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Quantifying *Orientia tsutsugamushi* and other Rickettsiales bacteria by real time qPCR V.1

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Protocol status: Working

We use this protocol and it's working



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Abstract

The objective of this protocol is to calculate how many bacteria you have in a certain volume of cultured cells using real time PCR (qPCR). The protocol uses a gene that is present in only one copy in the bacterial genome. Since *Orientia tsutsugamushi* is slow growing there is likely only one genome per cell. Therefore we expect one genome copy to be a good approximation of bacterial copy number. Whilst this protocol was originally developed for *Orientia tsutsugamushi*, we have included primer/probe/standard sequences for other Rickettsiaceae (OTR) and Anaplasmataceae (AWE) species. Primer/probe/standard sequences are also given for human and mouse genes, allowing the simultaneous quantification of bacteria and host cell copies to be determined. This can be used to calculate the average number of bacteria per host cell.

Protocol materials

 TE Buffer

 qPCRBIO Probe Mix Lo-Rox **PCR Biosystems Inc. Catalog #PB20.21-05**



Preparation of DNA standards for qPCR (once you have done this you can use the standards multiple times)

1 DNA standards

A	B	C	D
Standard name	Used for	Target gene	Sequence
tsa47	Orientia tsutsugamushi	tsa47	Check this
OTR16s	Orientia tsutsugamushi, Rickettsia spp	16s	GTCAAGTCATCATGGCCCTTACGGG TTGGGCTACACGCGTGCTACAATGG TGTTTACAGAGGGAAGCAAAACGGC GACGTGGAGCAAATCCCTAAAAGAC ATCTCAGTTCGGATTGTTCTCTGCAA CTCGAGAGCATGAAGTTGGAATCGC TAGTAATCGCGGATCAGCATGCCGC GGTGAATACGTTCTCGGGCCTTGTA CACACTGCCCCGTCACGCCATGGGAG TTGGTTTTACCTGAAG
AWE16s	Anaplasma, Wolbachia, Ehrlichia	16s	GGCACTTTAAGGAACTGCCAGTGA TAAACTGGAGGAAGGTGGGGATGAT GTCAAGTCAGCACGCGCCCTTATGGG GTGGGCTACACACGTGCTACAATGG CGACTACAATAGGTTGCAACGTCGC AAGGCTGAGCTAATCCGTAAAAGTC GTCTCAGTTCGGATTGTCCTCTGTA ACTCGAGGGCATGAAGTCGGAATCG CTAGTAATCGTGGATCAGCATGCCA CGGTGAATACGTTCTCGGGTCTT
Panrick 17kda	Rickettsia spp.	17 kDa (htrA)	ATGAAACTATTATCTAAAATTATGATT ATAGCTCTTGCAAGCTTCTATGTTACA AGCCTGTAAACAGTCCGGGCGGTATG AATAAACAAGGTACAGGAACACTTC TTGGCGGTGCTGGAGGTGCATTACT TGGTTCTCAATTCGGTAAGGGCAAA GGACAGCTTGTGGAGTAGGTGTAG GTGCATTACTTGGAGCAGTTCTTGG TGGACAAATCGGTGCAGGTATGGAT GAGCAGGATAGAAGACTTGC
Mouse Cfd	Mouse	Comple ment factor D	CCCACAGGCCCTGAATTTGCAGCC CCTCGGAAATGCACTTTACTGAGAT CGCTTTTGGGTCTGGGGCAACTGG GTTGGAGGTGTCTGTGGTCAGAGTG GGTTCTCTTTAGGTGCAACCCACCC CAACCCTTACAATACACCTACCCTC CTCAGCAGCACAGCCCCGAGGCCG GATTCTGGGTGGCCAGGA
Human GAPDH	Human	GAPDH	CTCACGTATTCCCCCAGGTTTACAT GTTCCAATATGATTCCACCCATGGCA AATTCCATGGCACCGTCAAGGCTGA



	A	B	C	D
				GAACGGGAAGCTTGTCATCAATGGA AATCCCATCACCATCTTCCAGGAGT GAGTGGAAGACAGAATGGAAGAAAT GTGCTTTGGGGAGGCAACTAGGATG GTGTGGCTCCCTTGGGTATATGGTA ACCTTGTGTCCCTCAATATGGTCCT GTCCCCATCTCCCCCCCACCCC

Sequences of DNA standards

- 2 It is very important that this work is done accurately as all your follow on work will depend on this. Spin/vortex/spin/vortex all tubes several times in between each addition to ensure that tubes are thoroughly mixed. Use correct pipetting technique.
- 3 Clean DNA hood with virkon (or equivalent) followed by 70% ethanol. Clean pipettes, tips, racks, waster containers etc and bring them into the hood.
- 4 Get a container of ice. Thaw the stock solution of the standard DNA on ice.
- 5 Get 35 × 0.6 ml eppendorf tubes (to make 5 batches of standards). These should be taken from a sterile container and you should not touch them with gloves. Label the tubes with 5 of each 10^7 , 10^6 , 10^5 , 10^4 , 10^3 , 10^2 , 10^1 . Add 1-5 on each so you know which is which.
- 6 Put  45 μ L of  TE Buffer in each of the prelabelled 0.6 ml tubes.
- 7 Add  5 μ L of the stock DNA standard (10^8) into your labelled 10^7 tube 1. Vortex the tube and spin down.
- 8 Serial dilutions: From 10^7 tube 1 take out 5uL into 10^6 tube 1. Vortex and spin. Continue until the 10^1 tube.
- 9 After the serial dilutions are done, aliquot  10 μ L of each on your tube 1s into tubes 2-5 of each concentration.
- 10 Store these standards at  -20 °C until use. Mark them when you use them so you can keep track of the number of freeze/thaws.



- 11 *Alternative option*. Note that standards can also be prepared fresh each time you run a qPCR reaction. In this case each lab member should prepare their own stock of 10^8 and use this to prepare standards whenever needed. This option is used preferable if a degradation of standard DNA is observed after storage. This will be apparent if your qPCR values for the standards are no longer presenting a nice linear distribution.
- 12 **Note** You should monitor the quality of your standard DNA samples through the qPCR data obtained (see below). The duplicate values should be close together and the values of the serial dilutions should appear along a clear line when plotted on a graph.

Preparation of 10 μ M probes and primers for qPCR (once you have done this you can use them multiple times)

- 13 This step is essential to obtaining good results. Work in a clean DNA hood. Note that the diluted samples degrade over time, even when stored in the freezer, so it is advisable to make multiple small aliquots and store them at -70 °C or -20 °C .

14 DNA standards

	A	B	C	D	E	F
	PCR name	Used for	Target gene	FW Primer sequence	RV primer sequence	Probe sequence
	tsa47	Orientia tsutsugamushi	tsa47	TCCAGAATTAAATGAGAAT TTAGGAC	TTAGT AATTA CATCT CCAG GAGC AA	[6FAM] TTCCA CATTG TGCTG CAGAT CCTTC [TAM]
	OTR16s	Orientia tsutsugamushi , Rickettsia spp	16s	GCTACACGCGCGTGCTAC AATGG	TGTGT ACAA GGCC CGAG AACG	[6FAM] ATCGC TAGTA ATCGC GGATC AGCAT GCC[T AM]
	AWE16s	Anaplasma, Wolbachia, Ehrlichia	16s	ACTGGAGGAAGGTGGGGA TG	TGATC CACG ATTAC TAGCG ATTCC	[6FAM] TGGG CTACA CACG TGCTA CAATG G[TAM]

	A	B	C	D	E	F
	Panric k 17kda	Rickettsia spp.	17 kDa (htrA)	AAACAAGGKCANGGHACA CTTCTTG	AAGTA ATGCR CCTAC ACCTA CTC	[6FAM] CCGA ATTGA GAAC CAAGT AATGC [TAM]
	Mouse Cfd	Mouse	Compl ement factor D	ACTGAGATCGCTTTTGGG TC	GGAG GGTAG GTGTA TTGTA AGG	[HEX] CTGG GTTG GAGG TGTCT GTGGT [BHQ1]
	Human GAPD H	Human	GAPD H	GTCAAGGCTGAGAACGGG AA	AGCC ACAC CATCC TAGTT GC	[6FAM] TCCA GGAG TGAGT GGAA GACA GAATG GAAG[TAM]

Sequences of DNA standards

- 15 The stock probes and primers should all be 100 μ M in concentration and stored at  -20 °C
- 16 It is very important that this work is done accurately as all your follow on work will depend on this. Spin/vortex/spin/vortex all tubes several times in between each addition to ensure that tubes are thoroughly mixed. Use correct pipetting technique.
- 17 Clean DNA hood with virkon (or equivalent) followed by 70% ethanol. Clean pipettes, tips, racks, waster containers etc and bring them into the hood.
- 18 Get a container of ice. Thaw the 100 μ M stocks solutions of primers and probes on ice.
- 19 Label sterile 1.5 ml eppendorf tubes for forward primer, reverse primer and probe.
- 20 Use sterile DNA-free and RNA-free PCR grade water. Put 90 μ l or 45 μ l of the water in the tube as below.



	A	B	C	D
		100 μ M stock (μ l)	PCR grade water (μ l)	Total (μ l)
probe	10	90	100	
primer	5	45	50	

- 21 Once they have fully thawed, spin down the stock primers and probes with microcentrifuge, vortex, and spin down again.
- 22 Add primer (5 μ l)/probe (10 μ l) stock solution into eppendorf tube according to the table above.
- 23 Mix by vortex and spin down.
- 24 Wrap the probe tube with aluminium foil.
- 25 Store these diluted primer and probe samples at $-20\text{ }^{\circ}\text{C}$ until use. Mark them when you use them so you can keep track of the number of freeze/thaws.

DNA extraction

- 26 Preheat the heat block or water bath to $95\text{ }^{\circ}\text{C}$.
- 27 If working in a multiwell culture plate: aspirate off the supernatant and add alkaline lysis buffer directly to each well of the plate. Alkaline lysis buffer: 25 mM NaOH, 0.2 mM EDTA.
- 28 If working in a different vessel: pellet the bacteria by centrifugation, aspirate off the supernatant and add alkaline lysis buffer directly to each well of the plate. Alkaline lysis buffer: 25 mM NaOH, 0.2 mM EDTA.
- 29 Note the volume of buffer you are adding so you can calculate back the original bacterial concentration. Appropriate volumes will vary, but for a 24-well plate add 200 μ l alkaline

lysis buffer and for 1ml starting volume in an eppendorf resuspend the pellet in 50 µl buffer.

- 30 Heat the sample for 30 minutes at  95 °C . If using an eppendorf tube, make sure the lid of the tube is tightly sealed to prevent evaporation or the lid popping open. Use plastic lid clamps if possible. If preparing the sample in a multiwell plate seal it with adhesive foil and use a water bath for the heating step.
- 31 Allow sample to cool to room temperature
- 32 Add an equal volume of neutralisation buffer (40 mM Tris-HCl, pH 7–8) to the volume of alkaline lysis buffer that was used. Note this step is optional and we often skip it.
- 33 The sample can be used immediately or stored for later use at  4 °C or  -20 °C

Setting up PCR reactions

- 34 Turn on qPCR machine and laptop.
- 35 Thaw the reagents on ice. These are: PCR-grade water, 2xPCRBIO mix
 qPCRBIO Probe Mix Lo-Rox **PCR Biosystems Inc. Catalog #PB20.21-05** 10 µM
primer and probe working stocks (prepared in sections above), DNA standard dilutions (prepared in sections above).
- 36 Use a clean DNA hood for preparing the master mix/primers but do not **ever** bring your DNA samples into this hood. Samples should be added on your bench (or a different hood used only for adding samples).
- 37 While the tubes are thawing: clean the DNA hood with virkon and 70% ethanol. Clean DNA hood with virkon (or equivalent) followed by 70% ethanol. Clean pipettes, tips, racks, waster containers etc and bring them into the hood. In the hood, label 1.5 ml PCR-grade tubes for the master mix.
- 38 Make calculations for dilutions that you will do. The number of reactions needs to include: duplicates of the 7 DNA standards, duplicates of a negative control (water in place of DNA), and duplicates of your sample(s). Take PCR plate or strip of PCR tubes and lids out and put them in an empty tip box in the hood.



- 39 Write down the planned layout of your PCR tube or strips. Do not keep this information in your head and assume you will remember later! Later the PCR plate or strip in one corner so it can correspond with your notes. When writing on the PCR strips/plate make sure you do not touch the tubes to avoid contamination. Try to just touch the edges.
- 40 Pulse spin down the reagent tubes, vortex them, and spin them down again. Put them back on ice.
- 41 Prepare master stock according to your own calculations (do not add the DNA yet!). You can add more or less DNA sample as needed and the volumes needed depend on the number of reactions being performed. An example is given here:

	A	B	C	D
	Reagent	Final concentration	Volume (1 reaction)	Volume (10 reactions)
	10 μ M forward primer	0.3 μ M	0.6 μ l	6 μ l
	10 μ M reverse primer	0.3 μ M	0.6 μ l	6 μ l
	10 μ M probe	0.2 μ M	0.4 μ l	4 μ l
	Sterile water	-	7.4 μ l	74 μ l
	2x qPCR probe mix Lo-Rox	1X	10 μ l	100 μ l
	DNA standard or sample	-	1 μ l	10 μ l
	Total	-	20 μl	200 μl

Example volumes used for setting up PCR reactions.

- 42 Spin, vortex, and spin again your master mix tube. Put the tube on ice. Return the 2xqPCRBIO, primers and probe tubes to the fridge/freezer.
- 43 Use a P20 pipette to add 19 μ l of master mix to each well including negative control.



- 44 Close the plate or strip with plastic lid.
- 45 Bring the ice bucket with PCR strips/plate out of the DNA hood to your bench or dedicated DNA hood. You now need DNA standard dilution tubes, PCR grade water, samples. Use two ice buckets: one for PCR plate/strip, the other for all your tubes of stocks and samples.
- 46 Clean the hood you have just been using, so the next person can use it.
- 47 Organise your tubes in the ice bucket in the order you want to load them. Note: you should first load your negative control, then your samples, and finally the diluted DNA standards.
- 48 Add DNA or water according to the table above (or your own calculations). 
1. Add 1 μ l DNase-free water as a negative control into the 2 appropriate duplicate reactions wells.
 2. Add 1 μ l of each DNA sample into the 2 appropriate duplicate reactions wells.
 3. Add 1 μ l of each diluted DNA standard into the 2 appropriate duplicate reactions wells.
- 49 It is very important to pipette accurately. Here are some tips: 
1. Make sure the pipette is set to the correct volume, and look to see you are pulling up liquid and not air.
 2. When loading, take the tips out of the box in the same pattern and DO NOT touch the pipette or tip with anything. If you are unsure, clean the pipette with ethanol again.
 3. When releasing liquid, push the pipette all the way down . Keep holding the pipette until you can look and see you have loaded everything. Then discard the tip.
 4. Work slowly and try to perform the best possible technique and avoid any confusion (don't chat to colleagues at this moment) or contamination. qPCR is very sensitive!
- 50 If using PCR strips:
1. Close the strip with lid and press tightly, holding it just at the edges.
 2. Label the lip of the strips so it matches your map.
- Spin down the PCR strip for 15 seconds, vortex, and spin down again. You may need to use an adapter to fit it to the microcentrifuge.
- 51 If using PCR plate use a large centrifuge to spin down the plate by setting to 1,000 rpm and pressing pulse.

Running PCR reaction

- 52 We use the CFX manager software and the CFX connect real-time system.

53 Open qPCR run protocol or set up new protocol.

	A	B	C	D	E	F
	OTR 16s	tsa47	AEW 16s	Panrick 17kDa	Cfd	GAPDH
	1. 50°C for 2:00 mins 2. 95°C for 2:00 mins 3. 95°C for 0:15 mins 4. 60°C for 0:30 mins (and plate read) 5. Repeat steps 3 and 4 x49 more times 6. End	1. 50°C for 2:00 mins 2. 90°C for 2:00 mins 3. 95°C for 0:15 mins 4. 60°C for 0:30 mins (and plate read) 5. Repeat steps 3 and 4 x40 more times 6. End	1. 50°C for 2:00 mins 2. 95°C for 2:00 mins 3. 95°C for 0:15 mins 4. 61°C for 0:30 mins (and plate read) 5. Repeat steps 3 and 4 x39 more times 6. End	1. 50°C for 2:00 mins 2. 95°C for 2:00 mins 3. 95°C for 0:15 mins 4. 60°C for 0:30 mins (and plate read) 5. Repeat steps 3 and 4 x40 more times 6. End		

54 At "Express Load" drop down, click "QuickPlate_96 wells_SYBR Only" and click next.

55 Click on "Open lid" to open qPCR machine lid and place the PCR strip or plate on the machine. Note: make a note of where you place the PCR strips, and pass a tissue paper over the surface of the lid of the strips and make sure that they are tightly sealed.

56 Make sure the qPCR machine is connected to the software (Run Status: Idle).

57 Click on "Close lid" to close qPCR machine lid.

58 Click "Start Run".

59 When the run is finished, click "Open lid" to open qPCR machine lid.



- 60 Remove and discard the PCR strip or plate from the machine.
- 61 Click "Close lid" to close qPCR machine lid.
- 62 Close the software and turn off the qPCR machine.