A Simplex Real-time PCR Method for Detection of Prohibited Materials in Animal Feed on the Applied Biosystems 7500 Fast System

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Abstract

A SmartCycler based real-time Polymerase Chain Reaction (rt-PCR) method is currently utilized in the FDA Bovine Spongiform Encephalopathy (BSE) program to identify prohibited materials in animal feed and feed ingredients. As the SmartCycler instrument was recently discontinued by its manufacturer Cepheid, a Single Laboratory Validation (SLV) platform extension study has been completed in ORA/ORS/Pacific Northwest Laboratory to evaluate the Applied Biosystems 7500 Fast (AB7500F) system as the replacement. The SLV study results demonstrated that the rt-PCR performance on the AB7500F was equal to or slightly better than that on the SmartCycler. Both the AB7500F software v1.4 and v2.3 worked for this rt-PCR method. The SLV study has been deemed successful by the FDA Food and Veterinary Medicine (FVM) Chemistry Research Coordination Group (CRCG) and the FDA Molecular ID Technical Advisory Group (MITAG) after review. This LIB describes the simplex rt-PCR method on the AB7500F platform and serves as a laboratory protocol.

Key words: platform extension, Bovine Spongiform Encephalopathy (BSE), simplex real-time PCR (rt-PCR), Applied Biosystems 7500 Fast (AB7500F), SmartCycler, Single Laboratory Validation (SLV).

Introduction

Bovine Spongiform Encephalopathy (BSE), or mad cow disease, is the bovine form of Transmissible Spongiform Encephalopathies (TSE) which cause fatal neurodegeneration in affected animals or humans (1). Besides cattle, TSE also affects goats, sheep, and certain other species. The disease is believed to be transmitted via consuming prion infected meat and bone meals (MBM) (2). To prevent the occurrence and spread of TSE in America's food supply, FDA bans ruminant MBM in animal feed and feed ingredients used for food-producing animals in interstate commerce under the regulations 21 CFR 589.2000 and 21 CFR 589.2001. The FDA compliance program 7371.009, "BSE/Ruminant Feed Ban Inspections" was put into effect in 2003 to assess the compliance of animal feed and feed producers to protect public health (3).

Three validated methods are being utilized in the FDA Laboratories: a simplex rt-PCR method published in the Laboratory Information Bulletin (LIB) 4486 (4, 5), a multiplex rt-PCR method in the LIB 4544A (6), and an Animal Feed Microscopy (AFM) method published in AOAC's Official Methods of Analysis (7). The multiplex rt-PCR method detects a homologous region of ruminant genomes, whereas the simplex rt-PCR method targets different genes in the bovine, caprine or ovine genome. ORA/ORS Laboratories often use a combination of the rt-PCR and the AFM as the screening and confirmation analytical methods (8-10). As Cepheid recently discontinued the manufacturing and services of the SmartCycler (11) used in BSE rt-PCR methods, a study was conducted to extend the instrument platform to the Applied Biosystems 7500 Fast (AB7500F) system (12) due to its universal availability across all ORS BSE service Laboratories.

To evaluate the method performance on the AB7500F system, a Single Laboratory Validation (SLV) study was designed following the FDA *Guidelines for the Validation of Analytical Methods for Nucleic Acid Sequence-Based Analysis of Food, Feed, Cosmetics and Veterinary Products* (13). After the design was approved by the FDA Food and Veterinary Medicine (FVM) Chemistry Research Coordination Group (CRCG) and the FDA Molecular Identification Technical Advisory Group (MITAG), this study was performed and completed at ORA/ORS/Pacific Northwest Laboratory. The FDA CRCG and MITAG reviewed the study results and deemed the platform extension successful.

This LIB describes the BSE simplex rt-PCR method on the AB7500F platform for the detection of prohibited materials in animal feed and feed ingredients. For the approved SLV study, please refer to a separate LIB published by our group (14).

Experimental

1. Equipment and supplies
Applied Biosystems 7500 Fast system (AB7500F)
Optical 96-well plates
Adhesive film for 96-well plates
Benchtop plate or strip centrifuge
Vortex mixer
Sterile 1.5-ml micro centrifuge tubes and centrifuge
Micro-pipettes (P2, P20, P200, and P1000)
Filter-barrier aerosol resistant (ART) pipette tips
iQ SYBR® Green SuperMix (Bio-Rad catalog #1708880)
ROX reference dye (Thermo Fisher catalog #12223012)
Bovine, caprine, and ovine primers (see below)
Internal amplification control (IAC) targeting the green florescence protein (GFP) (see below)
Genomic DNA from bovine, caprine, and ovine species (Zyagen catalog #GB-110F, GG-150, and GS-190F) as rt-PCR positive controls per LIB 4486

Molecular biology grade water (MGW)

2. Real-time PCR reactions, primers, and controls

As this is a simplex rt-PCR method, each set of master mix (bovine, caprine, ovine and IAC) was prepared separately. All DNA oligoes are commercially synthesized (Integrated DNA Technologies, Coralville, Iowa). The volume of each rt-PCR reaction is 25 µl. The final primer concentration is 400 nM for all target species and the IAC.

Bovine, Caprine and Ovine Primers:
The FDA's Center for Veterinary Medicine (CVM) originally developed the bovine, caprine and ovine primers without publicly releasing their sequences. ORA/ORS and CVM are negotiating with several companies to produce a commercial kit for this assay, but until a kit becomes commercially available, aliquots of the primers must be obtained directly from FDA. The current point of contact (POC) is Ms. Josefina Freeman from CFSAN-OARSA DFEM (email: Josefina.Freeman@fda.hhs.gov).

Internal Amplification Control (IAC):
The design and use of the GFP IAC is based on previous research (15). The IAC template is a 140 bp gBlock fragment including a 114 bp GFP amplicon and protection bases on both ends: 5'-GTGCTGCTGCCCGACAACCACTACCTGAGCACCCAGTCCGCCCTGAGCAAAGACCCCAACGAGAAGCGCGATCACATGG TCCTGCTGGAGTTCGTGACCGCCGCCGGGATCACTCTCGGCATGGACGAGCTGTACAAGTA-3'

IAC primer sequences:
GFP Forward: 5'-CGACAACCACTACCTGAGC-3'
GFP Reverse: 5'-TCCATGCCGAGAGTGATC-3'

Each IAC reaction uses 1 µl (approximately 0.6 pM) of the IAC template which equates to approximately 3.6×10^5 copies of the GFP fragment.

The IAC Ct values are usually ≤ 28 cycles. As it's a simplex rt-PCR assay, the IAC Ct is dependent on the concentration of IAC template and/or potential PCR inhibitors in feed DNA, and is not affected by the DNA concentration of target species (bovine, caprine or ovine).

Positive and negative controls of the rt-PCR:
Bovine, caprine and ovine gDNA from Zyagen are diluted with MGW for the optimal use in this assay as follows, and 2 µl for each control reaction.
Bovine: 1.0×10^{-3} µg/µl
Caprine: 1.0×10^{-5} µg/µl
Ovine: 1.0×10^{-5} µg/µl

The negative control is MGW, 2 µl for each reaction.

3. Master mix preparation and template addition

Note: All master mixes should be prepared in a designated Biosafety cabinet (BSC). The addition of template DNA should take place in a **separate** designated BSC.

3.1 Calculate the number of reactions for each primer set. Generally this will be the number of samples + 2 (for the positive and negative controls of the rt-PCR).

3.2 Multiply the number of reactions calculated in 3.1 with 20% extra to ensure that there is enough master mix. Please see Supplementary Information 1 for an example of calculation.

3.3   Thaw the reagents on ice, such as iQ SYBR® Green SuperMix, ROX reference dye, primer mixes (bovine, caprine, ovine and IAC), IAC template, and positive controls, etc.

3.4   Use **the designated BSC for master mix preparation**, add 5 µl (500×) thawed stock solution of the ROX reference dye to 1.25 ml thawed (2×) iQ SYBR® Green SuperMix to yield (2×) ROX in (2×) iQ SYBR® Green SuperMix, gently mix the reagents by inverting the tube 10 times, and spin the tube for ~10 seconds.

3.5   Prepare the rt-PCR master mixes following the recipes below. Use a 1.5-ml sterile centrifuge tube for each species (bovine, caprine, ovine and GFP IAC). There should be a total of four tubes of master mixes.

> 3.5.1   Bovine master mix (per reaction):
> | | |
> |---|---:|
> | (2×) iQ SYBR® Green SuperMix with ROX | 12.5 µl |
> | Bovine primer mix (5 µM forward and reverse) | 2.0 µl |
> | Molecular biology grade water (MGW) | 8.5 µl |
> | Total volume per reaction | 23.0 µl |
>
> 3.5.2   Caprine master mix (per reaction):
> | | |
> |---|---:|
> | (2×) iQ SYBR® Green SuperMix with ROX | 12.5 µl |
> | Caprine primer mix (5 µM forward and reverse) | 2.0 µl |
> | Molecular biology grade water (MGW) | 8.5 µl |
> | Total volume per reaction | 23.0 µl |
>
> 3.5.3   Ovine master mix (per reaction):
> | | |
> |---|---:|
> | (2×) iQ SYBR® Green SuperMix with ROX | 12.5 µl |
> | Ovine primer mix (5 µM forward and reverse) | 2.0 µl |
> | Molecular biology grade water (MGW) | 8.5 µl |
> | Total volume per reaction | 23.0 µl |
>
> 3.5.4   GFP IAC master mix (per reaction):
> | | |
> |---|---:|
> | (2×) iQ SYBR® Green SuperMix with ROX | 12.5 µl |
> | GFP primer mix (5 µM forward and reverse) | 2.0 µl |
> | Molecular biology grade water (MGW) | **7.5** µl |
> | Total volume per reaction | **22.0** µl |

Note: The MGW volume and total volume per reaction in the GFP IAC master mix are 7.5 µl and 22.0 µl, respectively (step 3.5.4), which is different from the other three master mixes (steps 3.5.1 – 3.5 3).

Please see Supplementary Information 1 for an example of rt-PCR master mix calculation.

After adding all master mix components to each tube, cap the tube lid, gently mix each tube by inverting at least 10 times, and spin the tube for ~10 seconds.

3.6   Label a 96-well plate with target species, template IDs, controls, etc., and pipet each master mix into the designated reaction wells. An example of a plate layout is shown in Figure 1. In **the designated BSC for master mix preparation**:

    3.6.1   Pipet 23.0 ul bovine, caprine and ovine master mixes into designated wells.

    3.6.2   Add 2.0 µl MGW to the bovine, caprine and ovine negative control wells.

    3.6.3   Pipet **22.0** µl GFP IAC master mix into the GFP negative control well, and add **3.0** µl MGW to the same well to reach a final volume of 25.0 µl.

    3.6.4   Add GFP template to the remaining GFP master mix in the original centrifuge tube (1.0 µl per reaction, for all samples and the GFP positive control, with 20% extra volume). Adding GFP template to the master mix, rather than adding it to individual wells will yield a more even IAC.

    Note: GFP template is not added to the GFP negative control well, so the reaction number for adding GFP template can be simply calculated by subtracting one reaction (the negative control) from the total reaction number calculated in step 3.2. Please see Supplementary Information 1 for an example of the calculation.

    3.6.5   Mix the GFP master mix again by inverting the centrifuge tube 10 times. Spin the tube for ~10 seconds.

    3.6.6   Pipet 23.0 µl GFP IAC master mix into designated sample wells and the GFP positive control well.

    3.6.7   Add 2.0 µl MGW to the GFP positive control well to bring the volume to 25.0 µl. No "GFP positive control" is needed for this well because the GFP template is already added into the IAC master mix (in 3.6.4).

    3.6.8   After pipetting master mixes and MGW, move the plate to **the template addition BSC**.

3.7 In **the template addition BSC**:

    3.7.1   Add 2.0 µl template into each reaction well (e.g., 2.0 µl of samples 1-4 in Figure 1). For each template, there are 4 separate reactions (1 bovine, 1 caprine, 1 ovine and 1 GFP IAC reactions). Store the templates on ice if they will be used again immediately or at -70°C for archival/long-term storage.

    3.7.2   Add 2.0 µl bovine, caprine or ovine positive control gDNA to its respective well on the 96-well plate. The concentrations of gDNA working solutions are as follows:
         Bovine: 1.0×10^{-3} µg/µl
         Caprine: 1.0×10^{-5} µg/µl
         Ovine: 1.0×10^{-5} µg/µl

    3.7.3   Seal the 96-well plate with an adhesive film and take the plate to your lab's designated PCR area (where the AB7500F instrument is located).

3.7.4 Spin the 96-well plate in a plate centrifuge for approximately 30 seconds prior to loading it on the AB7500F instrument.

Figure 1. An example of a 96-well reaction plate. Abbreviations: B = Bovine, C = Caprine, O = Ovine, G = GFP IAC, MM = master mix, neg ctl = negative control, pos ctl = positive control, w/o = without, w = with. Reactions using different primer sets are labelled in different colors. Volumes of master mix and template are included in each reaction. <u>Note</u>: the GFP negative control well contains 22 µl master mix and 3 µl water, which is different from other wells.

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B	B neg ctl well 23 µl B MM 2 µl H$_2$O		Sample1 23 µl B MM 2 µl template		Sample2 23 µl B MM 2 µl template		Sample3 23 µl B MM 2 µl template		Sample4 23 µl B MM 2 µl template			B pos ctl well 23 µl B MM 2 µl B gDNA
C												
D	C neg ctl well 23 µl C MM 2 µl H$_2$O		Sample1 23 µl C MM 2 µl template		Sample2 23 µl C MM 2 µl template		Sample3 23 µl C MM 2 µl template		Sample4 23 µl C MM 2 µl template			C pos ctl well 23 µl C MM 2 µl C gDNA
E												
F	O neg ctl well 23 µl O MM 2 µl H$_2$O		Sample1 23 µl O MM 2 µl template		Sample2 23 µl O MM 2 µl template		Sample3 23 µl O MM 2 µl template		Sample4 23 µl O MM 2 µl template			O pos ctl well 23 µl O MM 2 µl O gDNA
G												
H	G neg ctl well 22 µl G MM (w/o G template) 3 µl H$_2$O		Sample1 IAC 23 µl G MM (w G template) 2 µl template		Sample2 IAC 23 µl G MM (w G template) 2 µl template		Sample3 IAC 23 µl G MM (w G template) 2 µl template		Sample4 IAC 23 µl G MM (w G template) 2 µl template			G pos ctl well 23 µl G MM (w G template) 2 µl H$_2$O

4. Experiment setup on the AB7500F system

4.1 Turn on the AB7500F instrument and open the AB software.

4.2 Load the 96-well reaction plate on the AB instrument.

4.3 In the AB software, fill in the necessary fields for your experiment, select "sybrGreen" as the detector and "ROX" as the reference dye, then fill in the sample IDs as needed.

4.4 Set up the rt-PCR cycling conditions per Table 1. The conditions are the same for experiments with the AB7500F software v1.4 and v2.3.

4.5 Start the rt-PCR run and select the folder for saving data. The actual run time is approximately 1.8 hours on the AB7500F instrument.

<u>Note</u>: Two versions of the AB7500F software (v1.4 and v2.3) are validated in the platform extension study (14). Steps 4.1 - 4.5 are brief descriptions, and there are some differences in the software interface between the two versions. Please see Supplementary Information 2 and 3 for step-by-step instructions on how to use the two versions of AB software to set up rt-PCR experiments.

Table 1. The rt-PCR cycling conditions on the AB7500F platform.

Stage	Repetitions	Temperature	Time (min:sec)	Ramp Rate
1	1	94.0 °C	2:00	Auto
2	50	94.0 °C	0:10	Auto
		58.9 °C	0:15	Auto
		72.0 °C	0:40	Auto
3 (the **default** dissociation program)	1	95.0 °C	0:15	Auto
		60.0 °C	1:00	Auto
		95.0 °C	0:15	Auto
		60.0 °C	0:15	Auto
Mode: 7500 Fast **Data Collection:** Stage 2 Step 3 (72.0°C 40sec)				

5. Data processing and exporting

5.1   Turn off the instrument after the run is successfully completed.

5.2   In the "Analysis" pull-down menu, open "analysis settings" and select the **automatic** mode for the determination of threshold and baseline.

5.3   Click the "analyze" button to process data.

5.4   Save the processed data and export the results from the AB7500F computer to an IronKey or other laboratory computers as needed.

Note: Please see Supplementary Information 2 and 3 for detailed instructions on data processing and exporting with the AB7500F software v1.4 and v2.3, respectively.

6. Result reporting

Figure 2 summarizes the experimental procedures and key parameters in result reporting. For boxes 6.1 - 6.5 in the flowchart, the corresponding text descriptions are also included below on how to report results based on Ct, Tm, and melt curves. Additionally, some examples of acceptable/unacceptable melt curves are included in Supplementary Information 4 to help users interpret data using the melt curve analysis.

If target (B, C or O) Ct=0, re-extract DNA or dilute DNA for rt-PCR repeat.

If target Ct>0, follow steps 6.3-6.4 in the flowchart:
6.3) If target Tm outside range, **Result-=neg**;
6.4) If target Tm inside range & melt curve acceptable, **Result=pos** (despite PCR inhibition)

DNA extraction for rt-PCR.

6.1 Negative & positive controls correct?

No → rt-PCR failure. Repeat the rt-PCR with new reagents/supplies.

Yes

Significant PCR inhibition.

No ← **6.2** Sample IAC Tm within 86.4-88.5 °C & δCt≤3.3?

Yes

6.3 Target Tm outside range:
B: 81.8-83.3 °C;
C: 82.6-84.5 °C;
O: 78.5-80.3 °C.

6.4 Target Tm inside range & Ct>0:
B: 81.8-83.3 °C;
C: 82.6-84.5 °C;
O: 78.5-80.3 °C.

6.5 Target Tm inside range & Ct=0:
B: 81.8-83.3 °C;
C: 82.6-84.5 °C;
O: 78.5-80.3 °C.

Result=neg

Melt curve acceptable?
(e.g. a single peak without shoulder or multiple peaks)

Repeat the rt-PCR with 4ul template

Yes

No → Repeat the rt-PCR with 2ul template

Result=pos

Fig. 2 The flowchart in result reporting.

Result reporting:

6.1   The results of negative controls should be negative, and the results of positive controls should be positive. Otherwise the rt-PCR should be repeated until correct results are obtained for controls. This may require the use of new reagents and/or supplies.

6.2   The IAC Tm of each sample should be within the acceptable GFP Tm range (86.4-88.5 °C, as shown in Table 2); and the IAC Ct of each sample should not have a significant delay compared to the Ct of the GFP positive control. The cutoff of IAC Ct delay (δCt) is 3.3 cycles, i.e.,
IAC Ct $_{sample}$ – IAC Ct $_{GFP\ positive\ control}$ = δCt, which should be <3.3.
As $\log_2 10 \approx 3.3$, δCt⩾3.3 means that amplification is suppressed by at least ten times due to PCR inhibition.

6.2.1   For a sample with an acceptable IAC Tm **AND** δCt<3.3, there is no Significant PCR inhibition. Continue to step 6.3 below.

6.2.2   For a sample with an acceptable IAC Tm **AND** δCt≥3.3, PCR inhibition is present.

6.2.2a If the target Ct (bovine, caprine, and/or ovine) is "0" or "undetermined", the rt-PCR should be repeated using a diluted template [see LIB 4486 (4) for the dilution protocol] or a re-extracted template.

6.2.2b If the target Ct (bovine, caprine, and/or ovine) is > 0, continue to report the results **despite the PCR inhibition**, per steps 6.3-6.4 below.

6.3   After reviewing the IAC results of each sample, review the Tm results of the target species (bovine, caprine, and ovine). If the target Tm is out of the acceptable range (Table 2), the result is **negative** for that specific target species.

6.4   If the target Tm of a sample falls within the acceptable range (Table 2) **AND** the target Ct > 0, continue to review the melt curve.

    6.4.1   If the melt curve is acceptable, i.e., a single peak without shoulder, or without multiple peaks, the result is **positive** for the specific target species.

    6.4.2   If the melt curve is not acceptable due to an abnormal shape, such as a shoulder or multiple peaks (please refer to Supplementary Information 4 "Examples of acceptable/unacceptable melt curves"), the rt-PCR should be repeated with 2 µl template and follow 6.4.2a-c as below.

        6.4.2a If the repeated target Tm falls out of the acceptable range (Table 2), the result is **negative** for the specific target species.

        6.4.2b If the repeated target Tm is within the acceptable range (Table 2), the repeated target Ct>0, **AND** the repeated melt curve is acceptable, the result is **positive** for the specific target species.

        6.4.2c In rare cases, if the repeated target Tm falls within the acceptable range (Table 2), the repeated target Ct>0, but the repeated melt curve is not acceptable, please consult with the BSE SLV team at PNL (POC: Kun Connie Liu, kun.liu@fda.hhs.gov, 1-425-487-5388).

6.5   If the target Tm falls within the acceptable range (Table 2) **AND** the target Ct = 0, the rt-PCR should be repeated with 4 µl template. Adjust MGW volume accordingly in the rt-PCR reaction so that the total volume is still 25 µl. This repeat procedure is established in LIB 4486 (4).

    6.5.1   If the repeated target Tm falls outside of the acceptable range (Table 2), the result is **negative** for the specific target species.

    6.5.2   If the repeated target Tm is within the acceptable range (Table 2) **AND** the repeated target Ct=0, the result should be reported as **negative** for the specific target species.

    6.5.3   If the repeated target Tm is within the acceptable range (Table 2), the repeated target Ct > 0, **AND** the repeated melt curve is acceptable, the result is "**positive but under Limit of Detection (LOD)**" for the specific target species per LIB 4486 (4).

    6.5.4   If the repeated target Tm falls within the acceptable range (Table 2), the repeated target Ct>0, but the repeated melt curve is not acceptable, please consult with the BSE SLV team at PNL.

Table 2. The acceptable Tm ranges of the simplex rt-PCR method on the AB7500F platform.

Target	Acceptable Tm range (°C)
Bovine	81.8-83.3
Caprine	82.6-84.5
Ovine	78.5-80.3
GFP (IAC)	86.4-88.5

Note: In very rare cases, there may be Tm outliers due to the wide variability in feed and feed ingredients, as well as differences in instruments, reagent lots, and/or operators. If a target Tm is only slightly outside the current acceptable range, for example 0.1-0.2 °C outside it, but the PCR amplification is strong, i.e., the target Ct≤30 with an acceptable melt curve, the template may be positive. The rt-PCR can be repeated with 2 µl template to see if the same results are obtained again. If there is any question, please feel free to consult with the BSE SLV team at PNL.

Acknowledgments

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Supplementary Information 1. An example of the rt-PCR master mix calculation

In this example using feed sample # xxxx01, there are a total of 6 templates after DNA extraction: 1) the extraction blank control, 2) undiluted DNA extracted from sample # xxxx01, 3) 1:10 dilution of xxxx01 DNA, 4) 1:20 dilution of xxxx01 DNA, 5) 1:50 dilution of xxxx01 DNA, and 6) FTA™ blood punches as the extraction positive control (one punch from each target species). Once we add the positive and negative rt-PCR controls (Zyagen gDNA and MGW, respectively), there a total of 8 reactions per primer set (bovine, caprine, ovine or GFP IAC). Note: The number of diluted template and dilution factors can be adjusted in actual experiments.

To ensure that there is enough master mix for all reactions, multiply the reaction number by 120%: (8 reactions) * 120% = 9.6 reactions per primer set. The master mixes are as follows:

Bovine master mix:

(2×) iQ SYBR® Green SuperMix with ROX	12.5 µl x 9.6 = 120 µl
Bovine primer mix (5 µM forward and reverse)	2.0 µl x 9.6 = 19.2 µl
Molecular biology grade water (MGW)	8.5 µl x 9.6 = 81.6 µl

Caprine master mix:

(2×) iQ SYBR® Green SuperMix with ROX	12.5 µl x 9.6 = 120 µl
Caprine primer mix (5 µM forward and reverse)	2.0 µl x 9.6 = 19.2 µl
Molecular biology grade water (MGW)	8.5 µl x 9.6 = 81.6 µl

Ovine master mix:

(2×) iQ SYBR® Green SuperMix with ROX	12.5 µl x 9.6 = 120 µl
Ovine primer mix (5 µM forward and reverse)	2.0 µl x 9.6 = 19.2 µl
Molecular biology grade water (MGW)	8.5 µl x 9.6 = 81.6 µl

GFP IAC master mix:

(2×) iQ SYBR® Green SuperMix with ROX	12.5 µl x 9.6 = 120 µl
GFP primer mix (5 µM forward and reverse)	2.0 µl x 9.6 = 19.2 µl
Molecular biology grade water (MGW)	**7.5** µl x 9.6 = **72** µl

Note: The MGW volume in GFP IAC master mix is 72 µl for 9.6 reactions, which is different from the other three master mixes (81.6 µl).

For the GFP negative control, pipet 22 µl IAC master mix into the designated well, then add 3 µl MGW to the same well to reach a final volume of 25 µl.

Add working solution of the GFP template to the remaining IAC master mix (in the original centrifuge tube) to achieve the final concentration of 1 µl working solution per reaction. To calculate the volume of the GFP template solution, simply subtract the negative control from the total reactions. In this example, (9.6 – 1) = 8.6 reactions, so add 8.6 ul working solution of the GFP template to the remaining IAC master mix.

Supplementary Information 2. Experiment setup and data processing using the AB7500F software <u>v1.4</u>

1. Turn on the AB7500F instrument by pressing the power button (Fig. S2-1). Gently press the sample-loading button to open the plate tray (Fig. S2-2). Load the 96-well plate. Gently push the tray to retract it back into the instrument.

Fig. S2-1.   Fig. S2-2.

2. Open the 7500 Fast System software v1.4. A "Product Registration" window will open (Fig. S2-3). Fill in the fields and click "OK"; or leave the fields blank and click "Cancel". A "Quick Startup document" window will then open (Fig. S2-4).
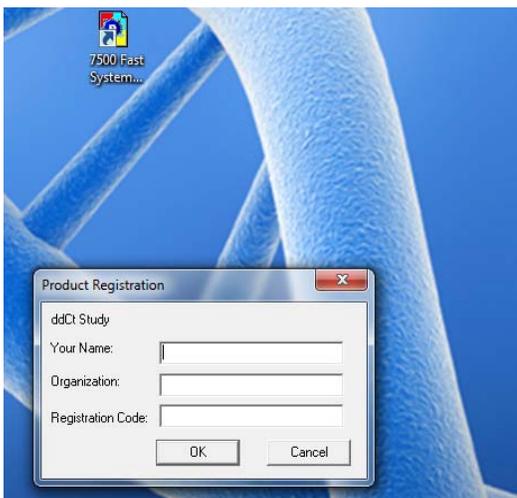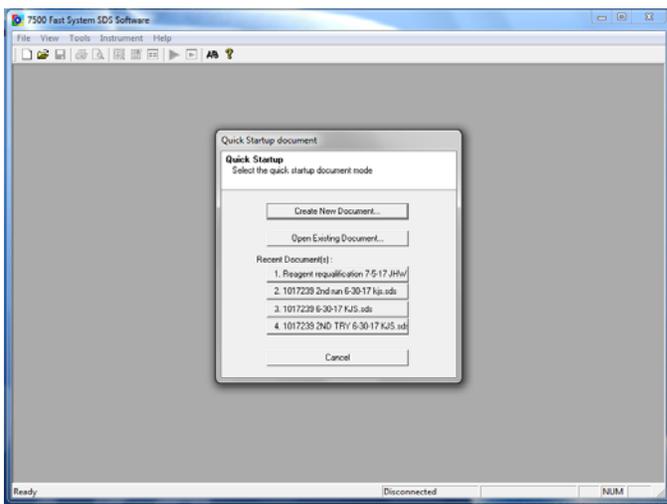
Fig. S2-3.   Fig. S2-4

3. Click "Create New Document" and a "Define Document" window will open (Fig. S2-5). Use the below parameters: Assay=Standard Curve (Absolute Quantitation), Container=96-Well Clear, Template=Blank Document or your desired template chosen by clicking "Browse", and Run Mode=**Fast 7500** (see the circle in Fig. S2-5). Fill in the "Operator", "Comments" and "Plate Name" fields, and click the "Next >" button. In the next "Select Detectors" window (Fig. S2-6) choose "**sybrGreen**" in the list on the left, then click the "Add>>" button to add this detector. Next, select "**ROX**" as the "Passive Reference" in the pull-down menu at the top on the right, and click "Next >" at the bottom of the window (see the arrows in Fig. S2-6).
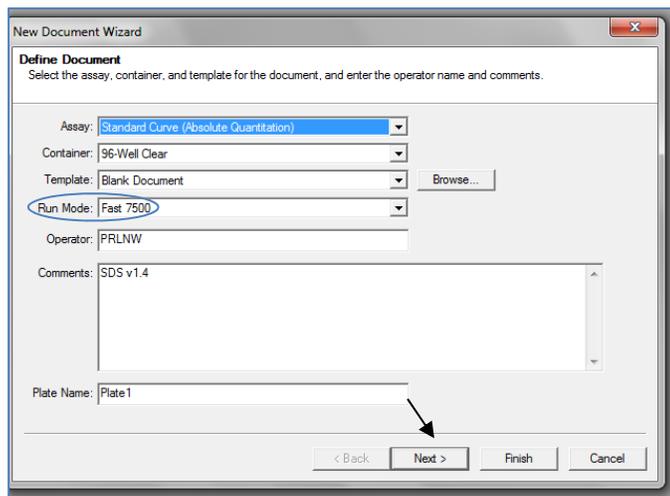
Fig. S2-5.
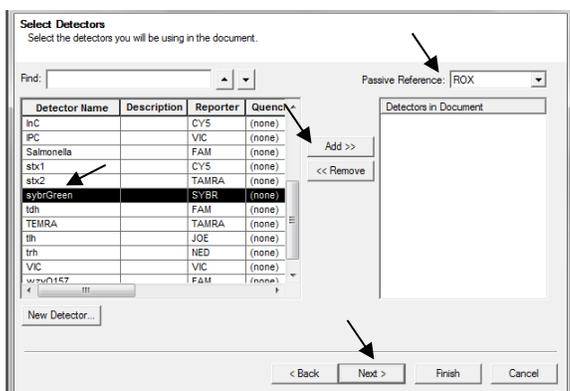
Fig. S2-6.

4. A "Set Up Sample Plate" window will then open (Fig. S2-7). Select the reaction wells that you are using for this run in the lower panel which represents the layout of the 96-well plate (use the "shift" or "ctrl" key + mouse left click to select multiple wells at the same time), then check the box on the left in the upper panel to assign "**sybrGreen**" as the detector to wells that you've selected. Click "Finish" to continue.

Note: If the instrument is not turned on, a reminder window will open (Fig. S2-8). Turn on the AB7500F instrument and then click "Retry" to continue.
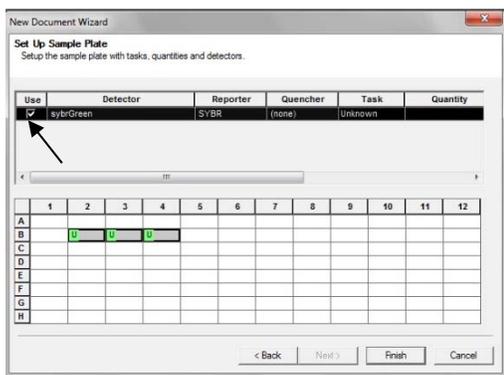
Fig. S2-7.
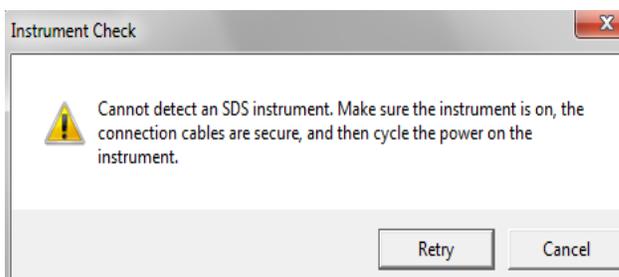
Fig. S2-8.

5. A new window will then open (Fig. S2-9). Under the "Setup" tab and the "Plate" sub-tab, the wells that you selected in step 4 should be labelled with the detector. To enter sample ID for a well, use mouse to double click the well, and fill the ID in the "Well Inspector" window that opens (Fig. S2-10). Then, click the "close" button on the bottom of the "Well Inspector" window. Enter other sample IDs in the same way.
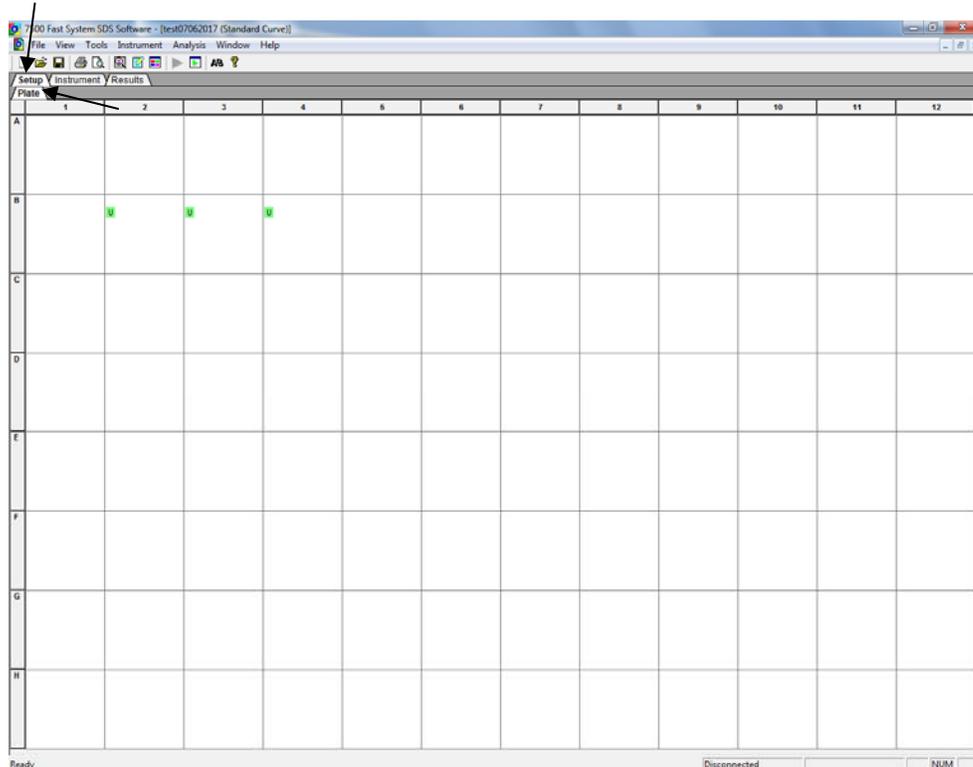
Fig. S2-9.
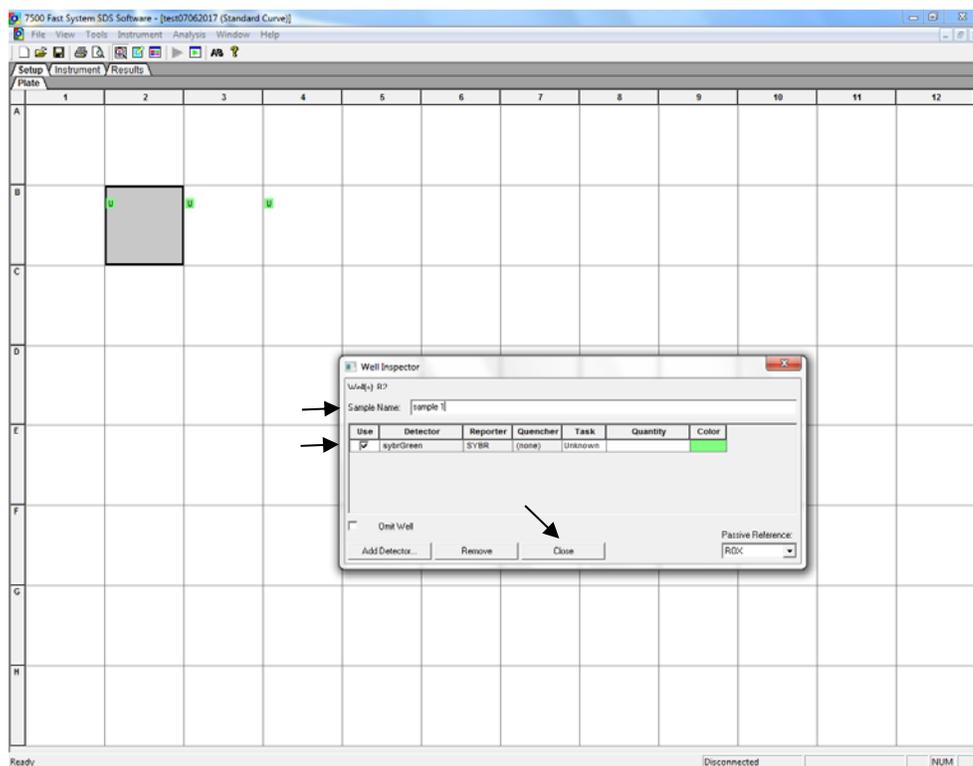
Fig. S2-10.

6. The selected wells should now be labeled with the sample IDs that you entered in step 5 (Fig. S2-11).
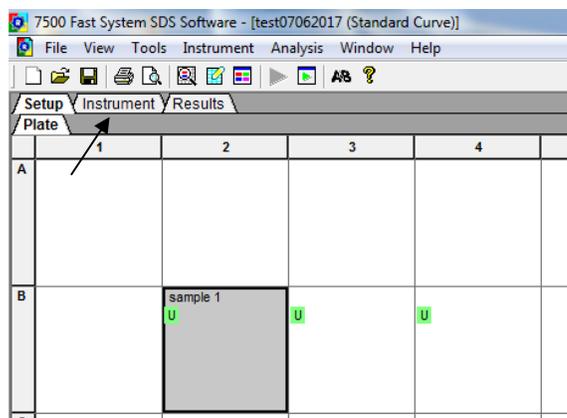
Fig. S2-11.

7. Next, click the "instrument" tab (the middle tab at the top, see the arrow in Fig. S2-11) and a new window will open (Fig. S2-12); this is where the rt-PCR cycling conditions are entered.

> 7.1 In the middle panel of the "instrument" tab, enter the AB7500F rt-PCR cycling program per Table S2-1 as below. Besides the temperature and duration time in each step, other key parameters include: "Stage 2 Reps"=**50**, "Sample Volume (μl)"=**25**, and "Run Mode"=**Fast 7500** (chosen from the pull-down menu). For "Data Collection", choose "**Stage 2, Step 3 (72.0 °C @ 0:40)**" from the pull-down menu, which is the extension stage (see the arrows in Fig. S2-12).

> Note: If the data collection stage is incorrectly chosen, the rt-PCR will not start because the denaturing or annealing time in Table S2-1 would be too short for the fluorescence detection on the AB7500F platform.
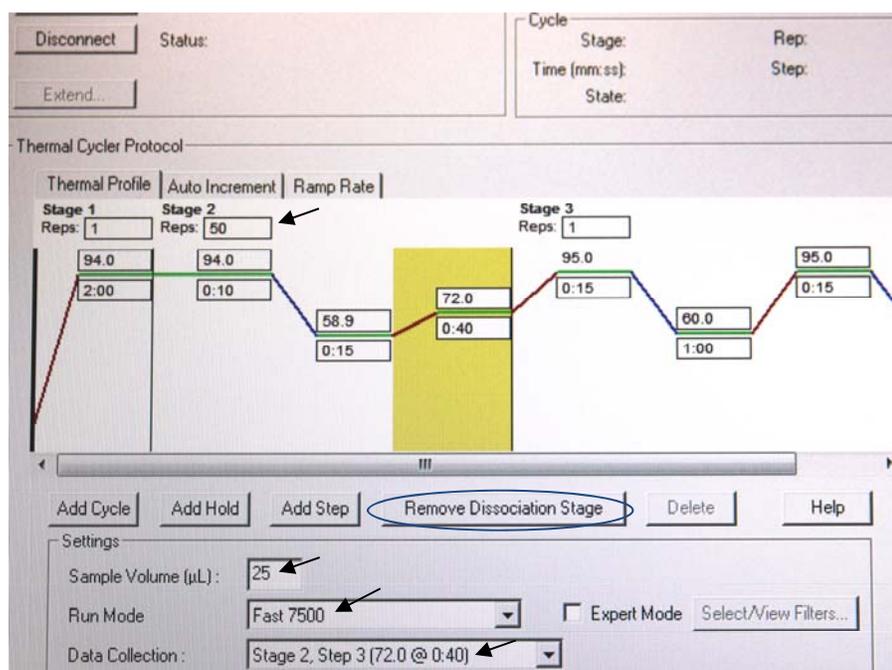
Fig. S2-12.

> 7.2 Click "Add Association Stage" in the same window (see the purple circle in Fig. S2-13 below), add the melt curve analysis using default settings provided by the AB7500F software v1.4 per Table S2-1.

After the melt curve analysis is added, this button will be changed to "Remove Dissociation Stage" (see the blue circle in Fig. S2-12 above).

Fig. S2-13.

Table S2-1. The cycling conditions used in the BSE simplex rt-PCR method on the AB7500F.

Stage	Repetitions	Temperature	Time (min:sec)	Ramp Rate
1	1	94.0 °C	2:00	Auto
2	50	94.0 °C	0:10	Auto
		58.9 °C	0:15	Auto
		72.0 °C	0:40	Auto
3 dissociation (default settings, "continuous" mode)	1	95.0 °C	0:15	Auto
		60.0 °C	1:00	Auto
		95.0 °C	0:15	Auto
		60.0 °C	0:15	Auto

8. Once steps 1-7 have been completed, click the "Start" button on the top left of the "Instrument" tab (see the arrow in Fig. S2-14). A small window will then open to remind you to save the document. Click "Save And Continue" to save the data in the folder of your choice (Fig. S2-14).
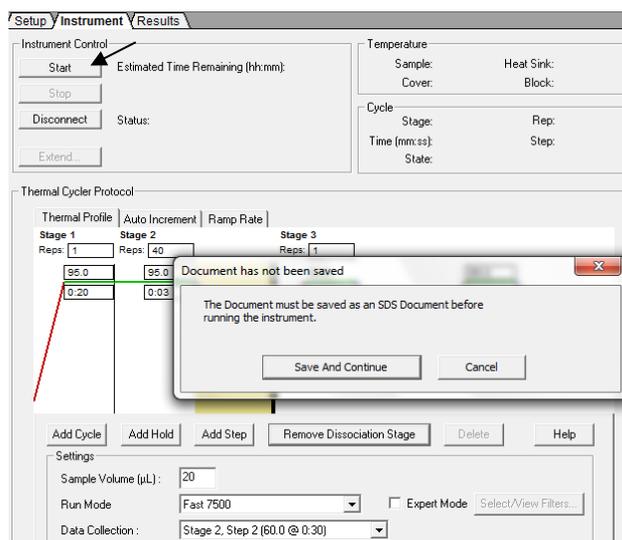
Fig. S2-14.

After this step, the AB7500F run is started. Data will be saved automatically once the run has completed. The on-instrument run time is initially estimated to be approximately 3 hours, but the actual run time is later adjusted to approximately 1.8 hours.

Note: It's ideal to wait until the run starts normally and the estimated run time appears on the screen.

9. Once the run has completed, the raw data will automatically be saved under the file name and a window will open stating that the run was completed successfully. Click the "OK" button to continue and turn off the instrument if no more runs are planned for the same day.

10. Next, click the "Analysis Settings" button which looks like a green triangle inside a box in the tool bar at the top, or choose "Analysis Settings" from the "Analysis" pull-down menu. Select "**Auto Ct**", then "**Automatic Baseline**" will be chosen by the software itself. Click "OK" to continue. Then click "Analysis → Analyze", or simply click the Analyze icon (a green triangle without box) to do data analyses in selected wells. If data are already analyzed, the Analyze icon is a grey triangle (Fig. S2-15).
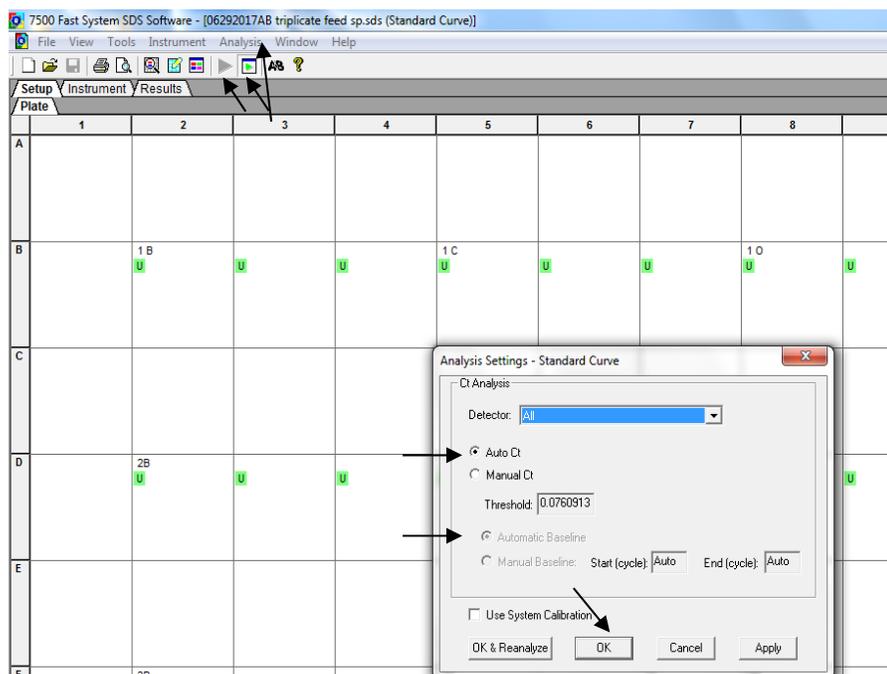
Fig. S2-15.

11. Click the "Results" tab on the top (see the circle in Fig. S2-16), click on each sample well to check the results that appear in the "Amplification Plot", "Dissociation" (melt curve analyses), and/or "Report" tabs (see the arrows in Fig. S2-16).

Note: Melt curves can be reviewed right after a run or at a later time. To review the results later, just open the software without turning on the AB7500F instrument.
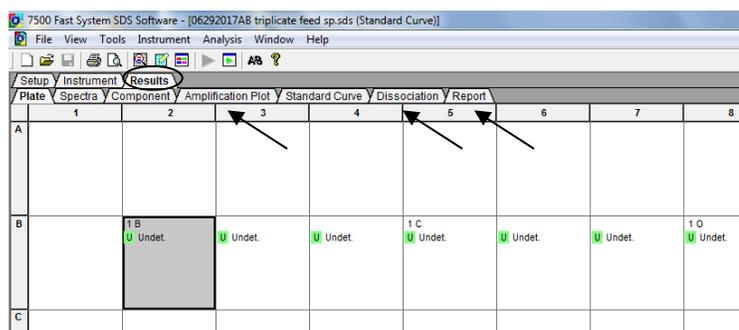
Fig. S2-16.

Below are two melt curve examples, one from an individual reaction, and the other from three reactions in three different wells (Fig. S2-17a and -17b). Use [CTL or Shift + left click] to select multiple wells.
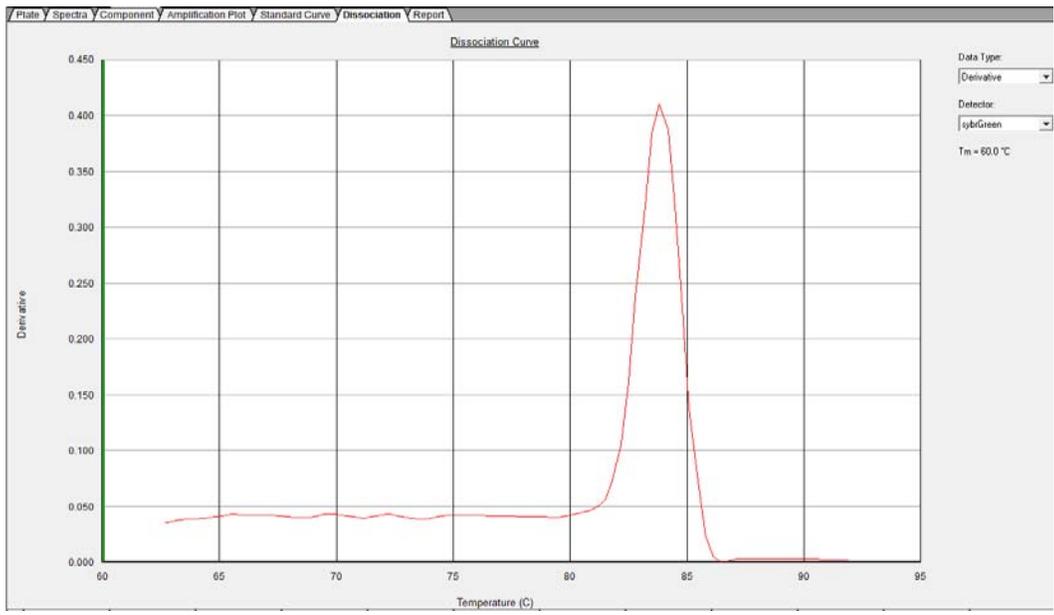
Fig. S2-17a.
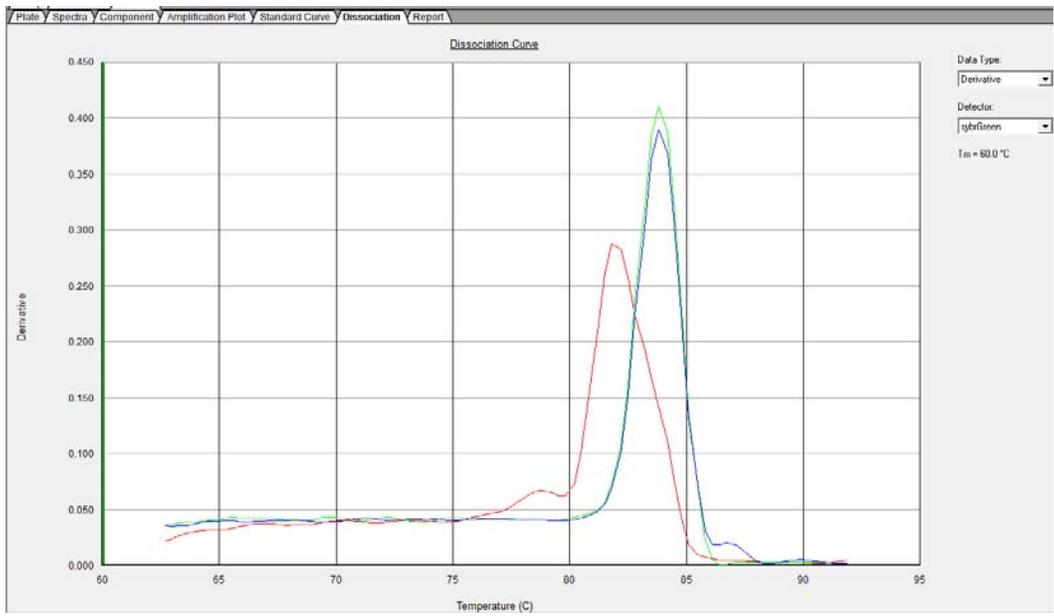
Fig. S2-17b.

12. To export data, select the target wells, then choose "File" -> "Export" -> "Results..." (Fig. S2-18). Select the folder where you would like for data storage, then check "export only selected wells" and click "OK" (Fig. S2-19). Data columns of report table can be customized, and "Apply Report Settings for Data Columns" can be checked for export.
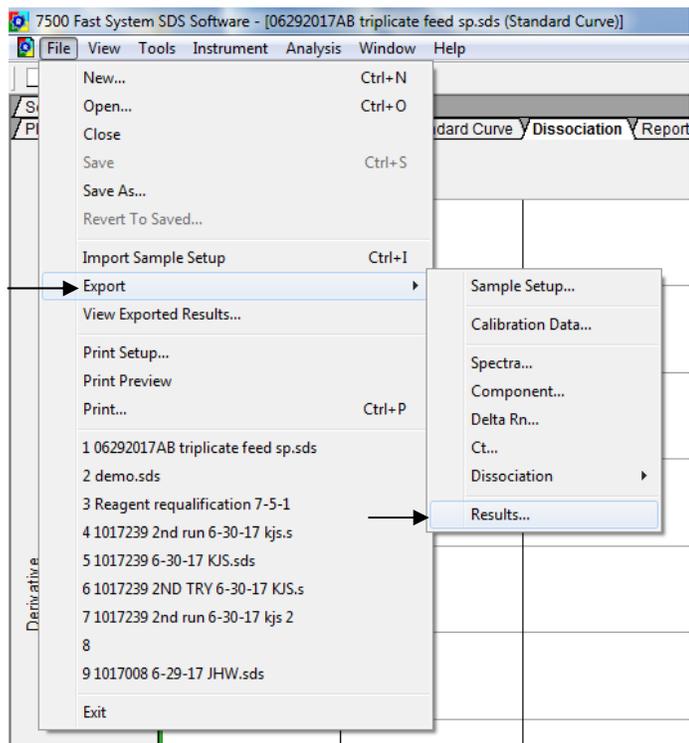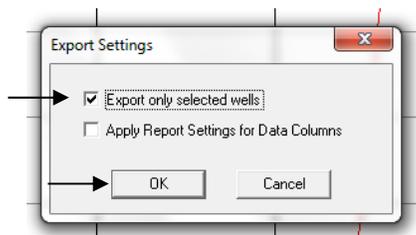

Fig. S2-18.

Fig. S2-19.

13. Save the changes to the run as needed, then close the AB7500F software v1.4.

Supplementary Information 3. Experiment setup and data processing using the AB7500F software <u>v2.3</u>

1. Turn on the AB7500F instrument by pressing the power button (Fig. S3-1a). Gently press the sample-loading button to open the plate tray (Fig. S3-1b). Load the 96-well plate. Gently push the tray to retract it back into the instrument. Open the AB software v2.3 by clicking "log in as Guest" (Fig. S3-1c).
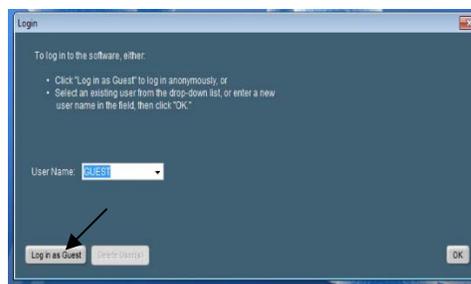
Fig. S3-1a.   Fig. S3-1b.   Fig. S3-1c.

<u>Note</u>: If the instrument is not turned on or the connection is lost, a "Fatal Error" message will open (Fig. S3-2). Click "OK" after checking the connection and/or turn on the instrument.
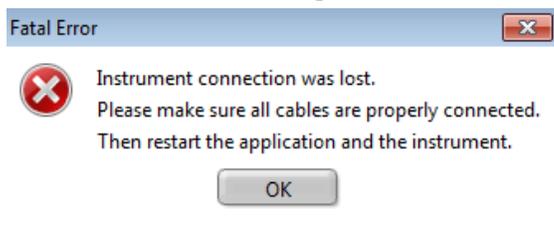
Fig. S3-2.

2. Click the "QuickStart" icon under the "Run" column (Fig. S3-3).

Fig. S3-3.

3. A new window will open (Fig. S3-4). In the "Experiment Properties" tab, enter the "Experiment Name", choose the desired folder ("Location"), and select the following parameters: 3a) "**Quantification-Standard Curve**"; 3b) "**SYBR® Green Reagent (With Melt Curve)**"; 3c) "**Fast (~ 40 minutes to complete the run)**"; 3d) "**cDNA (2-step RT-PCR) or gDNA**". Then, select the "Run Method" tab on top but **DO NOT** click "START RUN".

<u>Note</u>: **DO NOT** click "START RUN" before entering parameters in the "Run Method" tab as instructed below.
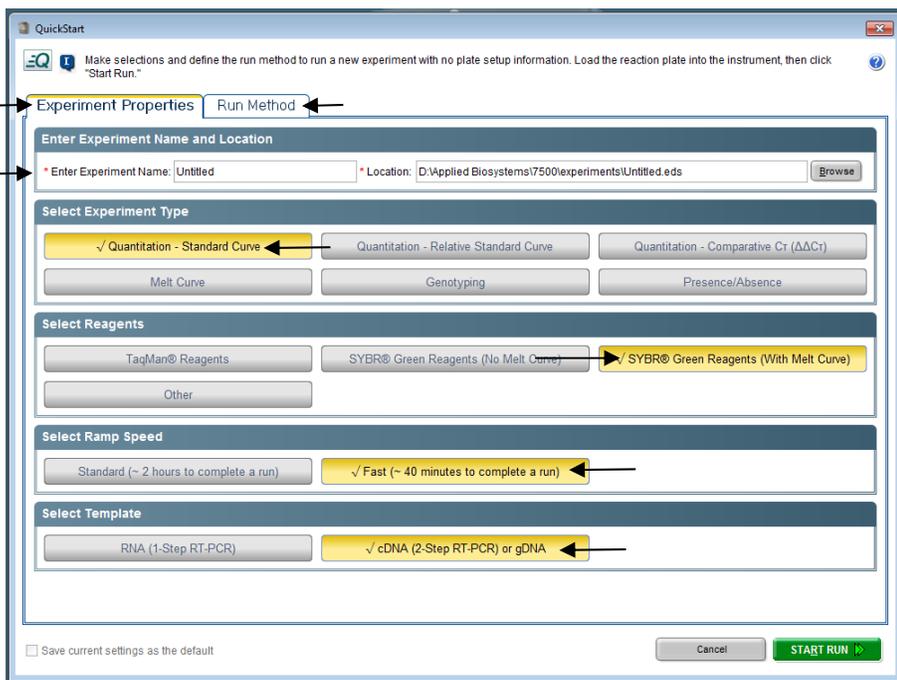
Fig. S3-4.

4. After clicking the "Run Method" tab, a new window will open (Fig. S3-5). Enter parameters: 4a) "Reaction Volume per Well" = **25** ul; 4b) "Number of Cycles" = **50**; 4c) Enter the cycling parameters per Table S3-1. Use mouse to highlight the Step 2 in "Cycling Stage", then add the Step 3 by clicking "Add Step after…" and enter cycling perimeters. Select **Cycling Stage Step 3 (72 °C 00:40)** for optic detection by clicking the optic icon off in Step 2 (greyed out) and clicking the icon on in Step 3 (with a solid frame, highlighted in yellow in Fig S3-5). 4d) Use the **default** parameters in "Melt Curve Stage" (Fig. S3-6). 4e) Click "START RUN" (the green button on the bottom right in Fig. S3-5).

Note: If the optic detection step is not selected correctly, there will be an error stating that 15 seconds in step 2 is too short for the instrument to detect signal. To save ~20 minutes of the run time, consider entering the target names and sample IDs after the run has started, which is described in this supplementary information. Other ways to create runs include using "Advanced Setup" or a template, please refer to AB user manual.
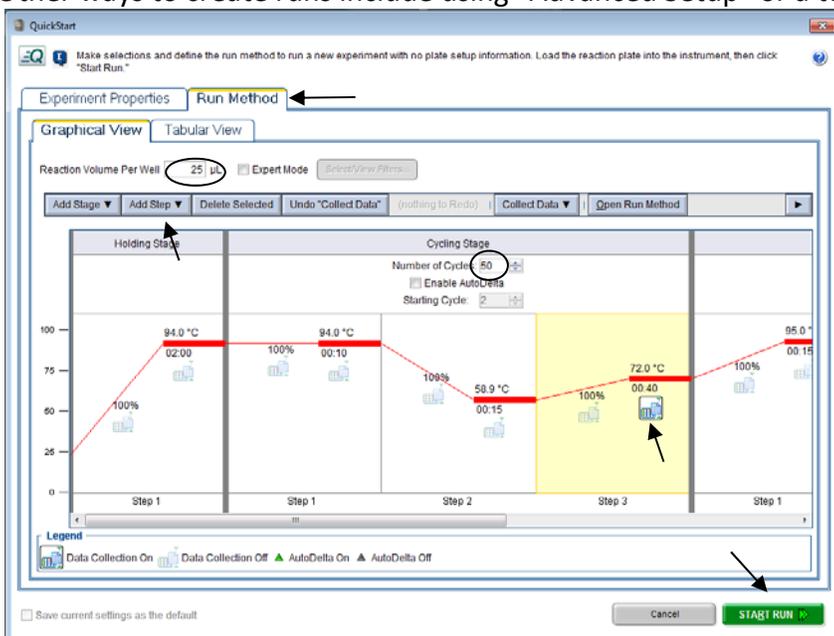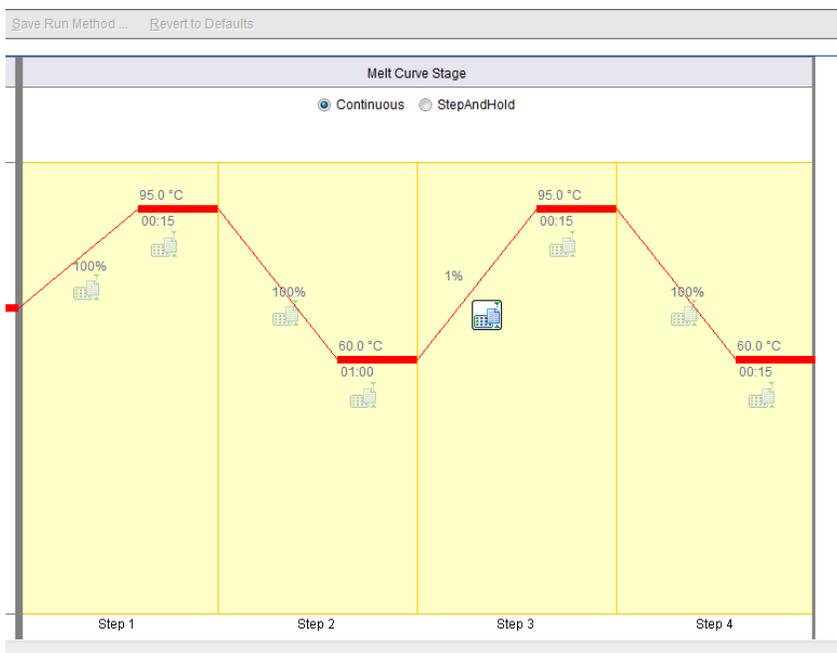

Fig. S3-5.

Fig. S3-6.

Table S3-1. The cycling conditions of the BSE simplex rt-PCR method on the AB7500F platform.

Stage	Repetitions	Temperature	Time (min:sec)	Ramp Rate
1	1	94.0 °C	2:00	Auto
2	50	94.0 °C	0:10	Auto
		58.9 °C	0:15	Auto
		72.0 °C	0:40	Auto
3 dissociation (default settings, "continuous" mode)	1	95.0 °C	0:15	Auto
		60.0 °C	1:00	Auto
		95.0 °C	0:15	Auto
		60.0 °C	0:15	Auto

5. After the run has started, click the "Setup" tab in the column on the left, then click "Plate Setup". A new window will open (Fig. S3-7). In the "Define Targets and Samples" tab, click "Add New Target", and enter the four targets in the "Define Targets" panel (for example, B for bovine; C for caprine; O for ovine; and G for GFP IAC). Make sure the "Reporter" for each target is "**SYBR**" and the "Quencher" is "**None**". Choose an arbitrary color for each target as desired.
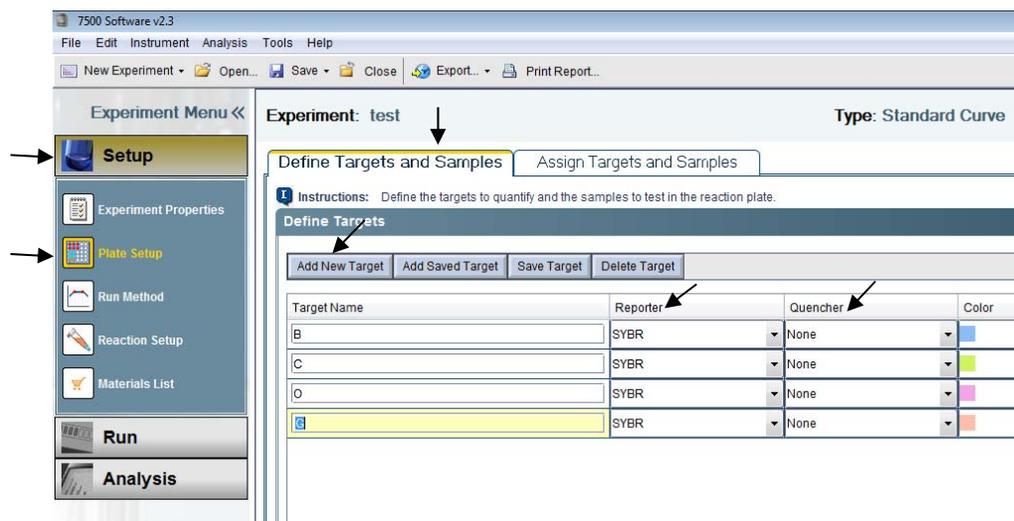
Fig. S3-7.

6. In the "Define Samples" panel on the right, click "Add New Sample" to include all the samples, enter each sample's ID as needed, and choose an arbitrary color for each sample (Fig. S3-8).
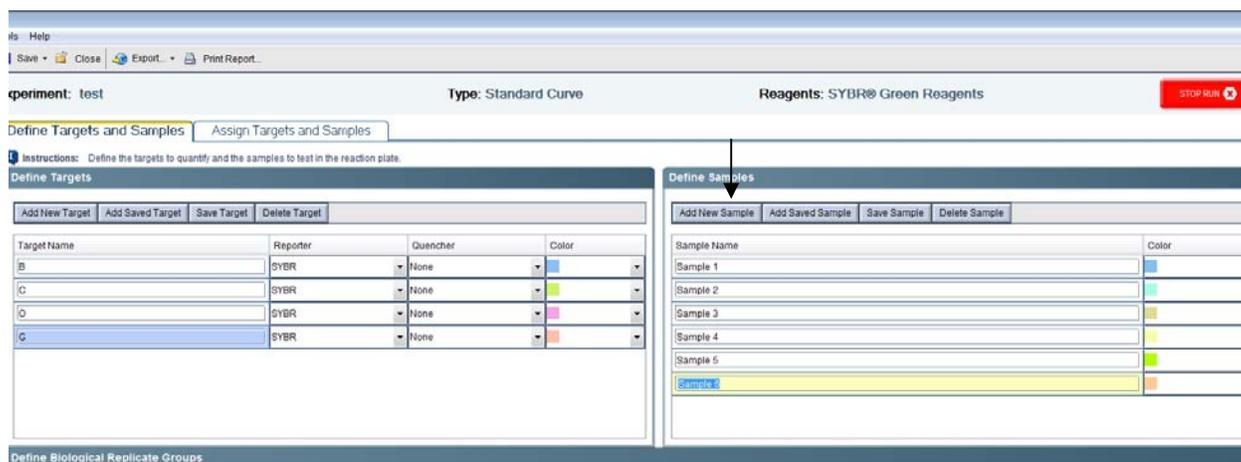

Fig. S3-8.

7. Select the "Assign Targets and Samples" tab in "Setup" (Fig. S3-9). In the new window, assign the target and sample ID for each experimental well. Multiple wells can be selected at the same time to assign the same target or the same ID by using [Ctrl + left click] or [Shift + left click].
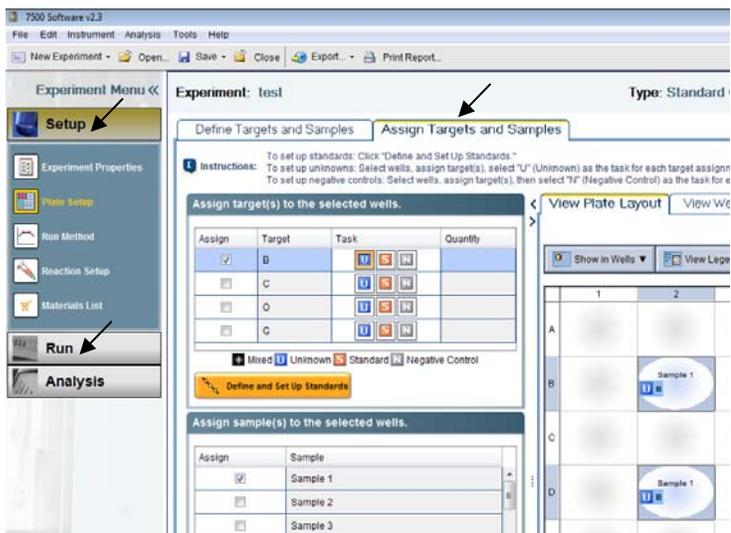
Fig. S3-9.

8. After assigning targets and sample IDs, click the "Run" tab (in the column on the left) to confirm that the run is still ongoing. If everything is fine, the remaining run time (estimated) is shown beside the red "STOP RUN" button (see the arrow in Fig. S3-10).

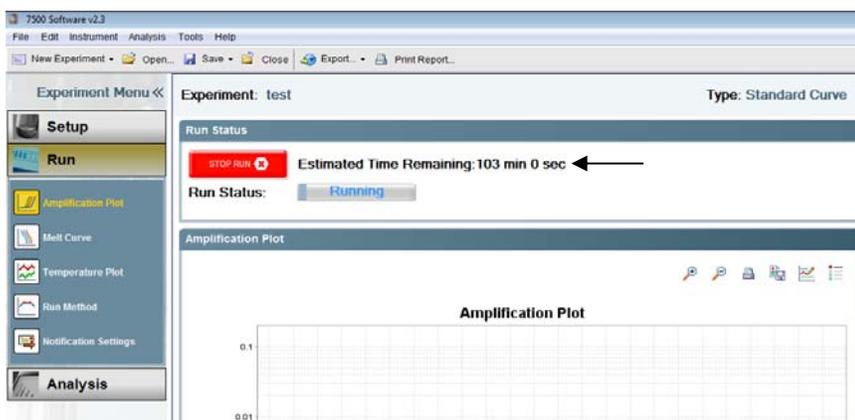Note: **DO NOT** click the "STOP RUN" button unless a run has to be terminated before completion.

Fig. S3-10.

9. After the run has completed, turn off the instrument if there are no more runs planned for the same day. Click the "Analysis" tab (in the column on the left), then click the "Analysis Settings" button on the top right to set up the analysis parameters (Fig. S3-11).
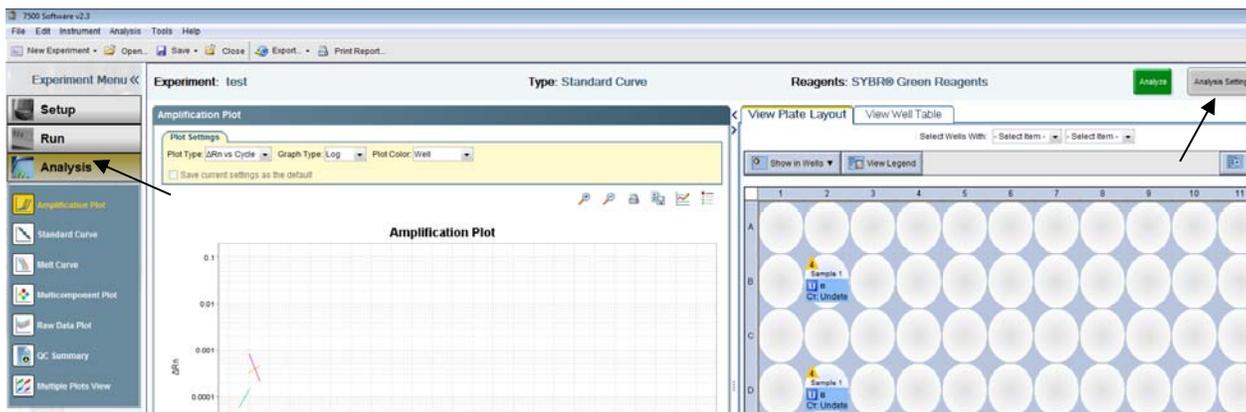
Fig. S3-11

10. After clicking the "Analysis Settings" button, an "Analysis Settings for test" window will open (Fig. S3-12). In the 1st tab, "C$_T$ Settings", make sure the "**Automatic Threshold**" and "**Automatic Baseline**" are selected as the default settings for all the targets. If not, click the "Edit Default Settings" button for parameter editing, then click "Apply Analysis Settings" on the bottom to confirm the updated parameters and close this window (see the arrows in Fig. S3-12).
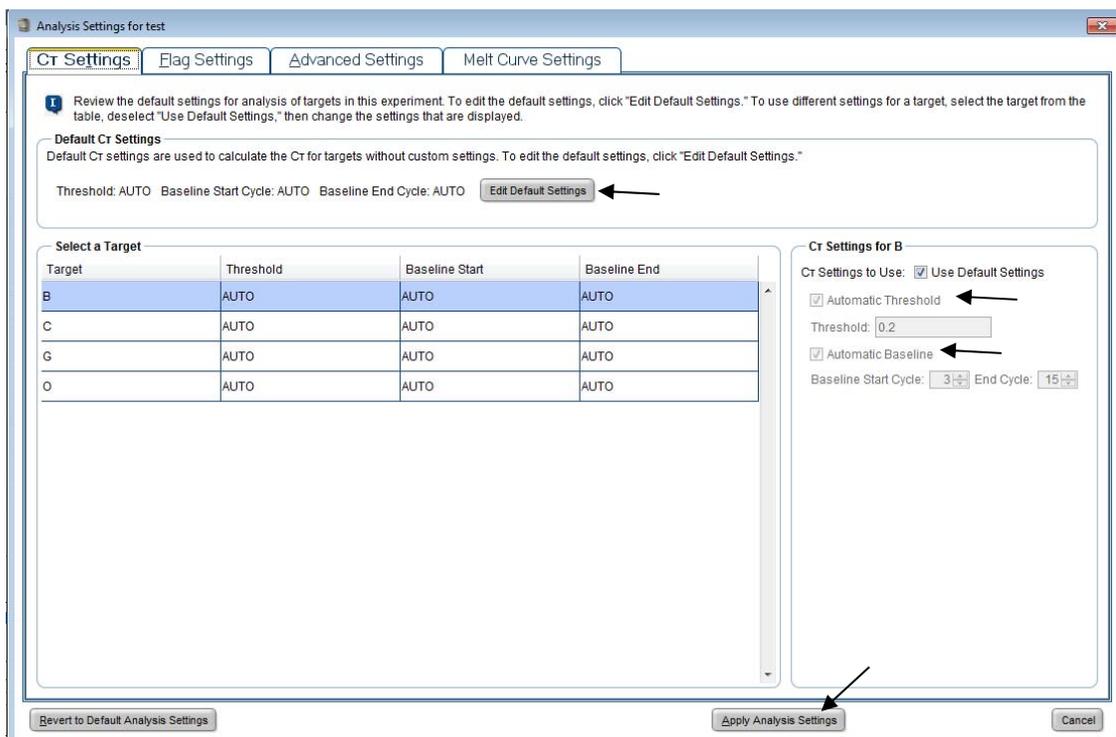

Fig. S3-12.

11. To process raw data, click the green "Analyze" button on the top right (see the arrow in Fig. S3-13).
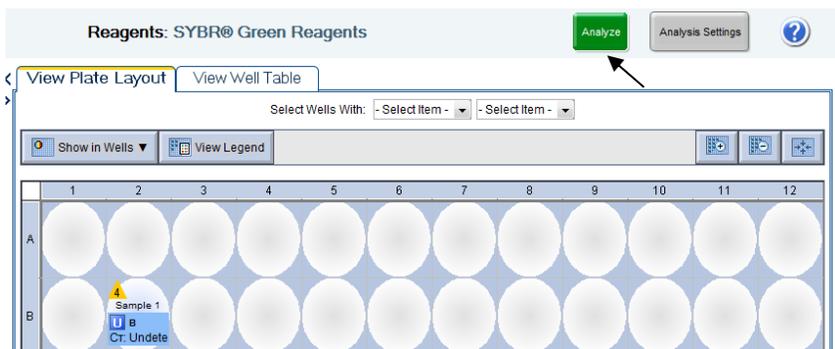
Fig. S3-13.

12. To view melt curve, select "Melt Curve" under "Analysis" (Fig. S3-14), then select a well in the "View Plate Layout" window on the right. The melt curve and Tm of that target well will be shown in the "Melt Curve" window. Similarly, other types of plots can be viewed by choosing different tabs in the column on the left, such as "Amplification Plot", "Raw Data Plot", etc. (see the arrows in Fig. S3-14).

One of the differences between the AB7500F software v1.4 and v2.3 is that Tm of a selected well is shown on the Melt Curve plot with v2.3, but not with v1.4.

Note: The review of melt curves can be performed right after a run is completed or any time later. For data analysis of a previous run, just open the AB7500F software v2.3 without turning on the instrument.
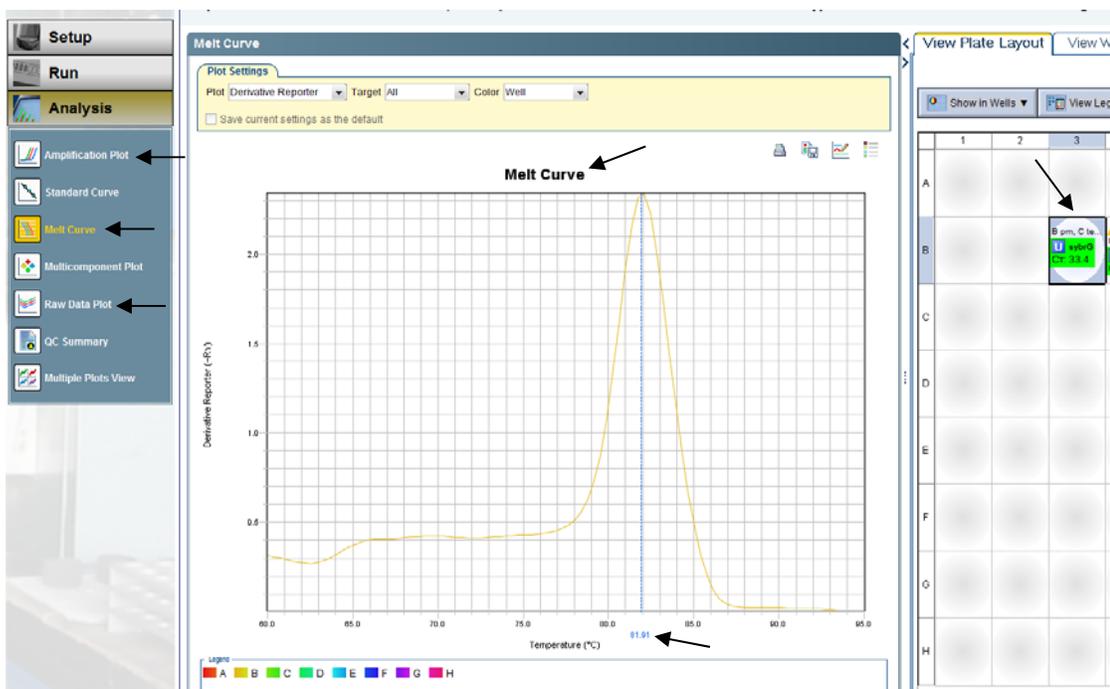

Fig. S3-14.

13. To export analyzed data, click "File"->"Export...." and an "Export Data" window will open (Fig. S3-15). In the "Export Properties" tab, select data to export, fill in "Export File Name", select "Export File Location", then click "Start Export".
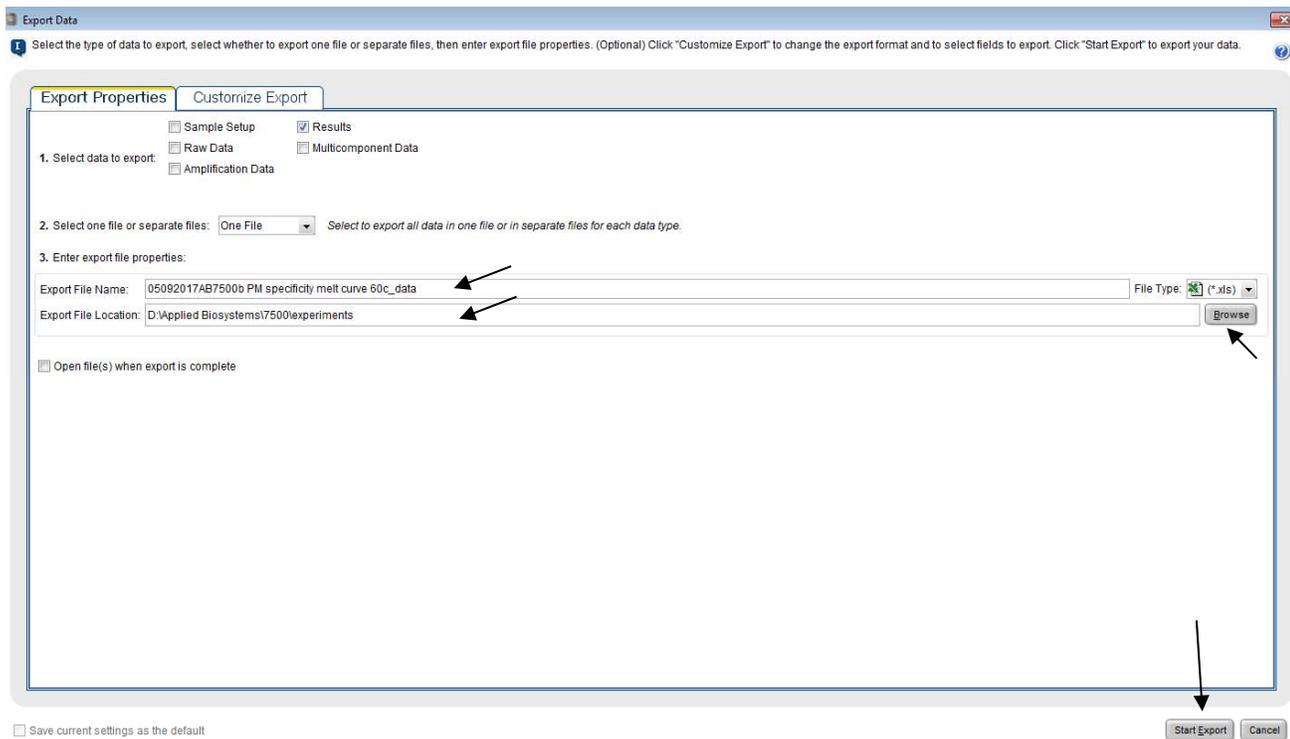

Fig. S3-15.

14. After the data has been exported to the designated folder, another window will open stating "Your file has been exported to..." (Fig. S3-16). Click "Export More Data" if you need to export additional data, or click "Close Export Tool" if there is no more data to be exported.


Fig. S3-16

15. Save the changes to the run as needed, then close the AB7500F software v2.3.

Supplementary Information 4. Examples of acceptable and unacceptable melt curves

Sometimes, a Tm value determined by the AB7500F software falls within the acceptable temperature range for the target species, but the sample may not be a true positive because the shape of the melt curve is not acceptable. For a Tm falling within the acceptable range, the respective melt curve needs to be visually inspected prior to making a positive call. Below are examples of acceptable and unacceptable melt curves. When a melt curve is unacceptable, a repeat of rt-PCR is usually needed to draw a conclusion.

**1)** Examples of acceptable melt curves, one for each target species on the AB7500F with software v1.4.

1a) Below are the melt curve results using bovine primers (Fig. S4-1a). Curve #1 is the negative control (red), #2 is the positive control (blue), and #3 is the sample (green). The sample Tm is 82.1 °C with Ct= 27.8. The sample Tm falls into the acceptable bovine range. The sample melt curve is acceptable as there is a single sharp peak on the curve. Therefore, the result is bovine positive. Sometimes there is a small peak on the melt curve of the negative control like the one on curve #1. This is acceptable as long as the negative control is negative (Tm outside of the respective range). The weak peak is probably caused by primer dimer.
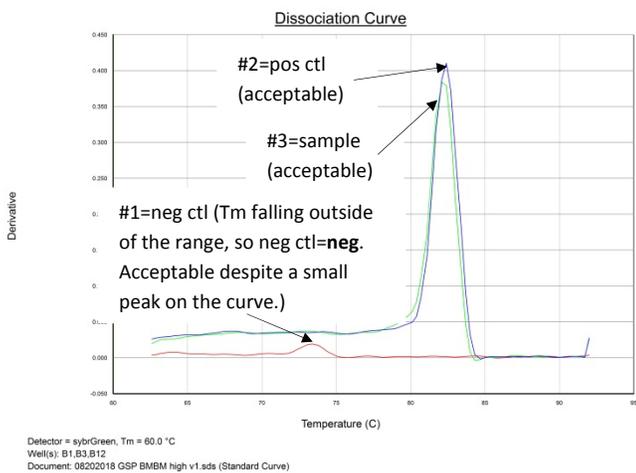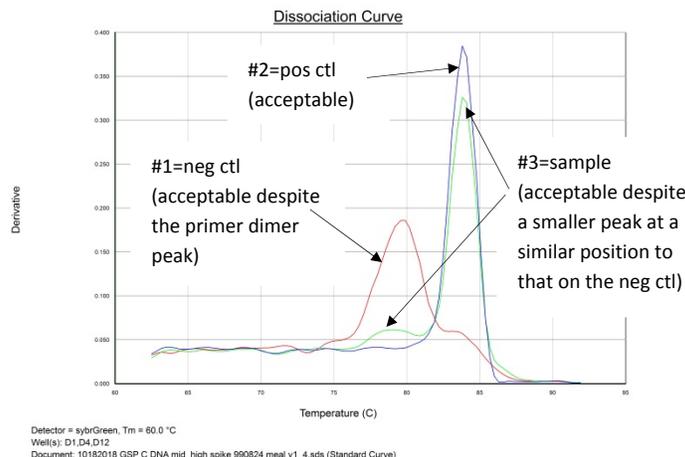

Fig. S4-1a

1b) Below are the melt curve results using ovine primers (Fig. S4-1b). Curve #1 is the negative control (green), #2 is the positive control (blue), and #3 is the sample (red). The sample Tm is 78.9 °C with Ct = 21.7. The sample Tm falls into the acceptable ovine range. After review, the sample melt curve is acceptable as there is a single sharp peak on the curve. Therefore, the
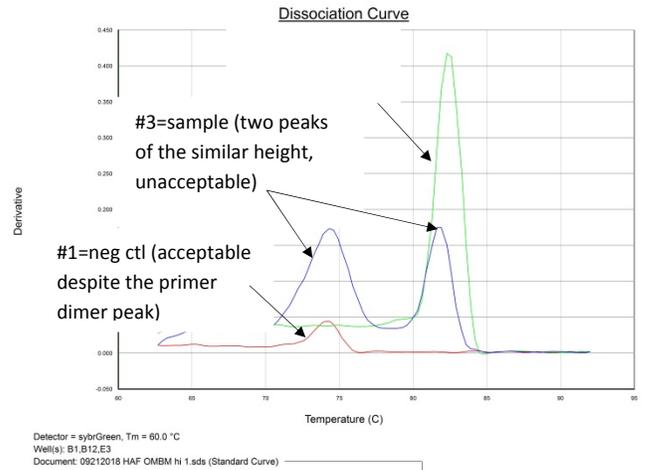
rt-PCR result is ovine positive.
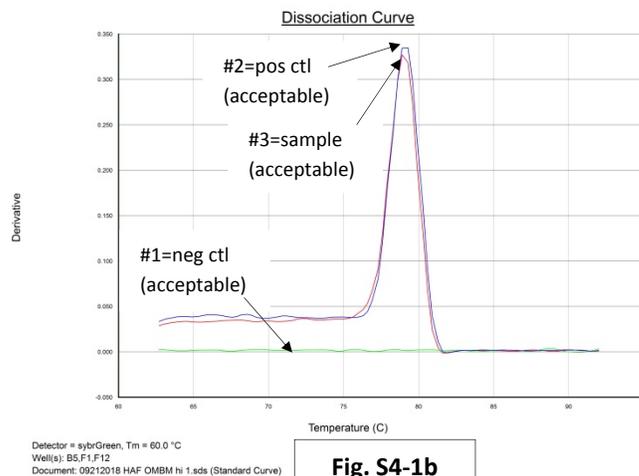
Fig. S4-1b

1c) Below are the melt curve results using caprine primers (Fig. S4-1c). Curve #1 is the negative control (red), #2 is the positive control (blue), and #3 is the sample (green). The sample Tm is 83.5 °C with Ct=28.0. The sample Tm falls into the acceptable caprine range, so the sample melt curve has been reviewed. To the left of the larger peak on the sample curve, there is a smaller peak at a similar position to that on the negative control curve. As the sample Tm (the larger peak) is not affected by the smaller peak, this melt curve is acceptable, and the result is caprine positive.
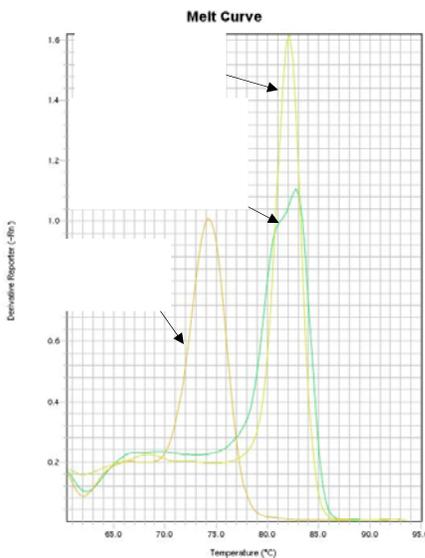

Fig. S4-1c

2) Below is an unacceptable melt curve example using ovine primers (Fig. S4-2). Curve #1 is the negative control (green), #2 is the positive control (blue), and #3 is the sample (red). The sample Tm is 79.1 °C with Ct=46.6. This Tm falls into the acceptable ovine range, but there are three peaks on the sample melt curve, which is unacceptable. After the rt-PCR is repeated with 2 µl template, the repeated sample Tm is 82.6 °C, falling outside of the acceptable ovine range. Therefore, the result is ovine negative. The template is 1.0% (v/v) BMBM DNA, so a false positive result has been avoided.

Detector = sybrGreen, Tm = 60.0 °C
Well(s): C9,F1,F12
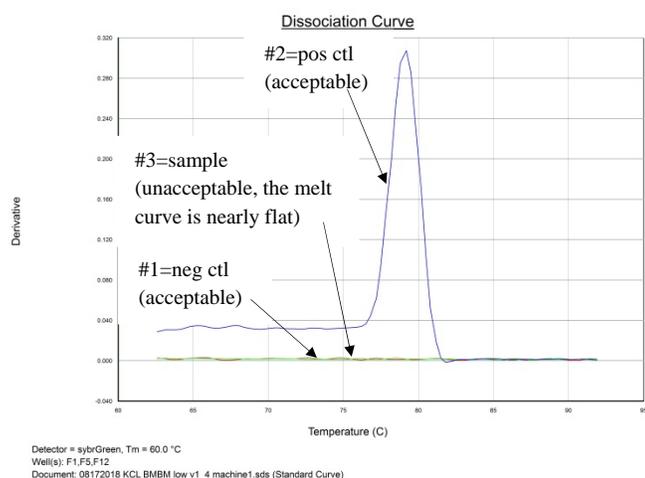Document: 08202018 GSP BMBM high v1.sds (Standard Curve)

3) Below is an unacceptable melt curve example using bovine primers (Fig. S4-3). Curve #1 is the negative control (red), #2 is the positive control (green), and #3 is the sample (blue). The sample Tm is 81.9 °C with Ct=34.9. This Tm falls into the acceptable bovine range, however, there are two peaks of similar height on the sample melt curve, which is unacceptable. After the rt-PCR is repeated with 2 µl template, the repeated sample Tm is 80.3°C which falls outside of the acceptable bovine range. Therefore, the result is determined to be bovine negative. The template is 1.0% (v/v) OMBM DNA, so a false positive result has been avoided.

#3=sample (two peaks of the similar height, unacceptable)

#1=neg ctl (acceptable despite the primer dimer peak)

Detector = sybrGreen, Tm = 60.0 °C
Well(s): B1,B12,E3
Document: 09212018 HAF OMBM hi 1.sds (Standard Curve)

4) Below is an unacceptable melt curve example using bovine primers with the AB7500F software v2.3 (Fig. S4-4). Although there is some difference in the software interface between v 1.4 and v2.3, the review criteria of melt curves are the same.

Curve #1 is the negative control (yellow), #2 is the positive control (yellow), and #3 is the sample (green). The sample Tm is 82.8 °C with Ct=36.3. This Tm falls into the acceptable bovine range, but there is a shoulder near the main peak on the sample melt curve, which is unacceptable. After the rt-PCR is repeated with 2 µl template, the repeated sample Tm is 80.5 °C, falling outside of the acceptable bovine range. Therefore, the result is bovine negative. The template is 1.0% (v/v) OMBM DNA, so a false positive result has been avoided.

Fig. S4-4

5) Caution should be taken when the sample Ct is 0, the sample melt curve is nearly flat (similar to that of the negative control), but the software has calculated an apparent sample Tm that falls within the acceptable range. See the example below.
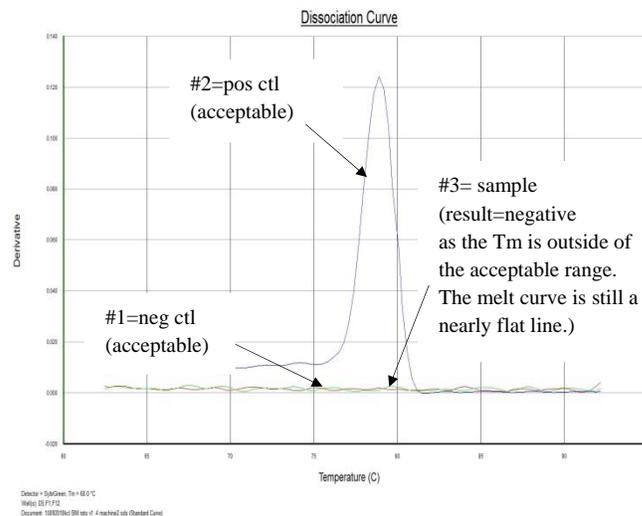
Curve #1 is the negative control (red), #2 is the positive control (blue), and #3 is the sample (green). Using ovine primers, the sample Tm is 78.5 °C, which falls into the acceptable ovine Tm range. However, the sample Ct=0, and the sample melt curve is nearly flat (Fig. S4-5a), indicating that there is probably no PCR amplicon produced in the reaction.

Detector = sybrGreen, Tm = 60.0 °C
Well(s): F1,F5,F12
Document: 08172018 KCL BMBM low v1_4 machine1.sds (Standard Curve)

Fig. S4-5a Melt curve results of the initial experiment in example #5.

The rt-PCR must be repeated with 4 µl template following the protocol in LIB 4486 when the sample Ct is 0. The repeated melt curve results are shown in Fig. S4-5b. Curve #1 is the negative control (red), #2 is the positive control (blue), and #3 is the sample (green). The repeated sample Tm is 84.1 °C with Ct=0. As the repeated sample Tm falls outside of the acceptable ovine range, the result is determined to be ovine negative. This template is a replicate of BMBM DNA at

the fractional level (0.0001%, v/v), and a false positive result using ovine primers has been avoided.

Note: In the repeated experiment, the sample melt curve is still a nearly flat line, like that of the negative control (Fig. S4-5b), indicating that there is probably no PCR amplification in the repeated experiment either.

Detector = SybrGreen, Tm = 60.0 °C
Well(s): D3,F1,F12
Document: 10082018kcl BM qtx v1_4 machine2.sds (Standard Curve)

Fig. S4-5b Melt curve results of the repeated experiment in example #5.