2nM, 5nM, 10nM, 20nM, or 30nM	1µL
dH ₂ O		Volume to make up
Final reaction		10µL

qPCR Instrument Protocol

1. Reactions - Generic Method

- A. Program wells on the qPCR machine.
- i. Select wells with samples and label.
 - ii. Select wells with controls and label.
 - iii. Set machine to read in the "FAM" channel.
 - iv. Set machine to read in the "ROX" channel (if possible).
 - v. Select any quencher in the programming.
 - vi. Program thermal profile:
 1. 24°C for 10 seconds
 - vii. Include one "read fluorescence" in step 1
 - viii. 95°C for 120 seconds
 - ix.. Cycle 5 times:
 1. 95°C for 5 seconds
 2. 60°C for 20 seconds
 - x. 24°C for 10 seconds
 - xi. Include one "read fluorescence" in step 4, after incubating at 24°C

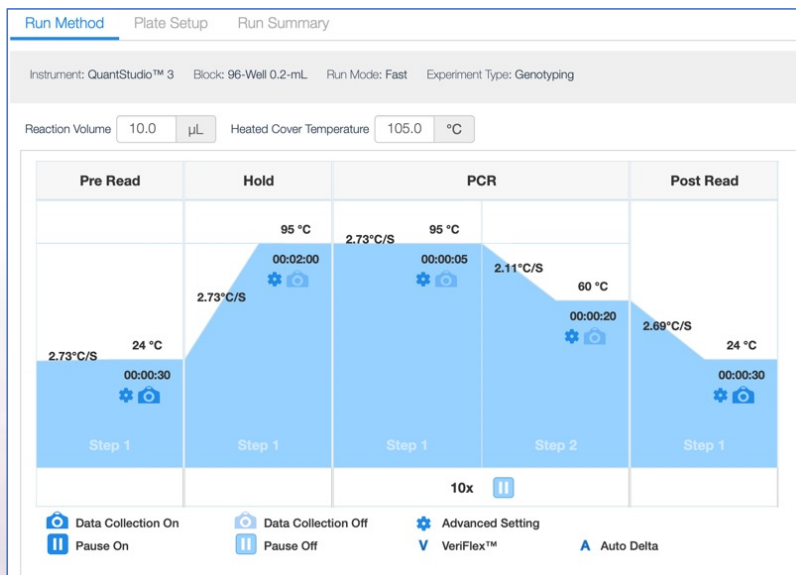


- B. Set the instrument to the fastest ramp rates.
C. Aliquot Assay Mix (including water) into an appropriate plate for your instrument.
D. Vortex Standards before use.
E. Add samples and Standards and gently mix.
F. Spin down as you would when routing qPCR.
G. Place plate into machine and start run.

qPCR Instrument Protocol

2. Reactions - QuantStudio Specific

- A. Program wells on the qPCR machine.
 - i. Select the app “Design and Analysis New”. Also known as DA2
 - ii. Select “Setup Plate”
 - iii. Select you Instrument, Block type, Fast Mode (if present), and “Genotyping”
 - iv. Select the App “Genotyping_Pre_PCR_Post”
- B. Program the App
 1. In the “Run Method” tab:
 - a. Set the reaction volume to 10uL
 - b. Change the temperature on the “PreRead” to 24 degrees (from 60)
 - c. Change the initial pre-reaction at 95 to 2 minutes (from 20 seconds)
 - d. Change the cycle numbers to 5 or 10 (from 40)
 - e. During the PCR, change the time at 95 degrees to 5 seconds (from 1 second if using Fast cycling)
 - f. Change the “Post Read” temperature to 24 degrees (from 60)
 - g. Deselect the “Camera” Icon from the PCR. An error window will pop up explaining that no amplification data will not be produced, select “OK”
 - h. You may get an error message (shown by a Red Exclamation mark at the top left next to “Run Method”, or an error box) asking you to change ramp rates. Change these to the fastest possible rate that is offered.
 - i. **The protocol should look like this (ramp rates may vary):**



2. In the “Plate Setup” tab
 - a. In the drop down menu for “Passive Reference” check that the preselected “ROX” is still there
 - b. select the wells you wish to use
 - c. In the “Sample” box select the “+” sign and select the radial button for “Sample 1”
 - d. In the “SNP Assay” box select the “+” sign and select the radial button for “SNP Assay 1”. (if you toggle to the right this should have “FAM” as “Allele Reporter 2”)
3. In the upper left use the “Actions” pull down menu:
 - a. Select “Save As” and give the app a name such as “BrightBox Reactions”
 - b. Select the folder you would like to use
 - c. And click “Save”
 - d. You will be able to use this template file for all further runs of the BrightBox Assay

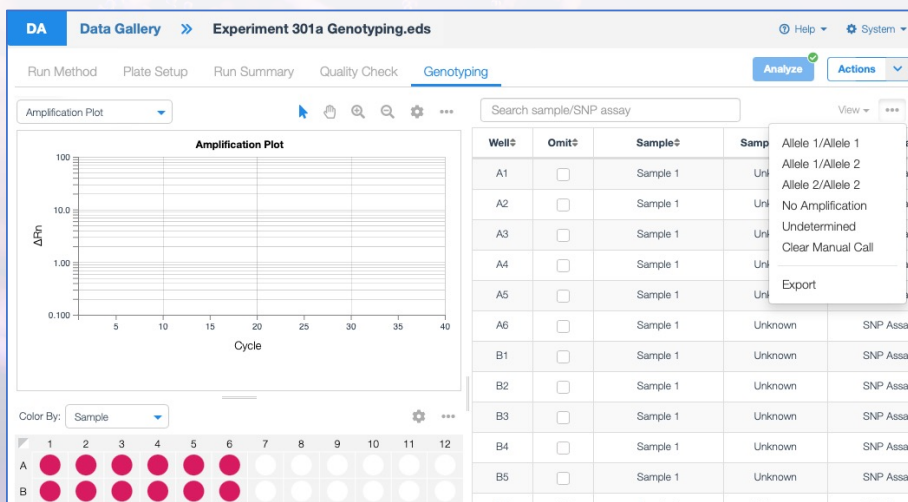
Analysis

1. Generic Data Analysis

- Export the raw fluorescence values from the instrument.
- Open the data into a spreadsheet.
- Select the data and sort the data based on “cycle number” then by well number.
- Copy the FAM and ROX values into Cells D18 and E18 and down in the provided Data Analysis Spreadsheet, with the standards at the top
- The FAM/ROX values (F18 and down) will be calculated
- If your instrument only provides FAM data, then copy these into the FAM/ROX column (F18 and down)
- In cells G18 and down perform background correction by removing the “Pre-reaction” fluorescent values from the “Post-reaction” values (Cycle number 2-1)
- Cells J18 and down will self-populate
- If triplicate values are far off from each other then remove outliers in column J
- Make note of the OnM control value (cell K18), which could be less than 0, other samples and Standards should be greater than this value
- The standard curve and trendline will self-populate.
- The R2 (cell K14) should be greater than 0.95
- Add the volumes of the Standards (K15) and your Samples (L36 and down) that you added to each reaction
- The concentration of your original library will be calculated and is in M36 and down

2. QuantStudio Specific Analysis

- Open the .eds file that is generated after your run
- The file will open on the “Quality Check” tab at the top, and you will not see anything in the “Amplification Plot”
- Go to the “Genotyping” tab and export the data table on the right.
 - Select the three dots at the right top of the table
 - Select “Export” from the pull-down menu.
 - It should look like this:



Analysis

- d. Open the downloaded file which will have a name something like “gt_reaction_results.csv”
- e. Column A has the well numbers
- f. Column J has the results for each well
- g. The sheet will look like this:

	A	B	C	D	E	F	G	H	I	J	K	L
1	Well	Omit	Sample	Sample Type	SNP Assay	Assay ID	Task	Confidence	Allele 1	Allele 2	Call	Manual Call
2	A1	FALSE	Sample 1	UNKNOWN	SNP Assay 1		UNKNOWN	1	-0.0091513	-0.0372669	NO_AMPLIFI	FALSE
3	A2	FALSE	Sample 1	UNKNOWN	SNP Assay 1		UNKNOWN	1	-0.0012698	-0.0738005	NO_AMPLIFI	FALSE
4	A3	FALSE	Sample 1	UNKNOWN	SNP Assay 1		UNKNOWN	1	-0.0029891	-0.049665	NO_AMPLIFI	FALSE
5	A4	FALSE	Sample 1	UNKNOWN	SNP Assay 1		UNKNOWN	1	-0.0027475	-0.1108108	NO_AMPLIFI	FALSE
6	A5	FALSE	Sample 1	UNKNOWN	SNP Assay 1		UNKNOWN	1	-0.0065368	-0.102129	NO_AMPLIFI	FALSE
7	A6	FALSE	Sample 1	UNKNOWN	SNP Assay 1		UNKNOWN	1	-0.0081719	-0.1063625	NO_AMPLIFI	FALSE
8	B1	FALSE	Sample 1	UNKNOWN	SNP Assay 1		UNKNOWN	1	0.00160329	-0.027375	NO_AMPLIFI	FALSE
9	B2	FALSE	Sample 1	UNKNOWN	SNP Assay 1		UNKNOWN	1	-0.0137838	-0.0472623	NO_AMPLIFI	FALSE
10	B3	FALSE	Sample 1	UNKNOWN	SNP Assay 1		UNKNOWN	1	-0.011738	-0.0732707	NO_AMPLIFI	FALSE
11	B4	FALSE	Sample 1	UNKNOWN	SNP Assay 1		UNKNOWN	1	-0.0129362	-0.0683052	NO_AMPLIFI	FALSE
12	B5	FALSE	Sample 1	UNKNOWN	SNP Assay 1		UNKNOWN	1	-0.0102067	-0.0595202	NO_AMPLIFI	FALSE
13	B6	FALSE	Sample 1	UNKNOWN	SNP Assay 1		UNKNOWN	1	-0.0142202	-0.0434701	NO_AMPLIFI	FALSE
14	C1	FALSE	Sample 1	UNKNOWN	SNP Assay 1		UNKNOWN	1	0.0012142	-0.0296173	NO_AMPLIFI	FALSE
15	C2	FALSE	Sample 1	UNKNOWN	SNP Assay 1		UNKNOWN	1	-0.0005785	-0.0678436	NO_AMPLIFI	FALSE
16	C3	FALSE	Sample 1	UNKNOWN	SNP Assay 1		UNKNOWN	1	-0.0066448	-0.0166729	NO_AMPLIFI	FALSE
17	C4	FALSE	Sample 1	UNKNOWN	SNP Assay 1		UNKNOWN	1	0.00650387	0.00563812	NO_AMPLIFI	FALSE
18	C5	FALSE	Sample 1	UNKNOWN	SNP Assay 1		UNKNOWN	1	-0.0500611	-0.0459448	NO_AMPLIFI	FALSE

- h. Put the standards at the top, and copy cells J2 to J289 (or all samples) into the provided Analysis Spreadsheet in cells G18 to G305
- i. Cells J18 and down will self-populate
- j. If triplicate values are far off from each other then remove outliers in column J
- k. Make note of the 0nM control value (cell K18), which could be less than 0, other samples and Standards should be greater than this value
- l. The standard curve and trendline will self-populate.
- m. The R2 (cell K14) should be greater than 0.95
- n. Add the volumes of the Standards (K15) and your Samples (L36 and down) that you added to each reaction
- o. The concentration of your original library will be calculated and is in M36 and down

Analysis continued

A set of videos has been created to guide you through instrument programming and data analysis using a variety of different 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 (contact customerservice@edgebio.com to request):
<https://www.youtube.com/watch?v=HtpLO-sK-jw>
- Agilent AriaMX: programming and data analysis:
<https://www.youtube.com/watch?v=nLro9kQchtl&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=Qd0yICU_dcw&t=12s

Limited Product Warranty

BrightBox Quantitation and/or its affiliate(s) warrant their products as set forth in the BrightBox Quantitation's General Terms and Conditions of Sale.

For support or questions please contact: customerservice@edgebio.com