Viral detection by fluorogenic one step real-time qPCR; proper dilution of RNA isolates is key to truly relative log-linear quantitative analysis

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A.) Trizol should be used to isolate RNA-based virions (like h5n1) from tissues since it inactivates the pathogen, rendering it inert by chaotropism; viral proteins are entirely denatured by the chaotropic GIT salt (within Trizol) while nucleic acids remain intact.

B.) 50 mg of tissue is all that is necessary, so needle-puncture collections are possible as long as the needle's contents can be extruded safely and immediately into Trizol.

C.) Trizol isolation of total tissue and viral RNA can be performed as follows:

TRIZOL virion RNA isolation from 0.05 grams of infected tissue;

In a 50 mL nuclease-free tube: 0.05 g tissue + 1 mL Trizol; The sample is then homogenized 30 sec. with an Omni TH Homogenizer (in a hood). Let sample sit for 5 minutes. Add 0.2 mL Chloroform, shake vigorously for 15 seconds. Let sit for 2-3 min. at room temp. Microfuge @ 4°C for 10 minutes. Transfer (top) aqueous layer to a new 1.5 mL tube (when gathering these water layers, avoid the white middle interface entirely! – this is protein and some genomic DNA). Add 0.5 mL nuclease-free isopropanol, mix, let stand for 10 minutes at room temp. Microfuge for 10 minutes @ 4°C. Dump isopropanol (should see nice pellets by now), (all microfuge procedures above are performed at 12,000 x g and 4°C).

Wash each pellet 2X with 1 mL pre-cooled -20C 75% Ethanol: add 1 mL -20°C ethanol, invert tubes to mix, spin at 12,000 x g for 10 minutes (pellets stick well to the bottom as you pour off the first 75% EtOH wash). Vortex with final 1 mL 75% EtOH wash (dislodge pellet during vortexing here so it is swirling in solution so the 25% water in the ethanol solution can wash away any left over GIT and other salts from the pellet etc., while the ethanol keeps the RNA precipitated), microfuge 5 minutes (at top speed), dump off final 75% EtOH wash. Air dry pellets for 20 minutes to an hour (don't over dry pellets – they are hard to redissolve).

Resolubilize pellets in a total of 80 uL of Ambion nuclease-free water with 0.1 mM EDTA pH 6.75, or HPLC-grade water with 0.1 mM EDTA (preferably <pH 7.0 in order to prevent divalent cation-dependent and pH-dependent base hydrolysis of RNA). Vortex, and warm samples to 65°C for 5 minutes (to ensure full resolubilization), vortex to mix, spin down, then:

Measure 260nm and 280nm absorbances of a 1:50 dilution of each RNA isolate in a spec. (i.e. 10 uL RNA sample + 490 uL of the same diluting buffer used for RNA sample solutions above); and zero the spec. with the same buffer you are diluting your samples with. Wash cuvette in between measurements with the same buffer also. (Spec readings from 0.085 to 0.85 are desirable; see Table 1).

***TurboDNase (Ambion) can be used for genomic DNA contamination of Trizol-isolated RNA samples (60 uL of RNA is used per reaction and 80 uL is recovered when complete). But, since samples will be used only to detect RNA virus, DNase treatment may be unnecessary unless one suspects qPCR signal from viral sequence incepted into genomic DNA. 60 uL of each RNA sample above can be used if DNase treatment is not performed. (Tissue genomic DNA contamination can occur from being careless when lifting aqueous layers during Trizol RNA extraction). Next:**

Once your RNA has been isolated, spec.'d @ 260nm, and *DNase treated or not, the most crucial thing is performed at this point – and that is to:

Dilute each RNA sample to 0.32 ng/uL (based on A_{260nm} readings). This particular dilution is often 1:1500 or more! The goal is to obtain in-well [RNA] of 0.083 ng/uL in each final qPCR reaction; perfect for tissue-viral qPCR.

(e.g. First dilute your 60 or 80 uL RNA samples from above to 0.32 ng/uL since another dilution is incurred merely by adding the RNA samples to the qPCR reaction mixtures talked about below. An "in-well" dilution of 7.8 uL RNA sample per each 30 uL qPCR rxn additionally achieves the desired final RNA concentration of "0.083 ng/uL" of each RNA sample by the time it exists in-well for the purpose of qPCR analysis; which represents a total overall dilution of most RNA pellets, post-resolubilization, of 1:5000 or higher; see Table 1)

For reasons too nebulous to go into (concerning template inhibition and/or chemical inhibition of RT and Taq DNA polymerase reactions), this is the single most important maneuver in the entire protocol in order to ensure that you will be in the truly quantitative range for fluorogenic one step real-time qPCR for viral signals. This detail gets overlooked or missed 95% of the time because it is counter-intuitive and appears to be a ridiculous thing to do to one's precious samples, i.e. use your samples at final in-well dilutions of 1:5000 or more to get better tissue-viral qPCR signals? Exactly.

Now, your total tissue RNA (containing viral RNA) is ready to participate in the following fluorogenic one step real-time qPCR reaction set-up:

Primers and 6FAM-TAMRA or 6FAM-MGBNFQ probes are designed using Primer Express version 2.0 from Applied Biosystems Inc. (ABI) and the TaqMan One-Step RT-PCR Master Mix Reagents Kit cat. no. 4309169 (ABI) is used as the Master Mix. 7.8 uL of each "0.32 ng/uL" RNA sample from above will be used per 30 uL qPCR reaction as prepared below.

Forward and reverse primers can both be used at 1 μM, while fluorogenic probes can be used at 150 nM; this will give you 100% guarantee of high-efficiency qPCR reactions virtually every time with any qPCR target (so long as you process and dilute your RNA samples as stated above).

The qPCR plates do not have to include standard curves as it will only be important to generate a 'yes' or 'no' from each sample suspected of harboring the potentially emergent human-to-human h5n1 variety of the avian flu virus.

Each 96-well plate can be used to examine 48 samples at a time (all samples represented in duplicate). Make primers and probe only to the Ncap (nucleocapsid) genetic sequence of h5n1 (or to the most highly-conserved genetic region of h5n1 viruses in general). Once a case of human-to-human h5n1 is suspected in a tissue sample, ascertain its presence by this qPCR method, then have it immediately sequenced and have the original tissue source (still harboring intact virus) used for vaccine-manufacturing purposes.

The Master Mix, primers-probe and sample set-up per 96-well plate is thus:

One Step Real-Time qPCR Set-Up

	One-Step MM 1826.67 uL		xtra ea.made	Well size prepared:	30 uL
30 uL prepared/Well	RT 91.33 uL	Set 1/sample	122.10 uL	RNA added/well:	7.80 uL
25 uL used/Well	Total MMRT prepared: 1918.00 uL	Set 1/MM ea	2253.30 uL	Duplicates	48 samples prepared
	Total MMRT needed: 1598.63 uL	Set 2/sample	0.00 uL		96 wells prepared
		Set 2/MM ea	0.00 uL	Other	

h5n1			
Fwd primer	304.50 uL	split	319.38 uL extra made
Rev primer	304.50 uL	into	
Probe	45.68 uL	48	
MMRT:	1598.63 uL	44.40 uL amounts	
Water:	0.00 uL	then add 15.60 uL RNA to each	

(Bear in mind that a 384-well plate format is also available)

Notice that 30 uL reactions are prepared in duplicate for each sample, but only 25 uL is actually added to each well of the final qPCR reaction plate. ABI real-time qPCR machine model numbers 5700, 7300, 7500 and 9700 can all be used to run these reactions; so can the Stratagene Mx4000 and Mx3005P qPCR machines.

The thermocycling parameters are: A.) 48°C for 35 minutes (RT),
B.) 95°C for 10 minutes (hot start Taq),
C.) then 40 to 50 cycles of:
95°C for 15 seconds/1 minute at 58°C
(melting and annealing-extension etc.)

Table 1.

Example dilutions of total tissue RNA isolates to attain the optimal dilution range for qPCR of viral RNA co-isolated during Trizol isolation of total RNA from infected tissue samples

Sample	260nm spec. readings of RNA at 1:50 dilution	Total RNA isolate initial concentration	Required 1st RNA dilution	Achieved pre-well RNA concentration	Final in-well RNA sample qPCR concentration	Total dilution incurred by each RNA sample since isolation into 80 uL
A	0.01	20 ng/uL	1: 62.5	0.32 ng/uL	0.0832 ng/uL	1: 240.4
B	0.05	100 ng/uL	1: 312.5	0.32 ng/uL	0.0832 ng/uL	1: 1201.9
C	0.085	170 ng/uL	1: 531.25	0.32 ng/uL	0.0832 ng/uL	1: 2043.3
D	0.1	200 ng/uL	1: 625	0.32 ng/uL	0.0832 ng/uL	1: 2403.8
E	0.2	400 ng/uL	1: 1250	0.32 ng/uL	0.0832 ng/uL	1: 4807.7
F	0.3	600 ng/uL	1: 1875	0.32 ng/uL	0.0832 ng/uL	1: 7211.5
G	0.4	800 ng/uL	1: 2500	0.32 ng/uL	0.0832 ng/uL	1: 9615.4
H	0.5	1000 ng/uL	1: 3125	0.32 ng/uL	0.0832 ng/uL	1: 12019.2
I	0.6	1200 ng/uL	1: 3750	0.32 ng/uL	0.0832 ng/uL	1: 14423.1
J	0.7	1400 ng/uL	1: 4375	0.32 ng/uL	0.0832 ng/uL	1: 16826.9
K	0.8	1600 ng/uL	1: 5000	0.32 ng/uL	0.0832 ng/uL	1: 19230.8
L	0.85	1700 ng/uL	1: 5312.5	0.32 ng/uL	0.0832 ng/uL	1: 20432.7
M	1	2000 ng/uL	1: 6250	0.32 ng/uL	0.0832 ng/uL	1: 24038.5
N	1.5	3000 ng/uL	1: 9375	0.32 ng/uL	0.0832 ng/uL	1: 36057.7

Formula for calculating ng/uL concentrations of total tissue RNA samples from A_{260nm} spectrophotometer readings of 1:50-diluted samples:

$A = \varepsilon lcd$ absorbance = molar extinction coefficient for ssRNA (M-1cm-1) x path length (1cm) x concentration (ug/mL or ng/uL) x dilution

Then, solving for "c" gives: $c = A/\varepsilon ld$ or: $c = A \times \varepsilon^{-1} \times l^{-1} \times d^{-1}$

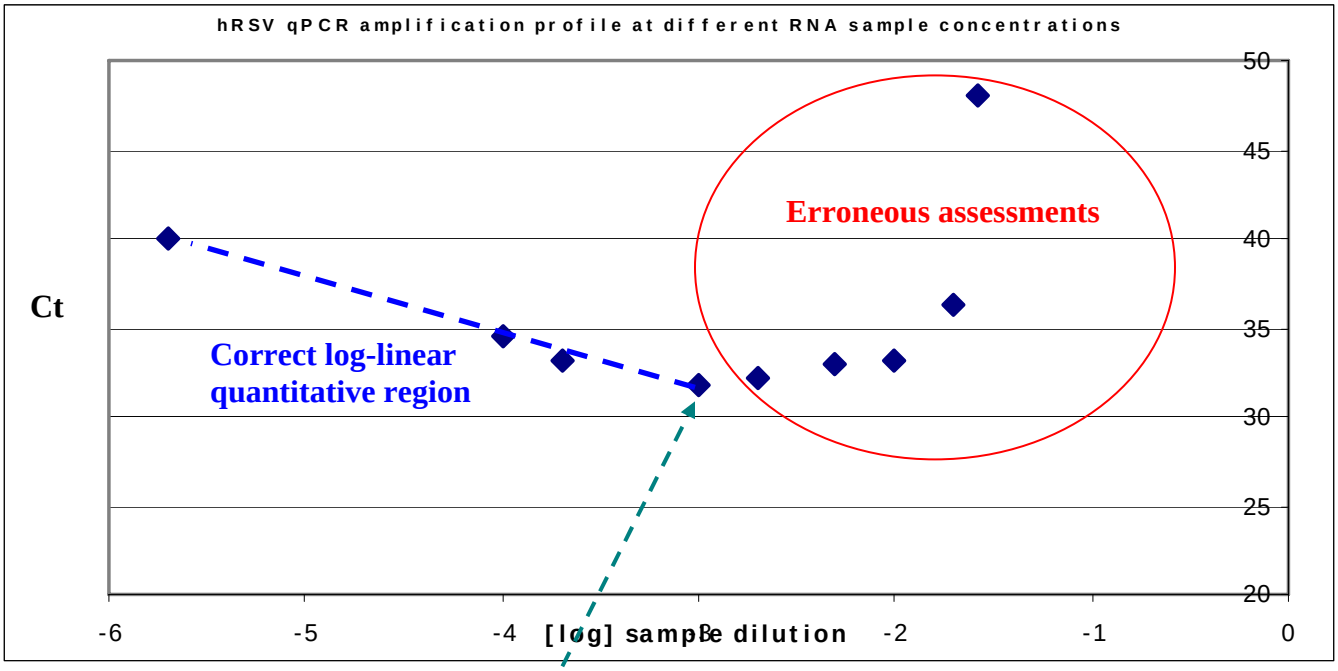
Thus: $ng/uL = A_{260nm} \times 40Mcm \times 1cm^{-1} \times 50$

Typically, Trizol-isolated total RNA from tissue samples yields A_{260nm} readings of 0.2 and above when diluted 1:50. Dilutions are all performed with certified nuclease-free water. It is always important to note that absorbance readings at or near 0.301 are the most accurate on any spectrophotometer, while absorbance readings of 1 and above become increasingly inaccurate because sample concentration calculations based on absorbance measurements of optically active substances in solution only demonstrate linearity for readings which fall in the range from 0.05 to about 0.9; noise and non-linearity is increasingly expected below and above these absorbance values, respectively.

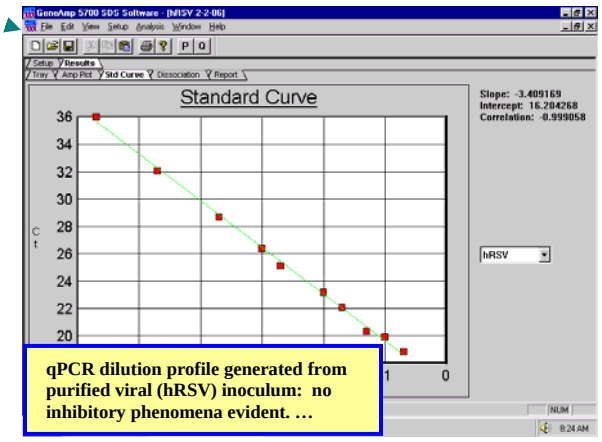
Multiple readings can be taken if necessary – then averaged.

If samples are not diluted "0.32 ng/uL" at the point stated above, one runs the risk of severely underestimating the amount of virus present in infected tissue samples.

See the example below which shows how hRSV RNA virus gives faulty values when tissue total RNA sample is used too concentrated in the qPCR application:



Note: the inhibitory phenomena illustrated above does not manifest itself with Trizol-isolated viral RNA from purified viral inoculum, only with RNA Trizol-isolated from tissue.



The red-circled points are erroneous and suggest much lower viral presence than is actually the case. The blue line indicates those dilutions of the RNA sample which yield true quantitative results in the assay. The first blue-lined point which begins to behave in the desired fashion represents an in-well [RNA] of 0.1248 ng/uL. For three viral signals so far (bRSV, hRSV and PCV-2, a DNA virus), we have found 0.083 ng/uL to be a very good concentration at which to start using total tissue RNA (containing virus) for qPCR analyses.

The goal is to let this assay be as sensitive as possible. Diluting RNA samples out beyond their ability to generate qPCR signal at all is just as bad as not diluting RNA samples far enough.

For any questions on this – please contact either Dr. Mark R. Ackermann or Jack M. Gallup at: mackerma@iastate.edu or eag@iastate.edu

We are located in Ames, Iowa in the Department of Veterinary Pathology at Iowa State University. We would be pleased to help in any way we can.

Please note that the Trizol RNA isolation approach discussed here can also be readily adapted to swab and PBS-lavage samples. In addition, Trizol RNA isolation is considerably cheaper than alternative methods. In cases where cost is not a factor, and tissues are being extracted for RNA, we would highly suggest using the Qiazol RNEasy Lipid Tissue Mini kit #74804 from Qiagen. For non-tissue samples (i.e. swabs and lavages) other RNA column-based isolation kits from Qiagen can be employed. A nice feature of these column-based RNA isolations is that inhibitory qPCR phenomena typically disappears from qPCR reactions when the RNA isolates are used after an in-well dilution of 1:50. Trizol-isolated RNA requires in-well dilutions of at least 1:200 before one can be confident that most tissue-related qPCR inhibitory phenomena is held at bay.

Cost:
The Trizol approach costs roughly $1.50 per each RNA sample isolated, while the Qiazol approach costs $5.40 per sample. So, realize that if you choose methods other than Trizol, your cost will increase by a factor of 3.5 or more.

j.m.g.