Laboratory of Medical Zoology
Pathogen Testing Standard Operating Protocol

Ver 3.1 Modified Date: 8/29/2016

Receipt and Custody of specimens

Ticks sent by US Mail arrive on weekdays in the morning. Ticks sent by courier (UPS or FedEx) may arrive throughout the business day. Ticks may also be dropped off at the Reception Office (Rm 101).

Incoming ticks:

- Ticks submitted to TickReport.com usually come one or two per shipment and have an accompanying TickReport order number (TR#) assigned to the tick through the system until the point when photographs (dorsal and ventral) are taken (In-Processing of specimens step 4 below). A unique tick ID number is assigned to each tick at this point.
- Ticks submitted by contractor agencies will come in large batches. Open the container(s) and ensure all samples listed on the manifest have been received and are in good condition (e.g., not broken or missing; labels are legible). Notify the Contract Officer immediately of any problems with the shipment within 48 hours of receipt.
- After reconciling with TickReport.com or contractor manifest, ticks are then moved forward for In-Processing. Ticks from contract agencies should be accompanied with Internal Routing Sheet (Appendix A) indicating service required (photos, tests, etc.)

In-Processing of specimens

1. Each tick should be individually processed.
2. Carefully remove the tick from the bag/container it was sent in (with sterile forceps) and place in a plastic weigh boat.
3. Place the weigh boat with the tick under a microscope and identify the species, life stage, and feeding state (fed, no signs of feeding) using James Keirans Identification key.
4. For specimens requiring photo-micrograph (including all TickReport.com submissions), take a picture of both the dorsal and ventral sides of the tick. Tick

images are labeled according to the filename template 20150202_01_v_1.5x (where 20150202 is the Monday of that week, 01 is the tick number, v=ventral, 1.5x being the magnification on microscope.). Save pictures in a file for the week by selecting "Browse."

5. Contract ticks (those not submitted to TickReport.com) should be labeled with a FT-XXXXX number. The FT tick data should be input into FT database (see Appendix B for curating rules of FT Database).
6. Place one tick into a 2.0 ml safe-lock tubes (Qiagen Cat. No. 990381) containing one 5mm metal bead (VXB, 3/16" inch Diameter Stainless Steel 440C G16 Bearing Balls, Code: Kit12748). (Must use Qiagen or Eppendorf 2.0 ml Safe-Lock Tubes, Cheap tubes will crack after shaking). Place the tick ID number that it was assigned on the lid of the tube.
7. The samples will be stored at -80C. Once the TickReports have been activated, these specimens can be placed in line to be extracted.
8. Input tick identification information into the online database TickReport.

Total Nucleic Acid Extraction

Things to do before starting:

- This protocol was modified from Epicentre MasterPure Complete DNA and RNA Purification Kit Bulk Reagents (See Appendix C).
- Total Nucleic Acid Extractions are performed in the "dirty" room, Room B1.
- Total Nucleic Acid extractions are carried out on a bench that is cleaned daily with 10% Bleach.
- All waste from the DNA extraction is placed in a plastic "trash" container on the bench and then emptied in to the biohazard trash.
- There should be 1 extraction control for each set of extractions. This is typically the 30th tube in the set. This no template control allows us to monitor for DNA contamination in the extraction reagents as well as DNA contamination between samples due to human error or a problem with the protocol.
- Turn on and warm Hybridization Oven to 65C.
- Turn on and cool centrifuge to 4C.

1. Place the tube into a TissueLyser reaction-tube holder (24 samples/holder).
2. Place the tube holder and tubes into a -80C freezer overnight (or at least 1 hour). **DO NOT** freeze top and bottom plate of the adaptor, only freeze tube holder. (If you want to continue, I suggest preparing step 9 reagents).
3. Assemble the TissueLyser Adapter Set 2 x 24 using a bottom plate, the reaction-tube holder (with 2.0 ml safe-lock tubes containing one tick and one 5mm bead), and a top plate with sealing strips.

4. Ensure that the top and bottom plate fit securely, and make sure that the reaction tubes are sealed securely.
5. Make sure that two TissueLyser adapter sets are balanced. Distribute the reaction tubes evenly across the TissueLyser. Balance the lid by ensuring 2.0 ml safe-lock tubes are in all 4 corners of both 24 sample holders.
6. Place the assembled TissueLyser Adapter Sets into the TissueLyser holders (arms), and close the handles tightly. **DO NOT** use the TissueLyser with only one TissueLyser Adapter Set, since this will cause machine imbalance and decrease homogenization efficiency.
7. Homogenize the ticks: 23Hz, 1.5 minutes. Fully engorged ticks may need a second homogenization.
8. Take out smashed ticks and centrifuge at 14000rpm for 1 minute at 4oC.
9. Dilute 1 ul of 50 ug/ul Proteinase K (http://www.epibio.com; Proteinase K; MPRK09250 µg/µl) into 300 ul of 1X Tissue and Cell Lysis Solution (http://www.epibio.com; 2X T&C Lysis Buffer should be diluted to 1X using molecular grade water. 2X T&C Lysis Buffer MTC085H) for each sample. For 30 ticks, prepare master mixture: 1X Tissue and Cell Lysis Solution: 300 ul X 31 = 9300 ul; 50 ug/ul Proteinase K : 1 ul X 31 = 31 ul;
10. Take 301ul master mixture for one smashed tick. Double master mixture for fully engorged ticks.
11. Mix thoroughly and incubate at 65o C for 15 minutes; vortex every 5 minutes.
12. Place the samples on ice for 10 min. For engorged ticks, centrifuge for 10 min and transfer 400 ul of supernatant to a new tube.
13. Add 150 ul (200 ul for fully engorged ticks) of MPC Protein Precipitation Reagent (MPC Protein Precipitation; MMP03750 Bulk order) to 301 ul of lysed sample and vortex vigorously for 10 seconds.
14. Pellet the debris by centrifugation at 4 C for 10 minutes at 14,000 RPM in a microcentrifuge.
15. Transfer 350 ul of supernatant to a tube containing 500 ul of ice cold isopropanol. Invert the tube several (30-40) times. Store the protein pellets in the 4C fridge until all testing is complete.
16. Pellet the DNA/RNA by centrifugation at 4 C for 10 minutes at 14,000 RPM in microcentrifuge.
17. Carefully pour off the isopropanol without dislodging the nucleic acid pellet. You can remove all of the residual isopropanol with a pipette.
18. Rinse with 1000 ul 75% ice-cold ethanol, being careful to not dislodge the pellet. Centrifuge briefly if the pellet is dislodged. Remove all of the residual ethanol with a pipette.
19. Air-dry your pellets overnight in the bio-safety cabinet, or in the 65C incubator for 20 minutes. Use tube openers to separate tubes to prevent cross contamination. Resuspend the DNA/RNA in 40 ul of molecular grade water.
20. Store samples (29 ticks and 1 extraction control in general) at -20C in the freezer box and proceed to the next section for real-time PCR setup.

Design of Assays and Master Mix

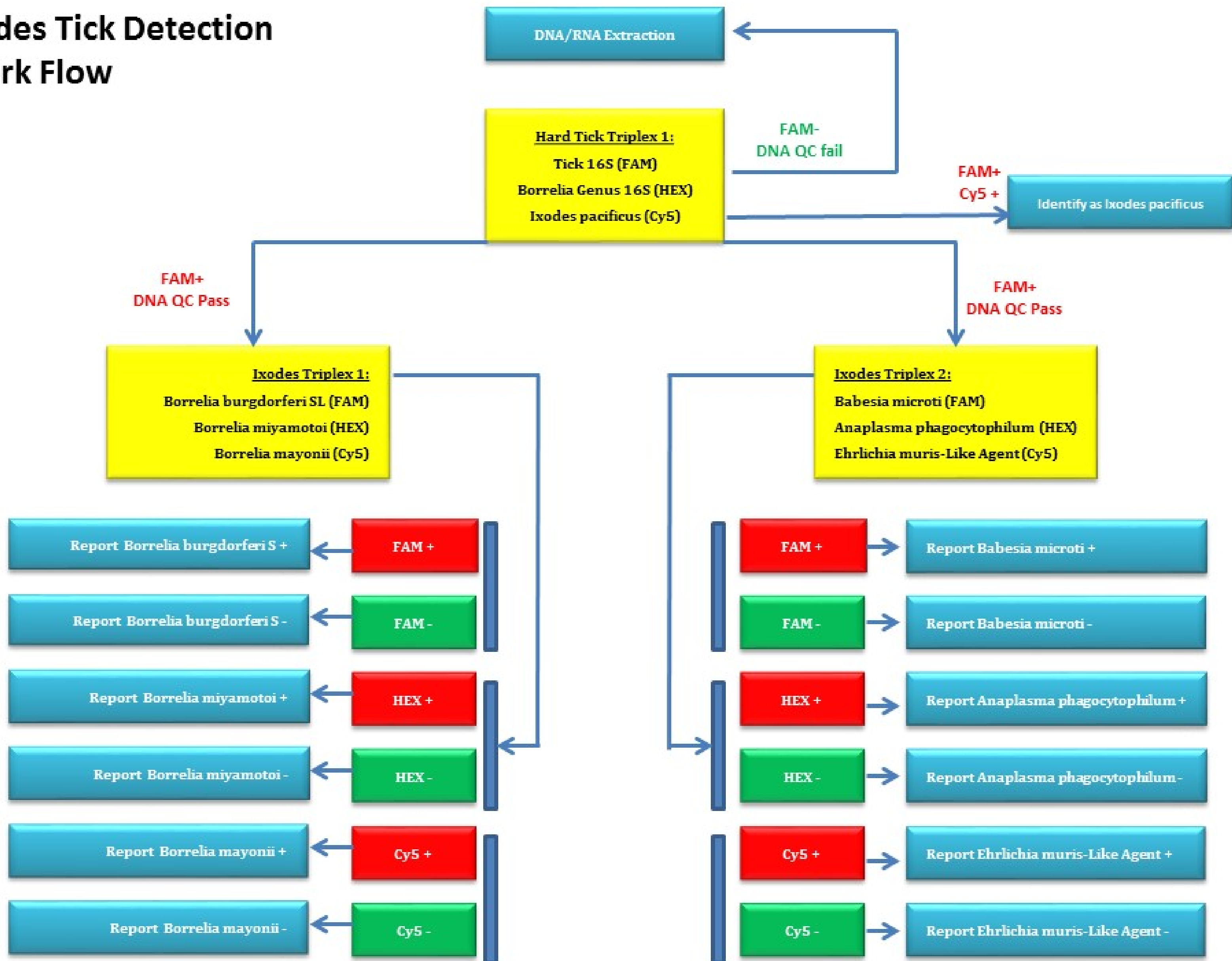
- We use triplex Taqman real-time PCR to detect tick pathogen DNAs: 3 triplex assays for Ixodes ticks and 3 triplex assay for non-Ixodes ticks.
- The multiplexing master mixes are using Agilent Brilliant III Ultra-Fast QPCR (Agilent Cat# 600880). The primers and dual-labeled probes are ordered from IDTDNA. Double-Quenched Probes contain a 5' fluorophore (FAM, HEX or Cy5); 3' IBFQ quencher; and proprietary, internal ZEN or TAO quencher.
- Hard tick 16S gene was used as an internal control for tick DNA quality control.
- Gene fragments (gBlocks) are ordered from IDTDNA as Taqman PCR positive QC controls. These gene fragments (gBlocks) are double-stranded, sequence-verified genomic blocks.
- Triplex master mix are made each in 50 ml conical tube. Six 15ul reaction from the master mix are tested QC at gBlock concentration 5 pg- 0.005pg for positive controls (3 reactions) and water for negative controls (3 reactions).
- If the master mixes pass the QC, they are aliquoted into 1.5 mL tubes and stored at -80C (About 100 reactions per tube, 15 ul per reaction). The master mixes that aren't used as frequently should be stored as smaller aliquots to limit the number of times each aliquot needs to be thawed and frozen (as this may reduce the efficacy of the master mix). Exposure to light can also damage the master mix. Each technician should have their own freezer box in the -20◦C freezer and they should remove a few tubes of the master mixes that they will need that week and place them at -20C. When preparing to run a plate, the appropriate mastermix tubes should be placed in a DNA-free tube rack at 4C to thaw.
- Tick pathogens for Ixodes and Non-Ixodes tick species, target genes and probe label dyes for each multiplexing are also listed below:

Ixodes and Nom-Ixodes tick pathogen detection assay

Tick species	Pathogen/tick Detection	Multiplexing	Target gene	Probe Label Dye
All hard ticks	DNA QC internal control	Hard Tick Triplex1	16S mtDNA	FAM
	Borrelia spp		16S	HEX
	Ixodes pacificus		COI	Cy5
Ixodes ticks	*Borrelia burgdorferi*	Ixodes Triplex1	ospA	FAM
	Borrelia miyamotoi		glpQ	HEX
	Borrelia mayonii		ospC	Cy5
Ixodes ticks	*Babesia microti*	Ixodes Triplex2	Tubulin	FAM
	Anaplasma phagocytophilum		MSP-2	HEX
	Ehrlichia muris-Like Agent		P13	Cy5
Non-Ixodes tick	*Ehrlichia chaffeensis*	Non-Ixodes Triplex1	dsb	FAM
	Borrelia lonestari		glpQ	HEX
	Ehrlichia ewingii		dsb	Cy5
Non-Ixodes tick	*Francisella tularensis*	Non-Ixodes Triplex2	ISFtu2	FAM
	Rickettsia rickettsii		RRi6	HEX
	Anaplasma phagocytophilum		MSP-2	Cy5

- Detection work flow are listed below:

Ixodes Tick Detection Work Flow



Non-Ixodes Tick Detection Work Flow

Setup a Sample Loading Map

- All sample are run as a 96-well plate format.
- Use a below sample as a template map. It contains 29 samples, 1 extraction control (EC), 1 super positive control (SPC) and 1 no template control (NTC) for 3 triplex assays (Hard Tick Triplex1, Ixodes Triplex1 and Ixodes Triplex 2).
- Print out the map and use this to determine which samples and master mixes need to be thawed at 4C in order to set up this plate.
- This printed map should also be labeled with the file name that contains the results and should be filed in the office according to month as well as a copy by contract/project name (if applicable).

  An example of sample loading map:

Sample ID	Well	Triplex	FAM	HEX	Cy5	Well	Triplex	FAM	HEX	Cy5	Well	Triplex	FAM	HEX	Cy5
1	A1	HTT1				A5	IT1				A9	IT2			
2	B1	HTT1				B5	IT1				B9	IT2			
3	C1	HTT1				C5	IT1				C9	IT2			
4	D1	HTT1				D5	IT1				D9	IT2			
5	E1	HTT1				E5	IT1				E9	IT2			
6	F1	HTT1				F5	IT1				F9	IT2			
7	G1	HTT1				G5	IT1				G9	IT2			
8	H1	HTT1				H5	IT1				H9	IT2			
9	A2	HTT1				A6	IT1				A10	IT2			
10	B2	HTT1				B6	IT1				B10	IT2			
11	C2	HTT1				C6	IT1				C10	IT2			
12	D2	HTT1				D6	IT1				D10	IT2			
13	E2	HTT1				E6	IT1				E10	IT2			
14	F2	HTT1				F6	IT1				F10	IT2			
15	G2	HTT1				G6	IT1				G10	IT2			
16	H2	HTT1				H6	IT1				H10	IT2			
17	A3	HTT1				A7	IT1				A11	IT2			
18	B3	HTT1				B7	IT1				B11	IT2			
19	C3	HTT1				C7	IT1				C11	IT2			
20	D3	HTT1				D7	IT1				D11	IT2			
21	E3	HTT1				E7	IT1				E11	IT2			
22	F3	HTT1				F7	IT1				F11	IT2			
23	G3	HTT1				G7	IT1				G11	IT2			
24	H3	HTT1				H7	IT1				H11	IT2			
25	A4	HTT1				A8	IT1				A12	IT2			
26	B4	HTT1				B8	IT1				B12	IT2			
27	C4	HTT1				C8	IT1				C12	IT2			
28	D4	HTT1				D8	IT1				D12	IT2			
29	E4	HTT1				E8	IT1				E12	IT2			
EC	F4	HTT1				F8	IT1				F12	IT2			
SPC	G4	HTT1				G8	IT1				G12	IT2			
NTC	H4	HTT1				H8	IT1				H12	IT2			

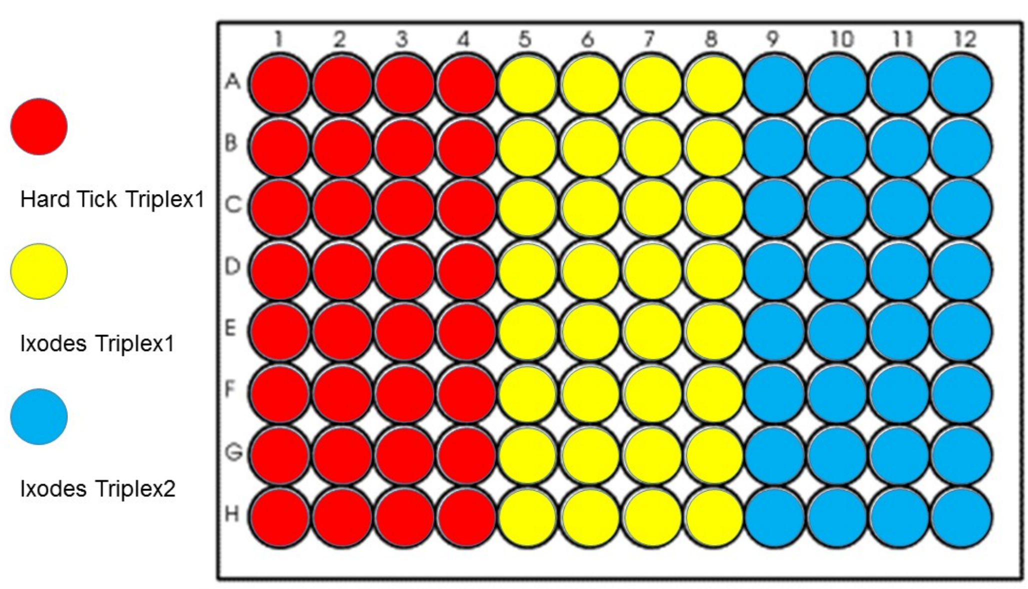
Date: Operator:

QPCR File Name: Note:

Real Time PCR Plate Setup

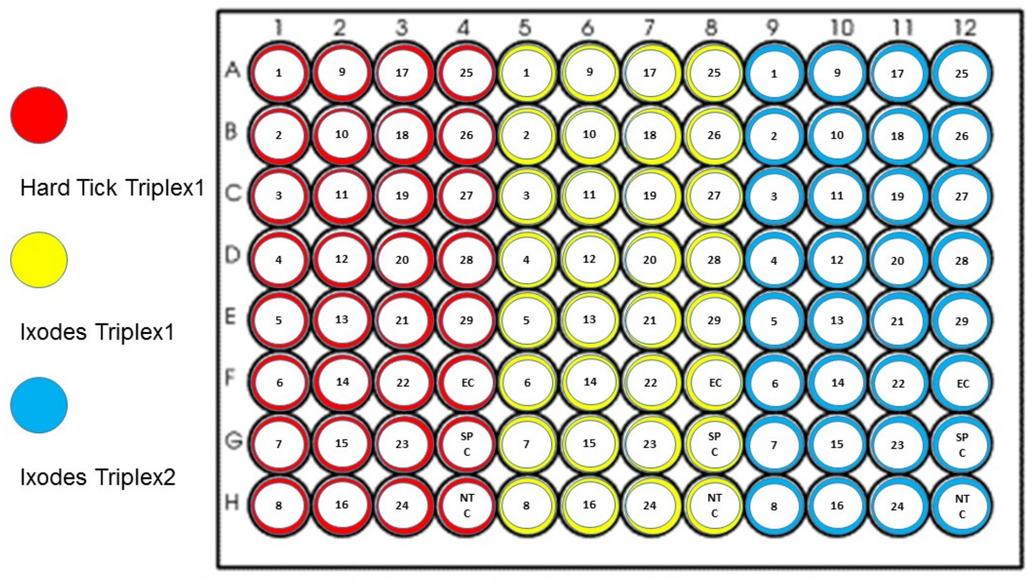
- This protocol was modified from Brilliant III Ultra-Fast QRT-PCR Master Mix MasterPure (See Appendix D).

1. Thaw the master mix.
2. Vortex briefly and spin down master mix. Put the master mix on ice.
3. Prepare a 96 well non-skirt PCR plate.
4. Add 15 µl of the master mix solutions, for example: Hard Tick Triplex1, Ixodes Triplex1 and Ixodes Triplex 2 into the appropriate wells based on the sample loading map, then bring the tubes back to their storage box at -20°C.



5. Add 1 ul of DNA template to each well, following the layout of the map, for example sample 1-29. When adding template, be sure NOT to open tubes of DNA over the plate. Pipette slowly to avoid contamination of other wells. Always hold pipette straight up and down (not tilted) to get the most accurate volume. Make sure that you are getting a NEW TIP for EVERY well and you are ejecting the tips ABOVE the waste container in the hood (don't let the pipette move below the edge of the container as this may cause contamination of the pipette with DNA). It is also helpful to move the template tube to a new tube rack and place in the spot corresponding to the last well it was added to on the 96-well plate.
6. Add 1ul extraction control (EC), 1 ul super positive control (SPC) and 1 ul water as NTC (No Template Control).

7. Seal the 96-well PCR plate with optically clear film. Make sure to press the film down around all edges and between wells to prevent sample evaporation.
8. Centrifuge the plate using a plate centrifuge, 800 g, 3 min. Proceed to MX3000P instrument instructions.



Instrument Settings and PCR run

Important notes before starting:

- The taqman detection is set up using the Agilent Mx3000P QPCR System. Standard filter sets include FAM/SYBR® Green I (492nm-516nm), HEX/JOE/VIC (535nm-555nm), ROX/Texas Red (585nm-610nm), Cy5 (635nm-665nm).
- ROX is used as a reference dye in all tests.

1. Open the “MxPro” program.
2. Select “Quantitative PCR (Multiple Standards)” as experiment/project type and select “Turn lamp on for warm up?” (warm-up takes about 20 minutes, so do this just before setting up your plate)
3. SETUP plate and thermal profile:
    a. Hit “All” to select all wells
    b. Well type- Unknown
    c. Select HEX, FAM, Cy5 and ROX under “Collect fluorescence data”
    d. Reference Dye: ROX

e. Thermal profile setup tab: Import from the file of the last plate run
f. 95 ◦C for 10 min (1 cycle)
g. 95 ◦C for 15 seconds, 60 ◦C for 60 seconds, END* (40 cycles). *Need endpoint to tell computer to read dye

4. File → Save As → C → Laboratory of Medical Zoology →
5. Name as: Date_Plate_# for day (20150105_Plate1)
6. You must write this plate name on qPCR map.
7. Carefully place the plate in the Agilent Mx3000P machine qPCR machine with A1 in the top left-hand corner.
8. Be sure to lock the metal cover down on top of the plate.
9. Verify correct settings in the template file.
10. Select RUN tab, be sure to specify if the light should turn off after the run. Select START. If the lamp is not warmed up yet, click warm-up then run.

Interpretation of results

Checklist for inspecting results:

- Hard Tick 16S internal control.
- Extraction control.
- Negative control.
- Super positive control.
- Amplification curve of each sample.

1. Once the program has finished, save the file and open to the Analysis tab.
2. Click on an individual well and then click Results.
    a. Check amplification curve and Ct values. The amplification curves of true positive targets are exponential. The results of the real-time PCR assays are based on cycle threshold (Ct) values obtained from the amplification curves of the targets. The Ct value represents the number of cycles required to reach a particular threshold fluorescence signal level. The fewer cycles it takes to obtain a detectable fluorescence level, the greater the amount of target in ticks.
    b. A positive sample should have an amplification curve and Ct values for FAM, HEX or Cy5. It is generally accepted that a 3-cycle difference in target Ct compared to the negative control Ct will reliably separate a true positive signal from a contamination result (Bustin, 2004). As the PCR protocol involves 40 cycles, Ct 37 is the appropriate 3 cycle different Ct value.

c. Ct values above 37, but below 40 may represent cases where target is present at very low levels. In this case, samples should be re-tested using 3 replicates.
d. Hard Tick 16S internal control: it will result in an amplification reaction if tick DNA was successfully extracted and PCR was correctly performed. The purpose of the Hard Tick 16S internal control is to confirm, for each sample and PCR reaction, that the DNA extraction quality and PCR reaction conditions were acceptable for the targets and that the plate setup (pipetting) was correctly performed.
e. Extraction control: it will result in an amplification curve if tick DNA contamination occurred during extraction step.
f. Negative control: it will result in no amplification cure. If the Ct value of the target in a negative control is less than 3 cycles apart from the Ct of the same target in a sample, that target in that sample fails to qualify as positive and must be disregarded in the analysis.
g. Super positive control: it consists of a control DNA template that will result in an amplification reaction if the PCR was correctly performed.

3. Record Ct value on the sample loading map.
4. Input the Ct values into the tick database along with tick identification data.

Appendix A: Statement of Work template

Agency Order #________

Statement of Work
PCR Testing for
Borrelia burgdorferi, Anaplasma phagocytophilum, and Babesia microti

University of Massachusetts – Amherst and CONTRACTOR AGENCY

The CONTRACTOR NAME and Laboratory of Medical Zoology, University of Massachusetts-Amherst (UMass), shall furnish the necessary personnel and otherwise do all things for or incidental to the performance of the work set forth in sections (a) through (c).

Tasks

(a) CONTRACTOR AGENCY shall coordinate the collection and submission of *Ixodes* and *Dermacentor* species ticks from environmental surveillance to UMass. CONTRACTOR AGENCY shall supply all necessary collection and shipping materials as well as covering the cost of sample shipment to UMass.

(b) CONTRACTOR AGENCY shall provide instruction on proper collection, packaging and shipping protocols to staff who conduct surveillance for ticks for testing at UMass. UMass shall notify CONTRACTOR AGENCY of discrepancies noted in packaging or shipping that impact the suitability of ticks for testing.

(c) UMass shall analyze all *Ixodes* spp. ticks received for *Borrelia burgdorferi*, *Borrelia miyamotoi*, *Anaplasma phagocytophilum,* and *Babesia microti* by PCR. UMass shall analyze all *Dermacentor* ticks received for *Francisella tularensis* and *Rickettsia rickettsii*. Should extra funds be available and if agreed upon by both parties,. UMass will report all tick test results to CONTRACTOR within two weeks of receiving tick pools.

Deliverable

UMass will report all findings to CONTRACTOR AGENCY within two weeks of receiving pools. Positive findings shall be reported to one of the following people, listed preferentially:

Dr. XXXX Phone: XXX.XXX.XXXX Email XXX@XXX.XXXX

Period of Performance

The period of performance under this statement of work shall be from Day/Month/Year through Day/Month/Year unless sooner terminated by either party upon 30 days written notice.

Payment

CONTRACTOR AGENCY will pay UMass the following rates per tick sample:

Tick Genus	Price per Sample
Ixodes ticks (pathogen testing for B. burgdorferi, B. miyamotoi, A. phagocytophilum, and Ba. microti	$XX.00

CONTRACTOR AGENCY will pay UMass within 30 days after receipt of an invoice voucher and verification of the number of pools tested from review of testing reports.

Dr. Stephen M. Rich
Director
Laboratory of Medical Zoology

Contract Administrator
CONTRACTOR AGENCY

Agency Order #________

Work Order Checklist

Sample condition when arriving at LMZ

☐ Live ☐ Dead

Additional solvents/liquids (water. EtOH)? __________

Identified by species? ☐ Yes ☐ No

Electronic data sheet from contractor? ☐ Yes ☐ No

Photomicrograph documentation of tick requested?

☐ No ☐ Yes(☐ Dorsal/ ☐ Ventral)

Tests to be applied:

☐ Borrelia burgdorferi sensu latu

☐ Borrelia miyamotoi

☐ Borrelia mayonii

☐ Ehrlichia muris-Like Agent

☐ Babesia microti

☐ Anaplasma phagocytophilum

☐ Rickettsia rickettsii

☐ Francisella tularensis

☐ Ehrlichia chaffeensi

☐ Ehrlichia canis

☐ Bartonella henselae

☐ Babesia divergens

☐ Powassan virus

☐ Other ____

Results on Contractor Form? ☐ Yes ☐ No

Report Due Date ______________________

Appendix B: FT Database sample

	Date Collected													Extraction Date									RLB Genotypes																				RLB Note	Pathogen Testing (qPCR Ct Values)					Project ID
tickID	Day	Month	Year	Collector	Page	Sample	TickSp.	Stage	Host	Location1	Town	State	Comments	Day	Month	Year	Extractor initials	Batch/ECID	DaughterPlate#	Results	Notes	PCR(+)	**ALL**	**A**	**B**	**D**	**E**	**F**	**G**	**H**	**I**	**J**	**K**	**L**	**M**	**N**	**O**	**T**	**U**	**V**	**W**	**null type**		Tick Internal	*B. burgdorferi*	*B. microti*	*A. phagocytophilum*	Treated/Control	

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Appendix C

MasterPure™ Complete DNA and RNA Purification Kit Bulk Reagents

Cat. Nos. MRC0912H, MTC096H, MTC085H, MMP095H, MMP03750, MRNA092, D9905K, MPRK092, MTE0970, and MBD092H

Connect with Epicentre on our blog (**epicentral.blogspot.com**), Facebook (**facebook.com/EpicentreBio**), and Twitter (**@EpicentreBio**).

1. Introduction

The MasterPure™ Complete DNA and RNA Purification Kit Bulk Reagents are provided in sufficient quantities to purify DNA from up to 2,000 samples using the MasterPure Complete DNA Purification Kit protocol (included in this product literature) or up to 1,000 RNA purifications using the MasterPure RNA Purification Kit protocol.

2. Product Specifications

Storage: Upon arrival, store the enzymes (Proteinase K, RNase A, and RNase-Free DNase I) at –20°C in a freezer without a defrost cycle. Store the remainder of the kit at room temperature. Dispense the 1X DNase Buffer aseptically.

Functional Assay: Reagents for the MasterPure Complete DNA and RNA Purification Kit are assayed by purifying total nucleic acid, DNA, and RNA from *E. coli* according to the protocols described in the MasterPure Complete DNA and RNA Purification Kit package insert. The purified nucleic acid is examined by electrophoresis, spectrophotometry, and fluorimetry for purity and concentration.

3. Kit Contents

Cat. #	Concentration	Quantity
Red Cell Lysis Solution		
MRC0912H		1,200 ml
Tissue and Cell Lysis Solution		
MTC096H		600 ml
2X T&C Lysis Buffer		
MTC085H		500 ml
MPC Protein Precipitation Reagent		
MMP03750		50 ml
MPC Protein Precipitation Reagent		
MMP095H		500 ml
RNase A		
MRNA092	@ 5 µg/µl	2 ml
(50% glycerol, 25 mM NaOAc [pH 4.6])		
DNase I, RNase-Free		
D9905K	@ 1 U/µl	5 ml
(50% glycerol, 10 mM Tris-HCl [pH 7.5],10 mM $CaCl_2$, 10 mM $MgCl_2$)		
Proteinase K		
MPRK092	@ 50 µg/µl	2 ml
(DNase- and RNase-Free)(50% glycerol, 50 mM Tris-HCl [pH 7.5], 0.1 M NaCl, 0.1 mM EDTA, 1 mM DTT, 10 mM $CaCl_2$, 0.1% Triton® X-100)		
TE Buffer		
MTE0970		70 ml
(10 mM Tris-HCl [pH 7.5], 1 mM EDTA)		
1X DNase Buffer		
MBD092H		200 ml
(33 mM Tris-HCl [pH 7.8], 66 mM KOAc,10 mM $Mg[OAc]_2$, 0.5 mM DTT)		

4. Related Products

The following products are also available:

– MasterPure™ DNA Purification Kits
– MasterPure™ RNA Purification Kits
– BuccalAmp™ DNA Extraction Kits
– MasterAmp™ PCR Optimization Kits
– MasterAmp™ *Taq, Tth, Tfl,* and AmpliTherm™ DNA Polymerases
– dNTP Solutions

5. MasterPure™ Complete DNA and RNA Purification Kit Protocol

The following protocols are from the MasterPure Complete DNA and RNA Purification Kit (Cat Nos. MC85200 and MC89010)

6. General Considerations

1. **Tissue Sources:** We have used the kit to isolate nucleic acid from a variety of sources including: bovine liver, human HL-60 tissue culture cells, paraffin-embedded breast tumor tissue (see below), human whole blood and plasma, saliva; mouse tail, corn and geranium leaf, *E. coli*, and lambda phage. Tissues other than those mentioned here are likely to be compatible with the kit with some optimization.
2. **Isolation of DNA from Paraffin-Embedded Tissue:** DNA isolated from preserved, paraffin-embedded tissues is generally of poor quality. The degree of degradation of these samples limits analysis mainly to techniques involving amplification. To obtain DNA from embedded tissues that is amenable to PCR, we recommend preserving the tissues in either acetone, 95% ethanol, or 95% buffered formalin, with fixation times of less than 24 hours.[2] Choose PCR primers such that the resultant amplification products are less than or equal to 300 bp in length. The use of xylene or Hemo-D to extract the paraffin has been shown to increase DNA yields, and an alternate protocol is provided. Generally, we do not recommend using the kit to isolate RNA from embedded tissues, as the RNA in these samples is likely to be substantially degraded. Nevertheless, you can use the kit for that application by following the protocol outlined on page 9.
3. **Sample Size:** You can purify nucleic acid from samples of various sizes by proportionally adjusting the amount of reagents to the amount of starting material. With larger samples, centrifugation conditions (time and speed) may also need to be adjusted.
4. **Proteinase K Treatment:** We recommend including the Proteinase K treatment to increase the efficiency of lysis, though for some samples this treatment is unnecessary (e.g., blood). If minimizing the time of purification is desirable, you may determine if Proteinase K treatment is required.
5. **Nuclease Treatment:** The removal of RNA from DNA preparations with RNase A, or the removal of DNA from RNA preparations with RNase-Free DNase I, is unnecessary for many applications. These steps may be eliminated from the protocol depending upon the intended use of the DNA or RNA. If the removal of contaminating nucleic acid is necessary, we recommend performing these steps as outlined in the protocol.

Note, however, for some samples, adjustments in nuclease concentration or time of incubation may improve the quality of the purified nucleic acid.

6. **Complete RNA Removal:** If complete removal of RNA is required for your application, refer to the Complete RNA Removal protocol (Part O).

7. Total Nucleic Acids Purification Protocols

The following protocol is provided for the purification of total nucleic acids from several biological sources (see General Considerations). Lyse the fluid or tissue as outlined in Part A, and then proceed with the remainder of the protocol as outlined in Part B. Additional purification protocols begin on page 11. If complete removal of RNA is required for your application, follow the protocol for Complete Removal of RNA (Part O).

A. Lysis of Fluid or Tissue Samples

Thoroughly mix the various Lysis Solutions to ensure uniform composition before dispensing.

Fluid Samples (e.g., saliva, semen)

1. Collect samples and either process immediately or freeze at –70°C.
2. Dilute 1 µl of Proteinase K into 150 µl of 2X T and C Lysis Solution for each sample.
3. Transfer 150 µl of the fluid sample to a microcentrifuge tube, add 150 µl of 2X T and C Lysis Solution containing the Proteinase K and mix thoroughly.
4. Incubate at 65°C for 15 minutes; vortex every 5 minutes.
5. Place the samples on ice for 3-5 minutes and then proceed with total nucleic acid precipitation in Part B.

Cell Samples (e.g., mammalian cell culture, buccal cells, *E. coli*)

1. Dilute 1 µl of Proteinase K, into 300 µl of Tissue and Cell Lysis Solution for each sample.
2. Pellet cells by centrifugation (0.5-1 x 10^6 mammalian cells; 0.1-0.5 ml of an overnight culture of *E. coli*) and discard the supernatant, leaving approximately 25 µl of liquid.
3. Vortex for 10 seconds to resuspend the cell pellet.
4. Add 300 µl of Tissue and Cell Lysis Solution containing the Proteinase K and mix thoroughly.
5. Incubate at 65°C for 15 minutes; vortex every 5 minutes.
6. Place the samples on ice for 3-5 minutes and then proceed with total nucleic acid precipitation in Part B.

Tissue Samples (e.g., plant or animal tissues)

1. Collect 1-5 mg of tissue and either process immediately or freeze the samples at –70°C.
2. Dilute 1 µl of Proteinase K into 300 µl of Tissue and Cell Lysis Solution for each sample.

3. Homogenize fresh tissue or grind frozen tissues in liquid nitrogen and transfer to a microcentrifuge tube.
4. Add 300 µl of Tissue and Cell Lysis Solution containing the Proteinase K and mix thoroughly.
5. Incubate at 65°C for 15 minutes; vortex every 5 minutes.
6. Place the samples on ice for 3-5 minutes and then proceed with total nucleic acid precipitation in Part B.

Whole-Blood Samples (with RBC lysis)

1. Draw 5 ml of blood into an EDTA Vacutainer® tube. Transfer 200 µl of whole blood into a microcentrifuge tube.
2. Add 600 µl of Red Cell Lysis Solution. Invert three times to mix and then flick the bottom of the tube to suspend any remaining material.
3. Incubate at room temperature for 5 minutes and then vortex briefly. Continue incubating at room temperature for an additional 5 minutes followed again by brief vortexing.
4. Pellet the white blood cells by centrifugation for 25 seconds in a microcentrifuge.
5. Remove most of the supernatant, leaving approximately 25 µl of liquid. Vortex to suspend the pellet.
6. Resuspend the white blood cells in 300 µl of Tissue and Cell Lysis Solution by pipetting the cells several times.
7. Place the samples on ice for 3-5 minutes and then proceed with total nucleic acid precipitation in Part B (below).

Formalin-Fixed, Paraffin-Embedded (FFPE) Tissues (see General Considerations)

1. Remove a section of tissue using a clean microtome blade; if possible, trim excess paraffin.
2. Place 1-50 mg of 10- to 35-µm thick paraffin sections into an appropriate tube. If using a larger amount of tissue, adjust the reagent volumes accordingly.
3. Dilute 2 µl of Proteinase K into 300 µl of Tissue and Cell Lysis Solution for each sample, and mix.
4. Add 300 µl of Tissue and Cell Lysis Solution containing the Proteinase K to the sample and mix.
5. Incubate at 65°C for 30 minutes; followed by a brief (10 seconds) vortex mix.
6. Place the samples on ice for 3-5 minutes and then proceed with total nucleic acid precipitation in Part B.

8. Alternate Protocol for Formalin-Fixed, Paraffin-Embedded (FFPE) Tissues

Note: *This protocol uses xylene or Hemo-D to extract the paraffin.*

1. Remove a section of tissue using a clean microtome blade; if possible, trim excess paraffin.
2. Place 10-50 mg of 10- to 35-µm thick paraffin sections into an appropriate tube. If using a larger amount of tissue, adjust the reagent volumes accordingly.
3. Add 1-5 ml of xylene or Hemo-D to extract the paraffin and incubate at room temperature for 10 minutes. Pour off the solvent.
4. Repeat Step 3.
5. Add 1-5 ml of 100% ethanol and incubate at room temperature for 10 minutes. Pour off the ethanol.
6. Repeat Step 5.
7. Aspirate all of the remaining ethanol.
8. Dilute 2 µl of Proteinase K into 300 µl of Tissue and Cell Lysis Solution for each sample, and mix.
9. Add 300 µl of Tissue and Cell Lysis Solution containing the Proteinase K to the sample and mix.
10. Incubate at 65°C for 30 minutes; followed by a brief (10 seconds) vortex mix.
11. Place the samples on ice for 3-5 minutes and then proceed with total nucleic acid precipitation in Part B.

B. Precipitation of Total Nucleic Acids (for all biological samples)

1. Add 150 µl of MPC Protein Precipitation Reagent to 300 µl of lysed sample and vortex vigorously for 10 seconds.
2. Pellet the debris by centrifugation at 4°C for 10 minutes at ≥10,000 x g in a microcentrifuge. If the resultant pellet is clear, small, or loose, add an additional 25 µl of MPC Protein Precipitation Reagent, mix, and pellet the debris again.
3. Transfer the supernatant to a clean microcentrifuge tube and discard the pellet.
4. Add 500 µl of isopropanol to the recovered supernatant. Invert the tube 30-40 times.
5. Pellet the total nucleic acids by centrifugation at 4°C for 10 minutes in a microcentrifuge.
6. Carefully pour off the isopropanol without dislodging the pellet.
7. Rinse twice with 70% ethanol, being careful to not dislodge the pellet. Centrifuge briefly if the pellet is dislodged. Remove all of the residual ethanol with a pipet.
8. Resuspend the total nucleic acids in 35 µl of TE Buffer.

9. DNA Purification Protocols

The following protocol is provided for the purification of DNA from several biological sources (see General Considerations). Lyse the fluid or tissue as outlined in Part C, and then proceed with the remainder of the protocol as outlined in Part D. Additional purification protocols begin on page 10. If complete removal of RNA is required for your application, follow the protocol for Complete Removal of RNA (Part O).

C. Lysis of Fluid or Tissue Samples

Thoroughly mix the various Lysis Solutions to ensure uniform composition before dispensing.

Fluid Samples (e.g., saliva, semen)

1. Collect samples and either process immediately or freeze at –70°C.
2. Dilute 1 µl of Proteinase K into 150 µl of 2X T and C Lysis Solution for each sample.
3. Transfer 150 µl of the fluid sample to a microcentrifuge tube, add 150 µl of 2X T and C Lysis Solution containing the Proteinase K, and mix thoroughly.
4. Incubate at 65°C for 15 minutes; vortex every 5 minutes.
5. Cool the samples to 37°C and add 1 µl of 5 µg/µl RNase A to the sample; mix thoroughly.
6. Incubate at 37°C for 30 minutes.
7. Place the samples on ice for 3-5 minutes and then proceed with total DNA precipitation in Part D.

Cell Samples (e.g., mammalian cell culture, buccal cells, *E. coli*)

1. Dilute 1 µl of Proteinase K into 300 µl of Tissue and Cell Lysis Solution for each sample.
2. Pellet cells by centrifugation (0.5-1 x 10^6 mammalian cells; 0.1-0.5 ml of an overnight culture of *E. coli*) and discard the supernatant, leaving approximately 25 µl of liquid.
3. Vortex for 10 seconds to resuspend the cell pellet.
4. Add 300 µl of Tissue and Cell Lysis Solution containing the Proteinase K and mix thoroughly.
5. Incubate at 65°C for 15 minutes; vortex every 5 minutes.
6. Cool the samples to 37°C and add 1 µl of 5 µg/µl RNase A to the sample; mix thoroughly.
7. Incubate at 37°C for 30 minutes.
8. Place the samples on ice for 3-5 minutes and then proceed with total DNA precipitation in Part D.

Tissue Samples (e.g., plant or animal tissues)

1. Collect 1-5 mg of tissue and either process immediately or freeze the samples at –70°C.
2. Dilute 1 µl of Proteinase K into 300 µl of Tissue and Cell Lysis Solution for each sample.
3. Homogenize fresh tissue or grind frozen tissues in liquid nitrogen and transfer to a microcentrifuge tube.

4. Add 300 µl of Tissue and Cell Lysis Solution containing the Proteinase K and mix thoroughly.
5. Incubate at 65°C for 15 minutes; vortex every 5 minutes.
6. Cool the samples to 37°C and add 1 µl of 5 µg/µl RNase A to the sample; mix thoroughly.
7. Incubate at 37°C for 30 minutes.
8. Place the samples on ice for 3-5 minutes and then proceed with total DNA precipitation in Part D.

Whole-Blood Samples (with RBC lysis)

1. Draw 5 ml of blood into an EDTA Vacutainer tube. Transfer 200 µl of whole blood into a microcentrifuge tube.
2. Add 600 µl of Red Cell Lysis Solution. Invert three times to mix and then flick the bottom of the tube to suspend any remaining material.
3. Incubate at room temperature for 5 minutes and then vortex briefly. Continue incubating at room temperature for an additional 5 minutes followed again by brief vortexing.
4. Pellet the white blood cells by centrifugation for 25 seconds in a microcentrifuge.
5. Remove most of the supernatant, leaving approximately 25 µl of liquid. Vortex to suspend the pellet.
6. Resuspend the white blood cells in 300 µl of Tissue and Cell Lysis Solution by pipetting the cells several times.
7. Add 1 µl of RNase A and mix thoroughly.
8. Incubate at 37°C for 30 minutes.
9. Place the samples on ice for 3-5 minutes and then proceed with total DNA precipitation in Part D.

Formalin-Fixed, Paraffin-Embedded (FFPE) Tissues (see General Considerations)

1. Remove a section of tissue using a clean microtome blade; if possible, trim excess paraffin.
2. Place 10-50 mg of 10- to 35-µm thick paraffin sections into an appropriate tube. If using a larger amount of tissue, adjust the reagent volumes accordingly.
3. Dilute 2 µl of Proteinase K into 300 µl of Tissue and Cell Lysis Solution for each sample, and mix.
4. Add 300 µl of Tissue and Cell Lysis Solution containing the Proteinase K to the sample and mix.
5. Incubate at 65°C for 30 minutes; followed by a brief (10 seconds) vortex mix.
6. Cool the samples to 37°C and add 1 µl of RNase A to the sample; mix thoroughly.
7. Incubate at 37°C for 30 minutes.
8. Place the samples on ice for 3-5 minutes and then proceed with total DNA precipitation in Part D.

Alternate Protocol for Formalin-Fixed, Paraffin-Embedded (FFPE) Tissues

Note: *this protocol uses xylene or Hemo-D to extract the paraffin.*

1. Remove a section of tissue using a clean microtome blade; if possible, trim excess paraffin.
2. Place 10-50 mg of 10- to 35-µm thick paraffin sections into an appropriate tube. If using a larger amount of tissue, adjust the reagent volumes accordingly.
3. Add 1-5 ml of xylene or Hemo-D to extract the paraffin and incubate at room temperature for 10 minutes. Pour off the solvent.
4. Repeat Step 3.
5. Add 1-5 ml of 100% ethanol and incubate at room temperature for 10 minutes. Pour off the ethanol.
6. Repeat Step 5.
7. Aspirate all of the remaining ethanol.
8. Dilute 2 µl of Proteinase K into 300 µl of Tissue and Cell Lysis Solution for each sample, and mix.
9. Add 300 µl of Tissue and Cell Lysis Solution containing the Proteinase K to the sample and mix.
10. Incubate at 65°C for 30 minutes; followed by a brief (10 seconds) vortex mix.
11. Cool the samples to 37°C and add 1 µl of RNase A to the sample; mix thoroughly.
12. Incubate at 37°C for 30 minutes.
13. Place the samples on ice for 3-5 minutes and then proceed with DNA precipitation in Part D.

D. Precipitation of Total DNA (for all biological samples)

1. Add 150 µl of MPC Protein Precipitation Reagent to 300 µl of lysed sample and vortex vigorously for 10 seconds.
2. Pellet the debris by centrifugation at 4°C for 10 minutes at ≥10,000 x g in a microcentrifuge. If the resultant pellet is clear, small, or loose, add an additional 25 µl of MPC Protein Precipitation Reagent, mix, and pellet the debris again.
3. Transfer the supernatant to a clean microcentrifuge tube and discard the pellet.
4. Add 500 µl of isopropanol to the recovered supernatant. Invert the tube 30-40 times.
5. Pellet the DNA by centrifugation at 4°C for 10 minutes in a microcentrifuge.
6. Carefully pour off the isopropanol without dislodging the DNA pellet.
7. Rinse twice with 70% ethanol, being careful to not dislodge the pellet. Centrifuge briefly if the pellet is dislodged. Remove all of the residual ethanol with a pipet.
8. Resuspend the DNA in 35 µl of TE Buffer.

10. RNA Purification Protocols

The following protocol is provided for the purification of RNA from several biological sources (see General Considerations). Lyse the fluid or tissue as outlined in Part E, and then proceed with the remainder of the protocol as outlined in Part F. If further purification of RNA (to remove contaminating DNA) is required, follow the protocol outlined in Part G. Use appropriate techniques to minimize degradation by exogenous ribonucleases. Additional purification protocols begin on page 11.

E. Lysis of Fluid or Tissue Samples

Thoroughly mix the various Lysis Solutions to ensure uniform composition before dispensing.

Fluid Samples (e.g., saliva, semen)

1. Collect samples and either process immediately or freeze at –70°C.
2. Dilute 1 µl of Proteinase K into 150 µl of 2X T and C Lysis Solution for each sample.
3. Transfer 150 µl of the fluid sample to a microcentrifuge tube, add 150 µl of 2X T and C Lysis Solution containing the Proteinase K, and mix thoroughly.
4. Incubate at 65°C for 15 minutes; vortex every 5 minutes.
5. Place the samples on ice for 3-5 minutes and then proceed with total nucleic acid precipitation in Part F.

Cell Samples (e.g., mammalian cell culture, buccal cells, *E. coli*)

1. Dilute 1 µl of Proteinase K into 300 µl of Tissue and Cell Lysis Solution for each sample.
2. Pellet cells by centrifugation (0.5-1 x 10^6 mammalian cells; 0.1-0.5 ml of an overnight culture of *E. coli*) and discard the supernatant, leaving approximately 25 µl of liquid.
3. Vortex for 10 seconds to resuspend the cell pellet.
4. Add 300 µl of Tissue and Cell Lysis Solution containing the Proteinase K and mix thoroughly.
5. Incubate at 65°C for 15 minutes; vortex every 5 minutes.
6. Place the samples on ice for 3-5 minutes and then proceed with total nucleic acid precipitation in Part F.

Tissue Samples (e.g., plant or animal tissues)

1. Collect 1-5 mg of tissue and either process immediately or freeze the samples at –70°C.
2. Dilute 1 µl of Proteinase K into 300 µl of Tissue and Cell Lysis Solution for each sample.
3. Homogenize fresh tissue or grind frozen tissues in liquid nitrogen and transfer to a microcentrifuge tube.
4. Add 300 µl of Tissue and Cell Lysis Solution containing the Proteinase K and mix thoroughly.
5. Incubate at 65°C for 15 minutes; vortex every 5 minutes.
6. Place the samples on ice for 3-5 minutes and then proceed with total nucleic acid precipitation in Part F.

Whole-Blood Samples (with RBC lysis)

1. Draw 5 ml of blood into an EDTA Vacutainer tube. Transfer 200 µl of whole blood into a microcentrifuge tube.
2. Add 600 µl of Red Cell Lysis Solution. Invert three times to mix and then flick the bottom of the tube to suspend any remaining material.
3. Incubate at room temperature for 5 minutes and then vortex briefly. Continue incubating at room temperature for an additional 5 minutes followed again by brief vortexing.
4. Pellet the white blood cells by centrifugation for 25 seconds in a microcentrifuge.
5. Remove most of the supernatant, leaving approximately 25 µl of liquid. Vortex to suspend the pellet.
6. Resuspend the white blood cells in 300 µl of Tissue and Cell Lysis Solution by pipetting the cells several times.
7. Place the samples on ice for 3-5 minutes and then proceed with total nucleic acid precipitation in Part F.

Formalin-Fixed, Paraffin-Embedded (FFPE) Tissues

1. Remove a section of tissue using a clean microtome blade; if possible, trim excess paraffin.
2. Place 2-30 mg of 10- to 35-µm thick paraffin sections into an appropriate tube. If using a larger amount of tissue, adjust the reagent volumes accordingly.
3. Dilute 2 µl of Proteinase K into 300 µl of Tissue and Cell Lysis Solution for each sample, and mix.
4. Add 300 µl of Tissue and Cell Lysis Solution containing the Proteinase K to the sample and mix.
5. Incubate at 65°C for 30 minutes; followed by a brief (10 seconds) vortex mix.
6. Place the samples on ice for 3-5 minutes and then proceed with total DNA precipitation in Part F.

F. Precipitation of Total Nucleic Acids (for all biological samples)

1. Add 150 µl of MPC Protein Precipitation Reagent to 300 µl of lysed sample and vortex vigorously for 10 seconds.
2. Pellet the debris by centrifugation at 4°C for 10 minutes at ≥10,000 x g in a microcentrifuge. If the resultant pellet is clear, small, or loose, add an additional 25 µl of MPC Protein Precipitation Reagent, mix, and pellet the debris again.
3. Transfer the supernatant to a clean microcentrifuge tube and discard the pellet.
4. Add 500 µl of isopropanol to the recovered supernatant. Invert the tube 30-40 times.
5. Pellet the total nucleic acids by centrifugation at 4°C for 10 minutes in a microcentrifuge.
6. Carefully pour off the isopropanol without dislodging the total nucleic acid pellet. If removal of contaminating DNA from the RNA is required, proceed with Part G. Otherwise, complete the remainder of Part F.
7. Rinse twice with 70% ethanol, being careful to not dislodge the total nucleic acid pellet. Centrifuge briefly if the pellet is dislodged. Remove all of the residual ethanol with a pipet.
8. Resuspend the total nucleic acids in 35 µl of TE Buffer.

G. Removal of Contaminating DNA from Total Nucleic Acids Preparations
(for all biological samples)

1. Remove all of the residual isopropanol with a pipet.
2. Prepare 200 µl of DNase I solution for each sample by diluting 5 µl of RNase-Free DNase I up to 200 µl with 1X DNase Buffer.
3. Completely resuspend the total nucleic acid pellet in 200 µl of DNase I solution.
4. Incubate at 37°C for 10 minutes.

   Note: *Additional incubation (up to 30 minutes) may be necessary to remove all contaminating DNA.*
5. Add 200 µl of 2X T and C Lysis Solution; vortex for 5 seconds.
6. Add 200 µl of MPC Protein Precipitation Reagent; vortex 10 seconds; place on ice for 3-5 minutes.
7. Pellet the debris by centrifugation at 4°C for 10 minutes at ≥10,000 x g in a microcentrifuge.
8. Transfer the supernatant containing the RNA into a clean microcentrifuge tube and discard the pellet.
9. Add 500 µl of isopropanol to the supernatant. Invert the tube 30-40 times.
10. Pellet the purified RNA by centrifugation at 4°C for 10 minutes in a microcentrifuge.
11. Carefully pour off the isopropanol without dislodging the RNA pellet.
12. Rinse twice with 70% ethanol, being careful to not dislodge the pellet. Centrifuge briefly if the pellet is dislodged. Remove all of the residual ethanol with a pipet.
13. Resuspend the RNA in 10-35 µl of TE Buffer.
14. Add 1 µl of RiboGuard™ RNase Inhibitor (optional).

11. Additional Purification Protocols

The following protocol is provided for the purification of total nucleic acid, DNA, or RNA from plasma. Lyse the plasma as outlined in Part H; if isolating DNA, users may add an optional RNase A step following treatment with Proteinase K (see Protocols for DNA Purification). Precipitate the nucleic acid as outlined in Part I. If further purification of RNA (to remove contaminating DNA) is required, follow the protocol outlined in Part N. For the purification of RNA, use appropriate techniques to minimize degradation by exogenous ribonucleases.

H. Lysis of Plasma

Thoroughly mix the Tissue and Cell Lysis Solution to ensure uniform composition before dispensing.

1. Collect plasma samples. Transfer 50 µl of plasma into a microcentrifuge tube.
2. Dilute 1 µl of Proteinase K into 400 µl of Tissue and Cell Lysis Solution for each sample.
3. Add 400 µl of Tissue and Cell Lysis Solution containing the Proteinase K and mix thoroughly.
4. Incubate at 65°C for 15 minutes; vortex every 5 minutes.
5. Proceed with total nucleic acid precipitation in Part I.

I. Precipitation of Total Nucleic Acids (from plasma lysis)

1. Place the samples on ice for 5 minutes.
2. Add 250 µl of MPC Protein Precipitation Reagent and vortex vigorously for 10 seconds.
3. Pellet the debris by centrifugation at 4°C for 10 minutes at ≥10,000 x g in a microcentrifuge.
4. Transfer the supernatant to a clean microcentrifuge tube and discard the pellet.
5. Add 600 µl of isopropanol to the recovered supernatant. Invert the tube 30-40 times.
6. Pellet the nucleic acid by centrifugation at 4°C for 10 minutes in a microcentrifuge.
7. Carefully pour off the isopropanol without dislodging the pellet. If removal of DNA from RNA preparations is required, proceed with the protocol in Part N. Otherwise, complete the remainder of Part I.
8. Rinse twice with 70% ethanol, being careful to not dislodge the pellet. Centrifuge briefly if the pellet is dislodged. Remove all of the residual ethanol with a pipet.
9. Resuspend the nucleic acid in 35 µl of TE Buffer.

The following protocol is provided for the purification of total nucleic acids, DNA, or RNA from whole blood without the initial lysis of the red blood cells. Lyse the blood as outlined in Part J; if isolating DNA, users may add an optional RNase A step following treatment with Proteinase K (see Protocols for DNA Purification). If further purification of RNA (to remove contaminating DNA) is required, follow the protocol outlined in Part N. For the purification of RNA, use appropriate techniques to minimize degradation by exogenous ribonucleases.

J. Lysis of Whole Blood (without RBC lysis)

Thoroughly mix the Tissue and Cell Lysis Solution to ensure uniform composition before dispensing.

1. Collect whole-blood samples. Transfer 12.5 µl of blood into a microcentrifuge tube.
2. Dilute 2 µl of Proteinase K into 400 µl of Tissue and Cell Lysis Solution for each sample.
3. Add 400 µl of Tissue and Cell Lysis Solution containing the Proteinase K and mix thoroughly.
4. Incubate at 65°C for 15 minutes; vortex every 5 minutes.
5. Proceed with total nucleic acid precipitation in Part K.

K. Precipitation of Total Nucleic Acids (from whole blood lysis)

1. Place the samples on ice for 5 minutes.
2. Add 225 µl of MPC Protein Precipitation Reagent and vortex vigorously for 10 seconds.
3. Pellet the debris by centrifugation at 4°C for 10 minutes at ≥10,000 x g in a microcentrifuge.
4. Transfer the supernatant to a clean microcentrifuge tube and discard the pellet.
5. Add 600 µl of isopropanol to the recovered supernatant. Invert the tube 30-40 times.

6. Pellet the nucleic acid by centrifugation at 4°C for 10 minutes in a microcentrifuge.
7. Carefully pour off the isopropanol without dislodging the pellet. If removal of DNA from RNA preparations is required, proceed with the protocol in Part N. Otherwise, complete the remainder of Part K.
8. Rinse twice with 70% ethanol, being careful to not dislodge the pellet. Centrifuge briefly if the pellet is dislodged. Remove all of the residual ethanol with a pipet.
9. Resuspend the total nucleic acids in 35 µl of TE Buffer.

The following protocol is provided for the purification of total nucleic acids, DNA, or RNA from buffy coat of blood. Prepare buffy coat and lyse the white cells as outlined in Part L, and then proceed with precipitation of total nucleic acids as described in Part M. If further purification of RNA (to remove contaminating DNA) is required, follow the protocol outlined in Part N. For the purification of RNA, use appropriate techniques to minimize degradation by exogenous ribonucleases.

L. Lysis of Buffy Coat

Thoroughly mix the various Lysis Solutions to ensure uniform composition before dispensing.

1. Draw 5 ml of blood into an EDTA Vacutainer tube. Separate fractions by centrifugation at 1,000 x g for 15 minutes and carefully transfer 600 µl of buffy coat (the white interface between the plasma and the red blood cells) to a microcentrifuge tube. The transfer of some red blood cells is not detrimental to the purification of nucleic acids from buffy coat. Vortex the buffy coat sample and transfer 300 µl of the sample to another microcentrifuge tube; process the two tubes in parallel.
2. Add 1.2 ml of Red Cell Lysis Solution to each tube, invert three times to mix, and flick the bottom of the tubes to suspend any remaining material.
3. Incubate at room temperature for 5 minutes and then vortex briefly. Continue incubating at room temperature for an additional 5 minutes, followed again by brief vortexing.
4. Pellet the white blood cells by centrifugation for 25 seconds in a microcentrifuge.
5. Remove most of the supernatant, leaving approximately 25 µl of liquid. Vortex to suspend the pellets.
6. Resuspend the white blood cells in 600 µl of Tissue and Cell Lysis Solution by pipetting the cells several times.
7. Place the samples on ice for 3-5 minutes and then proceed with total nucleic acid precipitation in Part M below.

M. Precipitation of Total Nucleic Acids (from buffy coat)

1. Add 300 µl of MPC Protein Precipitation Reagent and vortex vigorously for 10 seconds.
2. Pellet the debris by centrifugation at 4°C for 10 minutes at ≥10,000 x g in a microcentrifuge.
3. Transfer the supernatant to a clean microcentrifuge tube and discard the pellet.
4. Add 750 µl of isopropanol to the recovered supernatant. Invert the tube 30-40 times.
5. Pellet the nucleic acid by centrifugation at 4°C for 10 minutes in a microcentrifuge.
6. Carefully pour off the isopropanol without dislodging the pellet. If removal of DNA from RNA preparations is required, proceed with the protocol in Part N. Otherwise, complete the remainder of Part M.
7. Rinse twice with 70% ethanol, being careful to not dislodge the pellet. Centrifuge briefly if the pellet is dislodged. Remove all of the residual ethanol with a pipet.
8. Resuspend the total nucleic acids in 35 µl of TE Buffer.

N. Removal of Contaminating DNA from RNA Preparations

1. Remove all of the residual isopropanol with a pipet.
2. Prepare 200 µl of DNase I solution for each sample by diluting 10 µl of RNase-Free DNase I up to 200 µl with 1X DNase Buffer.
3. Completely resuspend the total nucleic acids pellet in 200 µl of DNase I solution.
4. Incubate at 37°C for 30 minutes.
5. Add 200 µl of 2X T and C Lysis Solution; vortex for 5 seconds.
6. Add 200 µl of MPC Protein Precipitation Reagent; vortex 10 seconds; place on ice for 3-5 minutes.
7. Pellet the debris by centrifugation for 10 minutes at ≥10,000 x g in a microcentrifuge.
8. Transfer the supernatant containing the RNA into a clean microcentrifuge tube and discard the pellet.
9. Add 500 µl of isopropanol to the supernatant. Invert the tube 30-40 times.
10. Pellet the purified RNA by centrifugation at 4°C for 10 minutes in a microcentrifuge.
11. Carefully pour off the isopropanol without dislodging the RNA pellet.
12. Rinse twice with 70% ethanol, being careful to not dislodge the pellet. Centrifuge briefly if the pellet is dislodged. Remove all of the residual ethanol with a pipet.
13. Resuspend the RNA in 10-35 µl of TE Buffer.
14. Add 1 µl of RiboGuard RNase Inhibitor (optional).

O. Complete Removal of RNA

1. Add 1 µl of RNase A to the sample; mix thoroughly.
2. Incubate at 37°C for 30 minutes.
3. Add 14 µl TE Buffer and 50 µl of 2X T and C Lysis Solution to each sample.
4. Place the samples on ice for 3-5 minutes. Add 100 µl of MPC Protein Precipitation Reagent and mix by vortexing vigorously for 10 seconds.
5. Pellet the debris by centrifugation at 4°C for 10 minutes at ≥10,000 x g in a microcentrifuge.
6. Transfer the supernatant to a clean microcentrifuge tube and discard the pellet.
7. Add 200 µl of isopropanol to the recovered supernatant. Invert the tube several (30-40) times.
8. Pellet the DNA by centrifugation at 4°C for 10 minutes in a microcentrifuge.
9. Carefully pour off the isopropanol without dislodging the DNA pellet.
10. Rinse twice with 70% ethanol, being careful to not dislodge the pellet. Centrifuge briefly if the pellet is dislodged. Remove all of the residual ethanol with a pipet.
11. Resuspend the DNA in 35 µl of TE Buffer.

12. Troubleshooting Nucleic Acid Purifications

Little or no nucleic acid after resuspension in TE buffer

1) **Increase the amount of tissue or biological fluid.** Use the recommended amount of starting material or use the recommended ratio of tissue:lysis buffer as indicated in the protocol. Increase the amount of tissue, particularly if purifying nucleic acids from a biological source other than those listed in the protocols.
2) **Increase the efficiency of cell lysis.** Either increase the amount of Proteinase K used during lysis or increase the time of incubation. In addition, vortex during Proteinase K treatment to facilitate lysis. If these adjustments fail, homogenize the tissue to more fully disrupt the cell membrane or wall.
3) **Decrease the amount of TE buffer.** Use less TE Buffer to resuspend precipitated nucleic acids.
4) **Avoid contamination by exogenous or endogenous nucleases.** Ensure that tissue or biological fluids were properly collected and stored. Use sterile technique. Add ribonuclease inhibitor to the TE Buffer before resuspension.
5) **Ensure that nucleic acids remain following isopropanol precipitation.** Make certain that the nucleic acid pellet adheres to the microcentrifuge tube during washing of the pellet with 70% ethanol.

A_{260}/A_{280} ratio is too low

1) **Decrease the amount of starting material.** The nucleic acid is contaminated with protein. Use less tissue or biological fluid; alternatively, dilute the nucleic acid to 300 µl with Tissue and Cell Lysis Solution, and follow the protocol for Total Nucleic Acid Purification.

A_{260}/A_{280} ratio is too high

1) **Treat with ribonuclease.** The DNA is contaminated with RNA. If RNase A treatment was omitted, treat with RNase A. Note that precipitation of nucleic acid is extremely efficient, resulting in the precipitation of small oligomers of ribonucleotides. If these are undesirable, treat the DNA with RNase I (available separately) to degrade these oligomers and precipitate the DNA.

Loose protein pellet

1) **Cool sample before protein precipitation.** Cool the sample thoroughly on ice before adding the MPC Protein Precipitation Reagent. If the pellet remains loose, centrifuge again. Carefully decant to minimize transfer of precipitated protein. Note that a small degree of transfer is generally not detrimental.

DNA rehydrates slowly

1) **Decrease drying time. Remove residual ethanol with a pipet and air dry briefly.** Suspend in TE Buffer and disrupt the DNA pellet gently with a pipet. If necessary, the DNA may be left at room temperature overnight to rehydrate. Use additional TE Buffer as required.

Residual RNA in DNA preparations

1) **Remove RNA.** If complete removal of RNA is required for your application, follow the protocol for Complete Removal of RNA (Part O).

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Brilliant III Ultra-Fast QRT-PCR Master Mix

Instruction Manual

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Brilliant III Ultra-Fast QRT-PCR Master Mix

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Brilliant III Ultra-Fast QRT-PCR Master Mix

MATERIALS PROVIDED

Catalog #600884 (single kit), #600885 (10-pack kit)

Materials Provided	Quantity [a,b]
2× Brilliant III Ultra-Fast QRT-PCR Master Mix	2 × 2 ml
RT/RNase Block	400 µl
100 mM DTT	100 µl
Reference dye [c], 1 mM	100 µl

[a] Sufficient reagents are provided for four hundred, 20-µl QRT-PCR reactions.
[b] Quantities listed are for a single kit. For 10-pack kits, each item is provided at 10 times the listed quantity.
[c] The reference dye is light sensitive and should be kept away from light whenever possible.

STORAGE CONDITIONS

All Components: Store at –20°C upon receipt. After thawing, the 2× master may be stored at 4°C for up to one month or returned to –20°C for long term storage

Note *The reference dye is light sensitive and should be kept away from light whenever possible.*

ADDITIONAL MATERIALS REQUIRED

Spectrofluorometric thermal cycler
Nuclease-free PCR-grade water

NOTICE TO PURCHASER

NOTICE TO PURCHASER: LIMITED LICENSE
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INTRODUCTION

Quantitative reverse transcription PCR (QRT-PCR) is a powerful tool for gene expression analysis. The Brilliant III Ultra-Fast QRT-PCR Master Mix was developed for the ABI StepOnePlus and Bio-Rad CFX96 real-time PCR instruments and other fast-cycling systems (such as the ABI 7900HT and 7500 Fast systems). It performs QRT-PCR in less time without compromising target detection sensitivity, specificity, or reproducibility. The master mix includes two key components that enable it to perform optimally under fast cycling conditions:

- A mutated form of *Taq* DNA polymerase that has been specifically engineered for faster replication
- An improved chemical hot start mechanism that promotes faster hot start release to improve amplification specificity while keeping the run time of the PCR protocol to a minimum

The master mix has been successfully used with fluorescent TaqMan® probes to amplify and detect a variety of high- and low-abundance RNA targets from experimental samples including total RNA, poly(A)$^+$ RNA, and synthetic RNA.

The kit includes the components necessary to carry out cDNA synthesis and PCR amplification in one tube and one buffer.* Brilliant kits support quantitative amplification and detection with multiplex capability. The single-step master mix format is ideal for most high-throughput QPCR applications where it is not necessary to archive cDNA.

Features of Kit Components

RT/RNase Block

The reverse transcriptase (RT) provided in the kit is a Moloney-based RT specifically formulated for the Agilent Brilliant III Ultra-Fast kits. This RT performs optimally at a reaction temperature of 50°C when used in 1-step QRT-PCR with the Brilliant III master mix. It is stringently quality-controlled to verify the absence of nuclease contaminants that adversely affect cDNA synthesis and to ensure sensitive and reproducible performance in QRT-PCR experiments with a broad range of RNA template amounts and a variety of RNA targets that vary in size, abundance, and GC-content. The RNase block, provided in the same tube, serves as a safeguard against contaminating RNases.

Brilliant III Ultra-Fast QRT-PCR 2× Master Mix

The 2× master mix contains an optimized RT-PCR buffer, $MgCl_2$, nucleotides (GAUC), stabilizers, and mutant *Taq* DNA polymerase. The DNA polymerase features a hot start capability that reduces nonspecific product formation.

* Primers, probes and template are not included.

DTT

A separate tube of 100 mM DTT is provided with the kit. Adding DTT to the reactions improves RT performance for more challenging targets.

Reference dye

A passive reference dye (an optional reaction component) is provided in the kit as an optional reagent that may be added to compensate for non-PCR related variations in fluorescence. Providing the reference dye in a separate tube makes the master mix adaptable for many real-time QPCR platforms.

Fluorescence Monitoring in Real-Time

When fluorescence signal from a PCR reaction is monitored in real-time, the results can be displayed as an amplification plot, which reflects the change in fluorescence during cycling. This information can be used during PCR experiments to quantify initial copy number. Studies have shown that initial copy number can be quantified during real-time PCR analysis based on threshold cycle (Ct).[1] Ct is defined as the cycle at which fluorescence is determined to be statistically significant above background. The threshold cycle is inversely proportional to the log of the initial copy number.[1] The more template that is initially present, the fewer the number of cycles it takes to reach the point where the fluorescence signal is detectable above background. Quantitative information based on threshold cycle is more accurate than information based on endpoint determinations because threshold cycle is based on measurements taken during the exponential phase of PCR amplification when PCR efficiency is not yet influenced by limiting reagents, small differences in reaction components, or accumulation of PCR inhibitors. Figure 1 shows an ABI StepOnePlus instrument amplification plot with Ct determination (top panel) and standard curve (bottom panel). In this experiment, the cyclophilin target was amplified and detected from total RNA using a TaqMan probe and the Brilliant III Ultra-Fast QRT-PCR master mix.

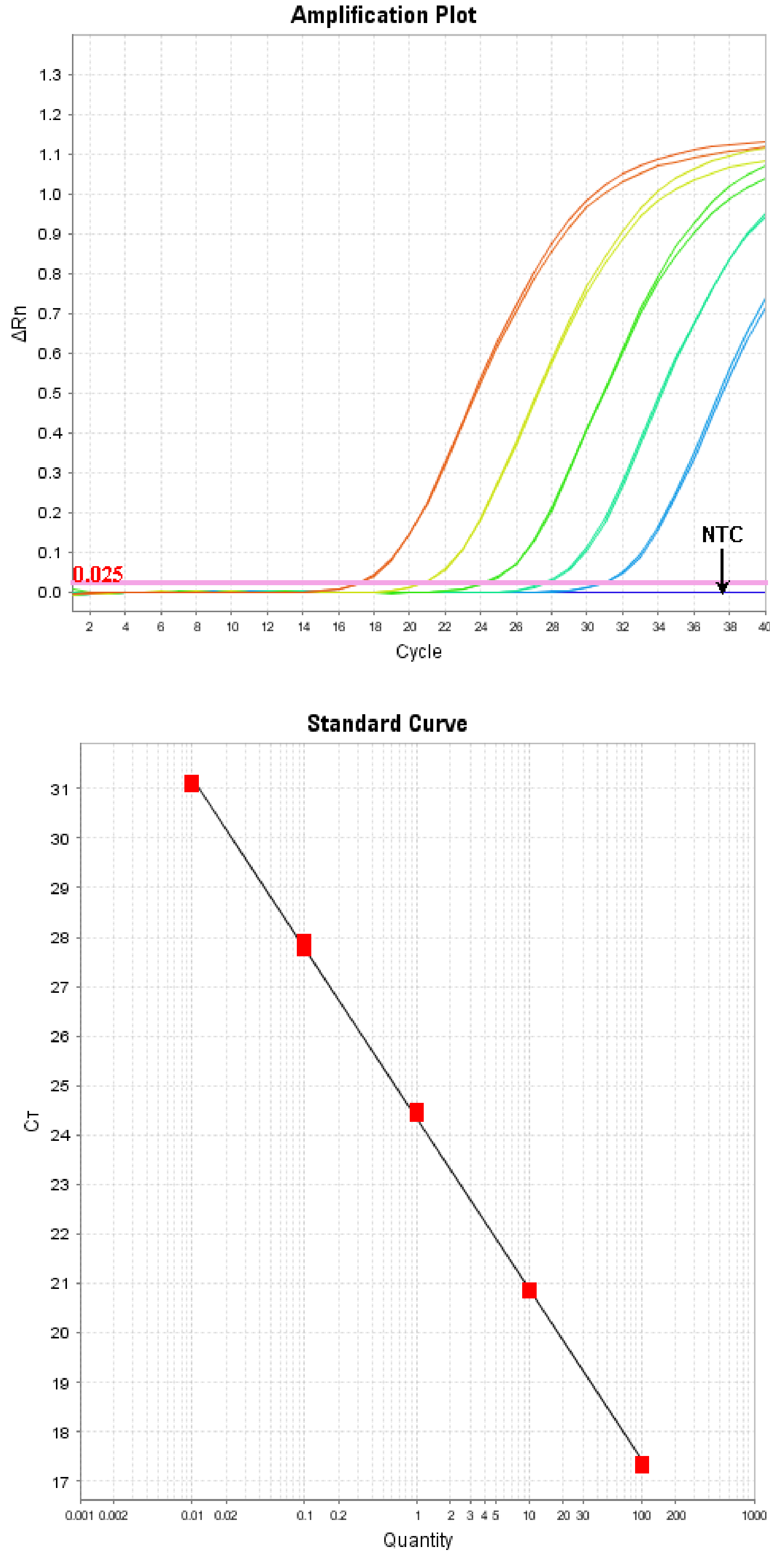

Figure 1 *Top panel*: StepOnePlus instrument amplification plot using a TaqMan® probe. A serial dilution of RNA template (ranging from 0.01–100 ng) was added to each reaction (set up in duplicate) . The fluorescence value used to determine Ct (the threshold line) is shown as a solid line. *Bottom panel*: Standard curve generated from amplification plot. An amplification efficiency of 93.2% and an R-squared value of 0.999 were obtained.

PREPROTOCOL CONSIDERATIONS

RNA Isolation

High-quality intact RNA is essential for successful synthesis of full-length cDNA. Total and poly(A)$^+$ RNA can be rapidly isolated and purified using Agilent Absolutely RNA isolation kits. Oligo(dT)-selection for poly(A)$^+$ RNA is typically not necessary, although including this step may improve the yield of specific cDNA templates. RNA samples with $OD_{260/280}$ ratios of 1.8–2.0 are optimally pure.

Preventing RNase Contamination

Take precautions to minimize the potential for contamination by ribonucleases (RNases). RNA isolation should be performed under RNase-free conditions. Wear gloves and use sterile tubes, pipet tips, and RNase-free water. Do not use DEPC-treated water, which can inhibit PCR. The RNase inhibitor that is included in the tube of RT/RNase Block provides additional protection against RNase contamination.

Preventing Genomic DNA Contamination

Contaminating DNA can be removed from the RNA preparation using an RNase-free DNase. Additionally, PCR primers may be designed to span adjacent exons in order to prevent amplification of the intron-containing genomic DNA.

Quantitative PCR Human Reference Total RNA

Agilent QPCR Human Reference Total RNA (Catalog #750500) is a high-quality control for quantitative PCR gene-expression analysis. Agilent QPCR Human Reference Total RNA is composed of total RNA from 10 human cell lines (see the table below), with quantities of RNA from the individual cell lines optimized to maximize representation of gene transcripts present in low, medium, and high abundance. The reference RNA is carefully screened for contaminating genomic DNA, the presence of which can complicate interpretation of QRT-PCR assay data.

Quantitative PCR Human Reference Total RNA Cell Line Derivations
Adenocarcinoma, mammary gland
Hepatoblastoma, liver
Adenocarcinoma, cervix
Embryonal carcinoma, testis
Glioblastoma, brain
Melanoma, skin
Liposarcoma
Histiocytic lymphoma; macrophage; histocyte
Lymphoblastic leukemia, T lymphoblast
Plasmacytoma; myeloma; B lymphocyte

The QPCR Human Reference Total RNA is ideally suited for optimizing QRT-PCR assays. Often only small amounts of experimental RNA template are available for setting up an expression profiling study. Using the extensive representation of specific mRNA species in the generic template, assays may be optimized for a variety of primer/probe systems. This eliminates the use of precious experimental RNA samples for assay optimization.

Probe Design

Probes should have a melting temperature that is 7–10°C higher than the annealing temperature of the primers. For additional considerations in designing TaqMan probes, refer to Primer Express® software from Applied Biosystems, the Primer3 program or a similar oligo design program.

Resuspend lyophilized custom TaqMan probes in buffer containing 5 mM Tris-HCl, pH 8.0, and 0.1 mM EDTA (low TE buffer).

Optimal Concentrations for Experimental Probes and PCR Primers

Probes

The optimal concentration of the experimental TaqMan probe should be determined empirically. The optimal concentration is the lowest concentration that results in the lowest Ct and an adequate fluorescence for a given target concentration. The TaqMan probe concentration can be optimized by varying the final concentration from 100 to 600 nM.

PCR Primers

The optimal concentration of the upstream and downstream PCR primers should also be determined empirically. The optimal concentration is the lowest concentration that results in the lowest Ct and an adequate fluorescence for a given target concentration. The primer concentration can be optimized by varying the concentration from 200 to 600 nM. The best concentrations of the upstream and downstream primers are not always of equal molarity.

Preventing Sample Contamination

Take precautions to minimize the potential for carryover of nucleic acids from one experiment to the next. Use separate work areas and pipettors for pre- and post-amplification steps. Use positive displacement pipets or aerosol-resistant pipet tips.

Treatment with Uracil-N-glycosylase (UNG) is NOT recommended for decontamination of single tube RT-PCR reactions since UNG would be active during the 50°C incubation necessary for reverse transcription.

Magnesium Chloride Concentration

Magnesium chloride concentration affects the specificity of the PCR primers and probe hybridization. The Brilliant III QRT-PCR master mix contains $MgCl_2$ at a concentration of 5.5 mM (in the 1× solution), which is suitable for most targets.

Reference Dye

A passive reference dye is included in this kit and may be added to compensate for non-PCR related variations in fluorescence. Fluorescence from the passive reference dye does not change during the course of the PCR reaction but provides a stable baseline to which samples are normalized. In this way, the reference dye compensates for changes in fluorescence between wells caused by slight volume differences in reaction tubes. The excitation and emission wavelengths of the reference dye are 584 nm and 612 nm, respectively. Although addition of the reference dye is not required when using the Bio-Rad CFX96 real-time PCR system, with other instruments (including the ABI StepOnePlus instrument) the use of the reference dye may be required for optimal results.

Reference Dye Dilution Recommendations

Prepare **fresh*** dilutions of the reference dye prior to setting up the reactions, and **keep all tubes containing the reference dye protected from light as much as possible**. Make initial dilutions of the reference dye using nuclease-free PCR-grade H_2O. If using a StepOnePlus or 7900HT Fast instrument, dilute the dye 1:50 for a final concentration of 300 nM in the reactions. For the Agilent Mx instruments or the ABI 7500 Fast instrument, dilute the dye 1:500 for a final concentration of 30 nM. The Bio-Rad CFX96, the Roche LightCycler® 480 and the QIAGEN Rotor-Gene Q instruments do not require the use of the reference dye.

Reaction Preparation

Setting Up Reactions on Ice

While setting up the reactions, keep the reagent mixture and reaction tubes on ice until the reactions are loaded into the instrument.

Preparing a Single Mixture for Multiple Samples

If running multiple samples containing the same primers and probes, prepare a single mixture of reaction components and then aliquot the mixture into individual reaction tubes using a fresh pipet tip for each addition. Preparing a common mixture facilitates the accurate dispensing of reagents, minimizes the loss of reagents during pipetting, and helps to minimize sample-to-sample variation.

Mixing and Pipetting Enzymes

Solutions that contain enzymes (including reverse transcriptase and DNA polymerase) should be mixed gently by inversion or gentle vortexing without generating bubbles. Pipet the enzymes carefully and slowly; otherwise, the viscosity of the buffer can lead to pipetting errors.

* The diluted reference dye, if stored in a light-protected tube at 4°C, can be used within the day for setting up additional assays.

Temperature and Duration of cDNA Synthesis Reaction

For cDNA synthesis, we recommend a 50°C incubation for use with the Brilliant III Ultra-Fast QRT-PCR master mix. A 10-minute incubation for the first-strand synthesis reaction is sufficient for most targets

Multiplex RT-PCR

Multiplex RT-PCR is the amplification of more than one target in a single polymerase chain reaction.[4] The Brilliant III Ultra-Fast QRT-PCR master mix has been successfully used to amplify two targets in a multiplex reaction without reoptimizing the concentrations of DNA polymerase, reverse transcriptase or dNTPs.

In a typical multiplex RT-PCR reaction, one PCR primer pair primes the amplification of the target of interest and another PCR primer pair primes the amplification of an endogenous control. For accurate analysis, it is important to minimize competition between concurrent amplifications for common reagents. To minimize competition, the limiting primer concentrations need to be determined.[5] Consideration should also be given to optimization of the other reaction components. The number of fluorophores in each tube can influence the analysis. The use of a dark quencher, which emits heat instead of light, might enhance the quality of multiplex RT-PCR results by reducing the background light emission. The following PCR primer and probe design guidelines are useful for multiplex RT-PCR.

PCR Primer Considerations for Multiplex RT-PCR

- Design primer pairs with similar annealing temperatures for all targets to be amplified.
- To avoid duplex formation, analyze the sequences of primers and probes with primer analysis software.
- The limiting primer concentrations are the primer concentrations that result in the lowest fluorescence intensity without affecting the Ct. If the relative abundance of the two targets to be amplified is known, determine the limiting primer concentrations for the most abundant target. If the relative abundance of the two targets is unknown, determine the limiting primer concentrations for both targets. The limiting primer concentrations are determined by running serial dilutions of those forward and reverse primer concentrations optimized for one-probe detection systems, but maintaining a constant target concentration. A range of primer concentrations of 50–200 nM is recommended. Running duplicates or triplicates of each combination of primer concentrations within the matrix is also recommended.[4]

TaqMan® Probe Considerations for Multiplex RT-PCR

- Label each TaqMan probe with a spectrally distinct fluorophore.
- Consider designing probes with dark quenchers.

Recommended Control Reactions

No Template Control (NTC)

We recommend performing no-template control reactions for each experimental sample to screen for amplicon contamination or false amplification.

No-RT Control

We recommend performing no-RT control reactions for each experimental sample by omitting the RT/RNase block from the reaction. The no-RT control is expected to generate no signal if there is no amplification of genomic DNA. No signal indicates that the RNA preparation is free of contaminating genomic DNA or that the primers are specific for the cDNA. See *Preventing Genomic DNA Contamination* in *RNA Isolation*.

Endogenous Control

Consider performing an endogenous control reaction to normalize variation in the amount of RNA template across samples. See Reference 3 for guidelines on the use of endogenous controls for QPCR.

PROTOCOL

Notes *Once the tube containing the 2× QRT-PCR master mix is thawed, store it on ice while setting up the reactions. Following initial thawing of the master mix, store the unused portion at 4°C for up to one month, or return to –20°C for long term storage.*

It is prudent to set up a no-template control reaction to screen for amplicon contamination or false amplification. Similarly, a no-RT control should be included to verify that the fluorescence signal is due to the amplification of cDNA and not of contaminating genomic DNA.

Consider performing an endogenous control reaction to normalize variations in the amount of RNA template across samples. See reference 3 for more information on endogenous controls.

Preparing the Reactions

1. If using the reference dye, dilute the provided dye using nuclease-free PCR-grade H_2O. **Keep all solutions containing the reference dye protected from light.**

   - For the ABI StepOnePlus instrument or the ABI 7900HT Fast instrument, dilute the dye **1:50** (for a final concentration of 300 nM in the reactions).

   - For the Agilent AriaMx, Mx3000P, or Mx3005P instrument or the ABI 7500 Fast instrument, dilute the dye **1:500** (for a final concentration of 30 nM in the reactions).

2. Prepare the experimental reactions by combining the following components *in order*. Prepare a single reagent mixture for replicate experimental reactions and replicate no-template controls (plus at least one reaction volume excess), using multiples of each component listed below. *Keep the reagent mixture on ice.*

   ### Reagent Mixture

   Nuclease-free PCR-grade H_2O to adjust the final volume to 20 µl (including experimental RNA)
   10 µl of 2× QRT-PCR master mix
   x µl of experimental probe (optimized concentration)
   x µl of upstream primer (optimized concentration)
   x µl of downstream primer (optimized concentration)
   0.2 µl of 100 mM DTT
   0.3 µl of the **diluted** reference dye (optional)
   1 µl of RT/RNase block

3. Mix the reagents well without creating bubbles, then distribute the mixture to individual PCR reaction tubes. *Keep the reactions on ice.*

4. Add *x* µl of experimental RNA to each reaction to bring the final reaction volume to 20 µl. The quantity of RNA depends on the RNA purity and the specific mRNA abundance. As a guideline, use 0.1 pg–100 ng of total RNA or 0.1 pg–1 ng of mRNA.

5. Gently mix the reactions without creating bubbles, then centrifuge the reactions briefly.

RT-PCR Cycling Programs

6. Place the reactions in the instrument. Based on the instrument you are using, select the appropriate PCR program from the tables below. Set the instrument to detect and report fluorescence at each cycle during the 60°C annealing/extension step.

Note *For optimal performance, the durations of the denaturation and annealing/extension steps may need to be adjusted for each probe/target system.*

Agilent AriaMx

Cycles	Duration of cycle	Temperature
1	10 minutes	50°C
1	3 minutes[b]	95°C
40	5 seconds	95°C
	10 seconds	60°C

Agilent Mx3000P and Mx3005P

Cycles	Duration of cycle	Temperature
1	10 minutes	50°C
1	3 minutes	95°C
40	5–20 seconds	95°C
	20 seconds	60°C

ABI 7500 Fast

Cycles	Duration of cycle	Temperature
1	10 minutes	50°C
1	3 minutes	95°C
40	12 seconds	95°C
	15 seconds	60°C

ABI 7900HT Fast

Cycles	Duration of cycle	Temperature
1	10 minutes	50°C
1	3 minutes	95°C
40	5 seconds	95°C
	15 seconds	60°C

ABI StepOnePlus

Cycles	Duration of cycle	Temperature
1	10 minutes	50°C
1	3 minutes	95°C
40	5 seconds	95°C
	10 seconds[c]	60°C

Bio-Rad CFX96

Cycles	Duration of cycle	Temperature
1	10 minutes	50°C
1	3 minutes[b]	95°C
40	5 seconds	95°C
	10 seconds	60°C

QIAGEN Rotor-Gene Q

Cycles	Duration of cycle	Temperature
1	10 minutes	50°C
1	3 minutes	95°C
40	5–20 seconds	95°C
	10–20 seconds	60°C

Roche LightCycler® 480

Cycles	Duration of cycle	Temperature
1	10 minutes	50°C
1	3 minutes	95°C
40	5 seconds	95°C
	10 seconds	60°C

TROUBLESHOOTING

Observation	Suggestion
Little or no increase in fluorescence with cycling	The probe is not binding to its target efficiently because the annealing temperature is too high. Verify the calculated melting temperature using appropriate software.
	The target is highly GC-rich. Raise the denaturation temperature to 98°C or titrate DMSO into the reactions in 1% increments.
	The PCR product is too long. Design the primers so that the PCR product is <150 bp in length.
	Taq DNA polymerase was not activated. Ensure that the 3-minute incubation at 95°C was performed as part of the cycling parameters.
	The DNA polymerase was activated for more than 3 minutes. Ensure that the initial 95°C incubation was not longer than 3 minutes.
	For multiplex PCR, the $MgCl_2$ concentration may be increased, if desired, by adding a small amount of concentrated $MgCl_2$ (not provided in this kit) to the 1× experimental reaction at the time of set up.
	The probe has a nonfunctioning fluorophore. Verify that the fluorophore functions by performing a nuclease digestion to ensure it is unquenching as expected.
	Redesign the probe using Primer Express or other software. Design a probe that performs well in reactions containing 5.5 mM $MgCl_2$.
	The reaction is not optimized and no or insufficient product is formed. Verify formation of the specific product by gel electrophoresis.
	The RNA template may be degraded. Ensure that the template RNA is stored properly (at –20°C or –80°C) and is not subjected to multiple freeze-thaw cycles. Check the quality of the RNA in the sample by gel electrophoresis or using an automated RNA population analysis system such as the Agilent 2100 Bioanalyzer.
	If the target RNA contains extensive secondary structure, increase the incubation temperature used during the first step of the RT-PCR program up to 55°C.
	For low-abundance targets or long amplicons, increase the duration of the cDNA synthesis step to 30-minutes while lowering the incubation temperature down to 42°C.
	Verify that all reagents and supplies are RNase-free.
	Where possible, increase the amount of template RNA. (Do not exceed the recommended amount of template.)
	For multiplex PCR of more than two targets, reactions may need to be supplemented with additional polymerase and dNTPs (not provided).
Increasing fluorescence in no-template control reactions with cycling	The reaction has been contaminated. Follow the procedures outlined in reference 6 to minimize contamination.
Ct reported for the no-template control (NTC) sample is less than the total number of cycles but the curve on the amplification plot is horizontal	Variation in fluorescence intensity. Review the amplification plot and, if appropriate, adjust the threshold accordingly.

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ENDNOTES

ABI PRISM® is a registered trademark of Applied Biosystems.
LightCycler® is a registered trademark of Roche.
Primer Express® is a registered trademark of The Perkin-Elmer Corporation.
TaqMan® is a registered trademark of Roche Molecular Systems, Inc.

MSDS INFORMATION

Material Safety Data Sheets (MSDSs) are provided online at *http://www.genomics.agilent.com*. MSDS documents are not included with product shipments.

BRILLIANT III ULTRA-FAST QRT-PCR MASTER MIX

Catalog #600884, #600885

QUICK-REFERENCE PROTOCOL

Prior to setting up the reactions, thaw the 2× QRT-PCR master mix and store on ice. Following initial thawing of the master mix, the unused portion may be stored at 4°C for up to one month, or returned to –20°C for long term storage.

1. If using the reference dye, dilute the provided dye with nuclease-free PCR-grade H_2O. For the ABI StepOnePlus instrument or the ABI 7900HT Fast instrument, dilute the dye 1:50 (for a final concentration of 300 nM in the reactions). For an Agilent Mx instrument or the ABI 7500 Fast instrument, dilute the dye 1:500 (for a final concentration of 30 nM in the reactions). Keep all solutions containing the reference dye protected from light.

2. Prepare the experimental reactions by adding the following components *in order*. Prepare a single reagent mixture for multiple reactions using multiples of each component listed below. *Keep the reagent mixture on ice*.

   Reagent Mixture

   Nuclease-free PCR-grade H_2O to bring the final volume to 20 µl (including experimental RNA)
   10 µl of 2× QRT-PCR master mix
   x µl of experimental probe (optimized concentration)
   x µl of upstream primer (optimized concentration)
   x µl of downstream primer (optimized concentration)
   0.2 µl of 100 mM DTT
   0.3 µl of diluted reference dye from step 1 (optional)
   1.0 µl of RT/RNase block

3. Mix the reagents well without creating bubbles, then distribute the mixture to individual PCR reaction tubes. *Keep the reactions on ice.*

4. Add x µl of experimental RNA to each reaction to bring the final reaction volume to 20 µl.

5. Gently mix the reactions without creating bubbles, and then centrifuge the reactions briefly.

6. Place the reactions in the instrument. Based on the instrument you are using, select the appropriate PCR program from the tables below. Set the instrument to detect and report fluorescence at each cycle during the 60°C annealing/extension step.

Note *For optimal performance, the durations of the denaturation and annealing/extension steps may need to be adjusted for each probe/target system.*

Agilent AriaMx

Cycles	Duration of cycle	Temperature
1	10 minutes	50°C
1	3 minutes[b]	95°C
40	5 seconds	95°C
	10 seconds	60°C

Agilent Mx3000P and Mx3005P

Cycles	Duration of cycle	Temperature
1	10 minutes	50°C
1	3 minutes	95°C
40	5–20 seconds	95°C
	20 seconds	60°C

ABI 7500 Fast

Cycles	Duration of cycle	Temperature
1	10 minutes	50°C
1	3 minutes	95°C
40	12 seconds	95°C
	15 seconds	60°C

ABI 7900HT Fast

Cycles	Duration of cycle	Temperature
1	10 minutes	50°C
1	3 minutes	95°C
40	5 seconds	95°C
	15 seconds	60°C

ABI StepOnePlus

Cycles	Duration of cycle	Temperature
1	10 minutes	50°C
1	3 minutes	95°C
40	5 seconds	95°C
	10 seconds[c]	60°C

Bio-Rad CFX96

Cycles	Duration of cycle	Temperature
1	10 minutes	50°C
1	3 minutes[b]	95°C
40	5 seconds	95°C
	10 seconds	60°C

QIAGEN Rotor-Gene Q

Cycles	Duration of cycle	Temperature
1	10 minutes	50°C
1	3 minutes	95°C
40	5–20 seconds	95°C
	10–20 seconds	60°C

Roche LightCycler® 480

Cycles	Duration of cycle	Temperature
1	10 minutes	50°C
1	3 minutes	95°C
40	5 seconds	95°C
	10 seconds	60°C