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Real-Time Quantitative PCR Analysis of Viral Transcription

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Summary

Whole-genome profiling using DNA arrays has led to tremendous advances in our understanding of cell biology. It has had similar success when applied to large viral genomes, such as the herpesviruses. Unfortunately, most DNA arrays still require specialized and expensive resources and, generally, large amounts of input RNA. An alternative approach is to query entire viral genomes using real-time quantitative PCR. We have designed such PCR-based arrays for every open reading frame of human herpesvirus 8 and describe here the general design criteria, validation procedures, and detailed application to quantify viral mRNAs. This should provide a useful resource either for whole-genome arrays or just to measure transcription of any one particular mRNA of interest. Because these arrays are RT-PCR–based, they are inherently more sensitive and robust than current hybridization-based approaches and are and ideally suited to query viral gene expression in models of pathogenesis.

Key Words: Real-time quantitative PCR; TaqMan; herpesvirus; microarray.

1. Introduction

Polymerase chain reaction (PCR) ***(1)***. has allowed many scientific fields, including virology, to develop assays for the detection of their template of interest. PCR has risen as the gold standard for detection of the presence of a pathogen in many instances in which cell culture or serological assays were once considered unsurpassed. However, post-PCR handling steps required to evaluate the product are a cumbersome part of PCR assays. The ability to track the amplification and quality of the product without post-PCR steps was first seen with the description of quantitative assays using replicatable hybridization probes ***(2)***. This technique has since become the foundation from which real-

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time quantitative PCR has been developed ***(3)***. Real-time quantitative (QPCR) PCR measures the amount of PCR product at each cycle of the reaction either by binding of a fluorescent, double-strand–specific dye (SYBRgreen™) or by hybridization to a third sequence-specific, dual-labeled fluorogenic oligonucleotide (molecular Beacon, TaqMan™). Since the introduction of real-time QPCR, many applications have arisen using this technology. The kinetics and chemistries of real-time QPCR are covered in detail by Mackay et al. ***(4)***.

Coupling reverse transcription (RT) to PCR yields the most sensitive method yet evolved to detect the presence of specific mRNAs. Unlike hybridization-based methods, RT-PCR can distinguish between various spliced mRNAs, through exon-specific primers. RNAse protection can also be used to distinguish between differently spliced messages, but this method is more difficult to adapt to high-throughput application. One of the many applications of real-time QPCR is its use in transcriptional profiling of DNA viruses. Because viruses encode on the order of 2–200 different mRNAs, many of which are coregulated, a limited number of PCR reactions can be used to query the entire viral transcriptome. This is in contrast to bacterial or mammalian genomes, which typically produce 1000–60,000 different mRNAs. We initially developed a real-time QPCR array to study the transcriptional profile of Kaposi's sarcoma-associated herpesvirus (KSHV) in culture and in different clinical samples ***(5,6)***. By designing and evaluating primers specific for every predicted open reading frame (ORF) in the KSHV genome, real-time QPCR arrived at essentially the same result as array studies for this virus, yet, at low throughput, no special training beyond good laboratory practices was required. In contrast to hybridization, we found real-time QPCR very forgiving with regard to sample quality, reagents, and handling (*see* **Note 1**).

This chapter covers the necessary guidelines and protocols for the development of a real-time QPCR assay for single genes or viral arrays. The guidelines listed in this chapter were adopted from the development of the KSHV genome-wide array ***(5,6)***.

2. Materials

1. TRI-Reagent™ (Sigma, to Louis, MO).
2. 1.5-mL Phase lock tubes (Eppendorf, Brinkman Instruments, NY).
3. 96-Well real-time PCR plates, skirted.
4. Tissue homogenizer.
5. Oligonucleotide primers/probes (MWG, NC).
6. SYBR Green Enzyme Mix (Applied Biosystems, CA).
7. TaqMan Enzyme Mix (Applied Biosystems).
8. Real-Time PCR thermocycler/equipment. (e.g., ABI Prizm 7700, ABI Prizm 5700).
9. 10X Reverse transcriptase buffer (Applied Biosystems).
10. 25 m*M* $MgCl_2$ (Applied Biosystems).
11. 10 m*M* dNTPs (Applied Biosystems).
12. Reverse transcriptase (SuperScript II, Invitrogen, CA).

13. Random hexamer RT primer (Applied Biosystems).
14. Oligotex dT beads (Qiagen).
15. 70% Isopropanol in diethyl pyrocarbonate (DEPC) water.
16. DEPC water.
17. *Primer3* (version 3.0.9) software ***(7)***.
18. *EMBOSS* (version 2.7.1) software ***(8)***.
19. Ruby (version 1.6.7) software ***(9)***.
20. PrimeTime (Vahrson and Dittmer, in preparation).
21. Excel (Microsoft, Redmond, WA).

3. Methods

The methods described below explain: (1) primer/probe design for QPCR, (2) mRNA isolation from tissues/cells, (3) reverse transcription of mRNA into DNA, (4) the setup of SYBR green-based QPCR, (5) the setup of probe-based QPCR, and (6) the setup of multiplex QPCR.

3.1. Primer Design

Primer/probe design is one of the most important aspects in achieving a successful QPCR assay. The following guidelines have been included to help attain the best primers possible for the assay. There are many computer programs and web-based applications available to assist in the design of primers and probes; for the purposes of this chapter, some have been listed in **Subheading 3.1.3.** and **3.1.4. Figure 1** exemplifies three primers and a possible nomenclature. Note that the primers are located toward the 3′-end of the ORF.

3.1.1. Primer Guidelines

1. The melting temperature (T_m) of the primers should be in the range of 59–2°C (*see* **Note 2**).
2. The maximal difference between two primers within the same primer pair should be ≤2°C.
3. Total guanidine (G) and cytosine (C) content within any given primer should be between 20 and 80%.
4. There should not be any GC clamp designed into any of the primers.
5. Primer length should fall into the range of 9–40 nucleotides.
6. Hairpins with a stem length of four residues or more should not exist in the primer sequence.
7. Fewer than four repeated G residues should be present within a primer
8. The resulting amplicon should be at least 50 nt in length, but typically no larger than 100 nt.

3.1.2. Probe Guidelines

Real-time QPCR products can be detected either by an intercalating dye or annealing of a third, specific probe. The guidelines for designing TaqMan

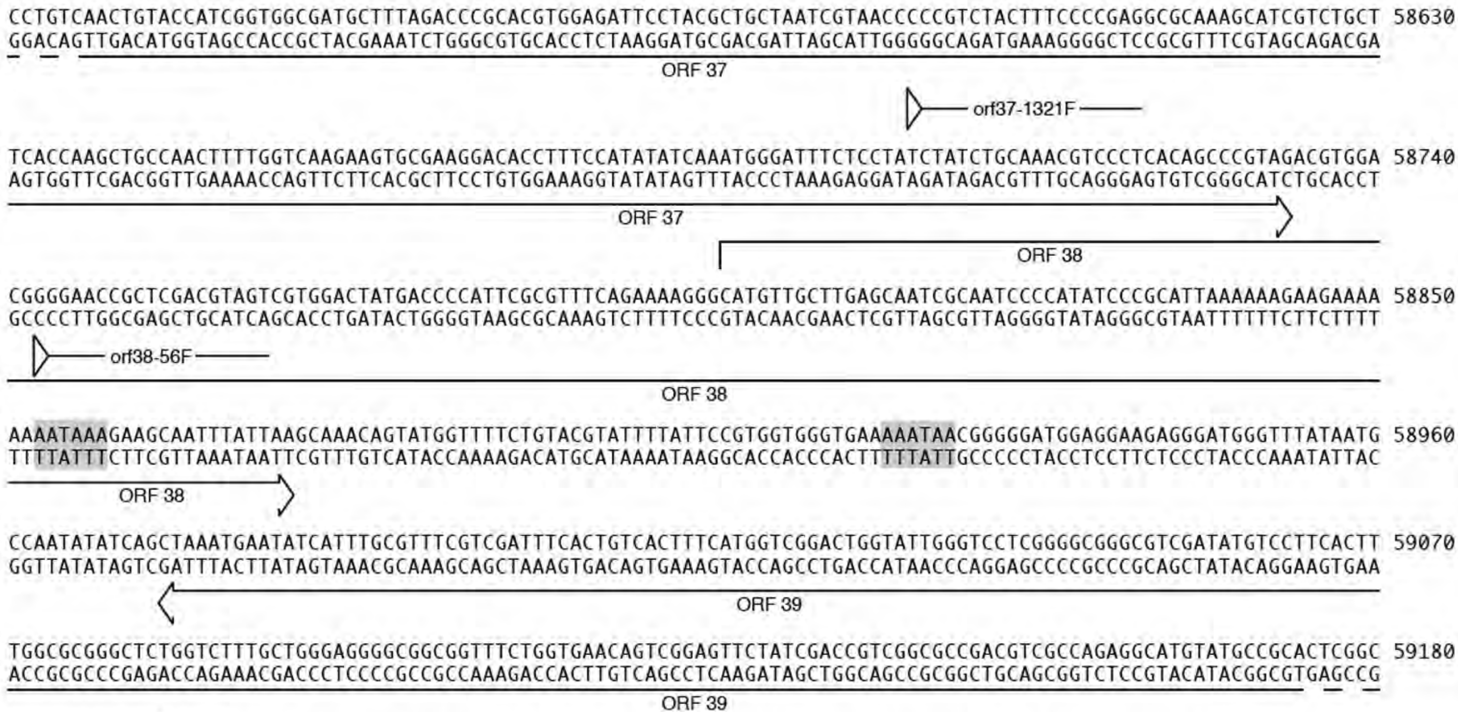

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Fig. 1. Illustration of primer position within an open reading frame. The diagram demonstrates open reading frames within a sequence and the primer placement within those open reading frames. Open reading frames are labeled ORF 37–39 and designated by the large arrows. Examples of primers are shown by the small arrows followed by the name of the primer. (Primers are named after the open reading frame and position they represent.) Shaded area signifies predicted mRNA termination signal).

probes follow those used for the construction of primers, with the following two exceptions:

1. The T_m of the probe should be greater than 10°C, compared with the T_m of the corresponding primer pair.
2. Their should not be a G residue should be at the 5′-end of the probe.

3.1.3. Primer Express®

The Primer Express® software v2.0 (Applied Biosystems, cat. no. 4330710) is available for Windows NT and Windows 2000. Prior versions (up to v1.5) were also available for Macintosh OS9. Hopefully, the program will eventually be ported to Windows XP, although no release date has been announced. This program does a good job at designing TaqMan-based primer and probe sets (as well as a host of other primers). It is easy to use and is well documented in the manual. We typically use the settings for TaqMan probes and primers to design SYBR-based primers (the other primers are identical).

There are three disadvantages to Primer Express: (1) the program must be purchased, although usually one copy is included with purchase of an ABI machine; (2) it has limitation in handling large batches of sequences or large genomes; at least version 1.5 was not able to design primers for one entire herpesvirus genome (120,000 bp) at a time; (3) at least version 1.5 scanned the sequence from the 5′-end, while we experienced better results when selecting TaqMan sets near the 3′-end of the open-reading-frame (ORF). This location allows detection in instances of lower quality RNA or of low-processivity reverse transcription.

Other commercial primer design programs are also available and should be suitable for design using the guidelines outlined above.

3.1.4. Emboss and Primer3

EMBOSS (European Molecular Biology Open Software Suite) ***(10)***. is a comprehensive collection of free open-source programs for sequence analysis. It represents a freely available and more robust alternative to proprietary programs such as PrimerExpress (Applied Biosystems) and others.

Eprimer3, its program for searching PCR primers, is based on the *Primer3* program ***(7)*** from the Whitehead Institute/MIT Center for Genome Research. It allows one to search a DNA Sequence for both PCR primers and oligonucleotide beacons. More than 60 parameters can be specified to adapt the program for various purposes. They include constraints on physicochemical properties of the primers, probes, and product, like T_M, GC content and size; constraints on sequence properties like the amount of self-complementarity and 3′-overlapping bases; positional constraints within the template sequence;

avoidance of sequences specified in a misspriming library; and many more (*see* **Note 3**).

3.1.4.1. Extracting ORFs From Database Entries

For many applications it is useful to restrict the target of the primer search to coding regions within a larger sequence. Using the UNIX *grep* command, you can inspect the annotations of GenBank or EMBL database entries for coding regions: *grep* 'CDS' *sequence-file.*

The EMBOSS *extractfeat* program lets you extract the respective sequences as individual sequences: extractfeat-type cds *sequence-file.*

As an alternative to extracting the coding sequences, one can use their positions as constraints for the primer search, as demonstrated below in **Subheading 3.1.4.2.**

3.1.4.2. Generating Primer Pairs for Real-Time PCR

Eprimer3 takes a vast number of parameters influencing the way primers are selected (*see* **Note 4**). When searching for primers suitable for real-time PCR, the most important parameters are the ones controlling T_M, sequence of the primers, and size of the product. Here is a sample invocation of *eprimer3* (explanations below):

```
eprimer3
  -otm 59.0 -mintm 57.0 -maxtm 61.0 -maxdifftm 2.0
  -mingc 20.0 -maxgc 80.0 -maxpolyx 4 -selfany 4
  -productosize 500 -productsizerange 200–800
  -includedregion 1736,5692 sequence-file
```

In the first line of the example, we specify the T_M for the primers: The optimal T_M (-otm) would be 59.0°C with a tolerance of –2°C (-mintm, -maxtm) and the additional constraint that the difference in T_M between the two primers must not exceed 2°C (-maxdifftm). On the next line parameters constraining the primer sequence are specified: the GC content must be 20–80% (-mingc, -maxgc), there must be no runs of identical nucleotides longer than four (-maxpolyx), and the maximal alignment score when testing for self-complementarity and for matches between forward and reverse primers must not be more than 4, which corresponds to an overlap of four nucleotides (-selfany). In the third line the desired optimal size of the product is given as 500 (-productosize)–300 (-productsizerange). Finally, in the last line, the portion of the sequence in which *eprimer3* searches for primers is restricted to a region between and including positions 1736 and 5692.

3.2. mRNA Isolation

The method described in this section outlines the purification of total RNA from either tissues **(Subheading 3.2.1.)** or cells **(Subheading 3.2.2.)** using

TRI-reagent (Sigma-Aldrich). Other companies offer similar chemicals that will yield similar results. A subsequent step using dT beads is then used for the selection of mRNA from the total RNA pool (3.2.3).

3.2.1. RNA Isolation From Tissues

1. Transfer 750 µL of TRI-reagent into the tube containing the tissue sample, and place the samples on ice.
2. Before one uses the homogenizer, clean it with TRI-reagent and ethanol in the following manner: TRI-reagent, 70% Ethanol, and then clean TRI-reagent.
3. Samples can then be homogenized one at a time and returned to ice. However, the homogenizer should be cleaned between every sample by the same method described in **step 2.**
4. Incubate the samples on ice for 5 min following homogenization.
5. Add 150 µL of chloroform to each sample, and mix well. This can be done by shaking the tubes, or by briefly vortexing the tubes.
6. After mixing, centrifuge the sample at full speed in a bench-top centrifuge for 15 min at 4°C.
7. Once the tube is removed from the centrifuge, three phases should be visible within the tube. *The upper phase contains the RNA and should be removed and placed into a new phase-lock tube* (Eppendorf, Brinkman Instruments, Westbury, NY). The middle phase contains the DNA from the sample, and the lower phase contains protein. The remaining two phases in the tube should be disposed of properly once the upper phase of interest is removed, as they are organic waste and must be handled as such.
8. Add 250 µL of phenol/chloroform/isoamyl alcohol to the sample in the phase-lock tube, and vortex the sample for 1 minute.
9. Incubate the samples on ice for 5 min.
10. Centrifuge the sample again at full speed for 10 min at 4°C.
11. After centrifugation, transfer 175 µL of the clear upper phase to a new tube, and mix with an equal volume (175 µL) of isopropanol.
12. Mix the sample thoroughly, and incubate at –80°C overnight.
13. Remove RNA in isopropanol from the freezer, and allow the sample to thaw.
14. Once the sample is thawed, centrifuge at full speed for 20 min at 4°C.
15. Aspirate the supernatant being cautious not to disturb the RNA pellet.
16. Add 1 mL of 70% ethanol (in DEPC water). *Do not* dislodge or attempt to redissolve the pellet.
17. Carefully aspirate the supernatant, and air-dry the tube for 10 min.
18. Resuspend the RNA pellet in 250 µL of DEPC-treated water. You can now proceed to the mRNA enrichment step **(Subheading 3.2.3.)**, or the total RNA pool can be frozen at –80°C for future use.

3.2.2. RNA Isolation From Cells

The process for extracting total RNA from cultured cells is identical to that for extracting total RNA from tissues **(Subheading 3.2.1.)**, with the exception

of using a tissue homogenizer. When one is isolating total RNA from cells, a tissue homogenizer is not needed; simply resuspending the cell pellet in TRI-reagent is sufficient to lyse the cells. The following protocol describes the isolation of total RNA from a cell pellet; cells should be pelleted for this procedure by centrifugation at 200*g* for 5 min at 4°C.

1. Transfer 750 µL of TRI-reagent into the tube containing the tissue sample, and place the samples on ice. If the samples are not already in an 1.5-mL centrifuge tube, they should be transferred once they are resuspended into TRI-reagent.
2. Vortex the samples for 30 s, and place them on ice for 5 min.
3. Add 150 µL of chloroform to each sample, and mix well. This can be done by shaking the tubes, or by briefly vortexing the tubes.
4. After mixing, centrifuge the sample at full speed in a bench-top centrifuge for 15 min at 4°C.
5. Once the tube is removed from the centrifuge, three phases should be visible within the tube. *The upper phase contains the RNA and should be removed and placed into a new phase-lock tube* (Eppendorf, Brinkman Instruments). The middle phase contains the DNA from the sample, and the lower phase contains protein. The remaining two phases in the tube should be disposed of properly once the upper phase of interest is removed, as they are organic waste and must be handled as such.
6. Add 250 µL of phenol/chloroform/isoamyl alcohol to the sample in the phased lock tube, and vortex the sample for 1 min.
7. Incubate the samples on ice for 5 min.
8. Centrifuge the sample again at full speed for 10 min at 4°C.
9. After centrifugation, 175 µL of the clear upper phase should be transferred to a new tube and mixed with an equal volume (175 µL) of isopropanol.
10. Mix the sample thoroughly, and incubate at –80°C overnight.
11. Remove the RNA in isopropanol from freezer, and allow the sample to thaw.
12. Once the sample is thawed, centrifuge at full speed for 20 min at 4°C.
13. Aspirate the supernatant, being cautious not to disturb the RNA pellet.
14. Add 1 mL of 70% ethanol (in DEPC water). *Do not* dislodge or attempt to redissolve the pellet.
15. Carefully aspirate the supernatant and air-dry the tube for 10 min.
16. Resuspend the RNA pellet in 250 µL of DEPC-treated water. You can now proceed to the mRNA enrichment step **(Subheading 3.2.3.)**, or the total RNA pool can be frozen at –80°C for future use.

3.2.3. Enrichment of mRNA

The Oligotex mRNA purification system (Qiagen) exploits the observation that cellular mRNAs contain a polyadenylated (poly[A]) tail of 20–250 adenosine residues ***(11)***. Since mRNAs are the only cellular RNAs that contain a poly(A) tail, this feature can be taken advantage of to purify and enrich mRNA exclusively from a total RNA pool. The Qiagen Oligotex system uses a dT oligomer coupled to a solid phase matrix to bind the poly(A) tail of mRNA

while the remaining RNA, which does not contain a poly(A) tail, is washed away. Hybridization of the poly(A) tail to the dT oligomer is dependent on high-salt conditions, so the complex can be easily disrupted by lowering the ionic strength. The protocol for purification of mRNA using the Oligotex system is covered in detail in the supplementary material of the kit and therefore is not included in this chapter.

3.3. Reverse Transcription

Reverse transcription takes advantage of reverse transcriptase, an enzyme found in retroviruses, to synthesize a strand of DNA that is complementary (cDNA) to the sequence of the RNA used in the reaction. This cDNA can then be used as a template in a PCR reaction. The following steps in this process involve the creation of cDNA from the previously isolated RNA/mRNA. Subheadings in this section describe the setup of the reverse transcription reaction **(Subheading 3.3.1.)**, the cycling conditions necessary for reverse transcription **(Subheading 3.3.2.)**, and the process to ready the sample for use as a template in a PCR reaction, including the digestion of the remaining RNA.

3.3.1. Setup of the Reverse Transcription Reaction

To begin setting up the reverse transcription reaction, a master mix should be created containing all the necessary reagents excluding the sample. Listed below are the reagents and the volume necessary for a 1X reaction. The total volume of master mix created should be sufficient for *n* + 1 reactions, where *n* equals the number of reactions you wish to carry out. As an example, the volume necessary for a 11X reaction master mix is also listed.

Reagents (µL) (µL)	1X	11X (10 + 1)
10X RT-buffer (ABI)	2	22
25 m*M* $MgCl_2$ (ABI)	4.4	48.4
10 m*M* dNTPs (ABI)	4	44
Reverse transcriptase (Invitrogen)	1	11
Random hexamer	1	11
RNase inhibitor (ABI)	0.4	4.4
Total	12.8	140.8

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Once the master mix is created, aliquot 12.8 µL of the master mix into a 0.2-mL thin-walled PCR tube for each reaction. Subsequently add 7.2 µL of RNA for each sample to a tube of aliquoted master mix. The total volume of the reaction is 20 µL (12.8 µL of master mix and 7.2 µL of RNA), and the amount of input RNA can be anywhere in the range of 3 to 4000 ng. The reaction is now ready for cycling; continue to **Subheading 3.3.2.**

3.3.2. Cycling Conditions for RT

The creation of cDNA using RT is a simple one-cycle, three-step reaction in the thermocycler. The times and temperatures are as follows: 42°C, 45 min; 52°C, 30 min; 70°C, 10 min.

After the cycling, continue to **Subheading 3.3.3.** or the reactions can be stored at 4°C until continuing with the sample preparation.

3.3.3. RNA Digestion and Sample Preparation

Following the RT reaction, the sample is prepared for PCR amplification by digestion of the remaining RNA and by increasing the volume of the sample. The RNA digestion is necessary to remove remaining RNA that might interfere with the subsequent PCR reaction. To perform the RNA digestion, simply add 1 U of RNase H to the sample, and incubate at 37°C for 30 min. This is sufficient to remove all the reaming RNA from any RNA/DNA hybrids. Since the total volume of the sample is only approx 20 µL, the volume should be increased using DEPC water to yield enough sample for multiple PCR reactions. The reaction should be diluted to a total of at least 200 µL, but it can be brought up to as much as 600 µL.

3.4. SYBR Green-Based QPCR

The setup and cycling of a QPCR reaction using SYBR Green as a detection system is covered in **Subheadings 3.4.1.** and **3.4.2.** This includes the preparation of the master mix, the concentration of reagents within the reaction, and the cycling conditions for QPCR (*see* **Note 5**).

One of the crucial aspects of PCR is to guard against contamination. Ideally all steps of the setup reaction are conducted in separate rooms: (1) a so-called white room to assemble the primers and reagents; (2) a so-called gray room to prepare the RNA and add the sample to the PCR; and (3) a post-PCR array black room, which is normal laboratory space. All surfaces should be washed with 10% bleach weekly, and if possible overhead ceiling UV lights should be installed.

3.4.1. Setup of the Reaction

1. The first step in setting up the reaction is creating the primer mix. The primer mix consists of both the forward and reverse primers at a concentration of 1 pmol/µL. However, individual primers are stored at 100 pmol/µL at –80°C and once a while enough combined and diluted to yield enough forward and reverse primer mix for 100 reactions. In the case of single-primer real-time QPCR, we did not find it necessary to test a range of primer concentrations and to optimize them individually.

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2. This primer mix is then combined with the SYBR Green 2X PCR mix (Applied Biosystems) to create the master mix. The volume of each mix that is added to cre-

96-well SYBR-Green QPCR Set-up

Reagents	1x	100x	
Primer Mix (1 μM each)	2.5μl	250μl	Master Mix
2xSYBR-Green PCR Mix	7.5μl	750μl	
Total	10μl	100μl	
		↓ 10μl/reaction	
Sample		5μl/reaction	
		15μl Total Volume	

Fig. 2. Diagram of SYBR-Green QPCPR setup. The diagram lists the reagents and volumes necessary for the setup of a 96-well reaction real-time QPCR. The master mix consists of primer mix and 2X SYBR-Green PCR mix. 1X, volumes necessary for one reaction; 100X, L volumes necessary for 96 reactions. Once the master mix is combined to a total volume of 100 μL, 10 μL is aliquoted per well (the volume for a 1X reaction), and 5 μL of sample is added later for a total volume of 15 μL.

ate the master mix depends on the final reaction volume you are trying to achieve and the number of reactions for which the master mix is being prepared. For the purposes of this chapter, a final volume of 15 μL will be used. This volume was chosen as we have previously demonstrated its efficacy ***(5,6)***. Depending on the individual equipment, smaller volumes may be possible, but we found that without automation the pipeting error becomes substantial. In comparison with the 50 L volume originally recommended by many manufacturers, a smaller volume lowers the cost of QPCR per reaction by 70%.

3. To create the master mix for a 15 μL final volume, 2.5 μL of the primer mix (166 n*M* final concentration) should be added to 7.5 μL of 2X SYBR Green PCR Mix (Applied Biosystems) for a 1X reaction. The amount of master mix created should be equal to $n + 1$, where n represents the number of reactions for which the master mix is being created.
4. Once the master mix is created, 10 μL of master mix should be aliquoted per reaction into a 96-well skirted PCR plate.
5. The plate is then moved into the next room and 5 μL of the sample is added to bring the final reaction volume to 15 μL.
6. **Figure 2** shows the contents and volumes necessary for the setup of 96-well QPCR reactions.

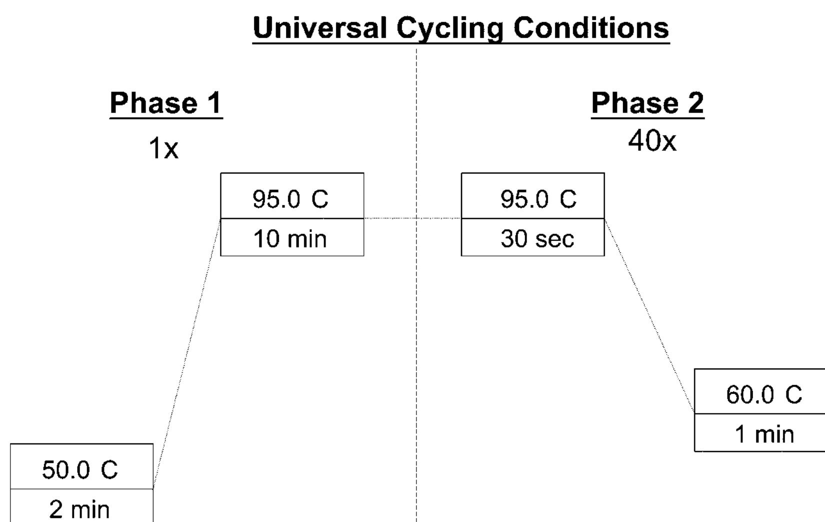


Fig. 3. Universal cycling conditions: representation of the cycling conditions necessary to conduct real-time QPCR. The cycling conditions are separated into two phases (1 and 2). The phases are divided by the dashed line, and the steps are represented by the boxes listing the temperature and the time of the step. The numbers listed below the phase (e.g., 40X in phase 2) shows the number of cycles for which the phase is run.

3.4.2. Cycling Conditions

Listed here are the universal cycling conditions for real-time QPCR (*see* **Note 6**). The cycling conditions consist of two phases **(Fig. 3)**. Phase one contains two steps. The first step, 2 min at 50.0°C, is an equilibration step used to bring all the samples to the same temperature and ready them for the reaction. The second step, 10 min at 95.0°C, is used to activate the polymerase within the PCR mix (hot-start PCR). Phase two is the cycling or amplification phase of the reaction. During this phase the first step is a denaturing phase, 15 s at 95.0°C, and the second step is the annealing and elongation phase, 1 min at 60.0°C. This second phase is run for 40 cycles (*see* **Note 7**).

3.5. Probe-Based QPCR

The setup and cycling of a QPCR reaction using a fluorogenic probe (*see* **Note 8**) as a detection system is covered in **Subheadings 3.5.1.** and **3.5.2.**

3.5.1. Setup of the Reaction

Setting up a reaction for probe-based QPCR is similar to the setup of SYBR Green-based QPCR as covered in **Subheading 3.4.1.**

1. First, a primer/probe mix is created. This mix, as the name suggests, contains the primer set and the probe that will be used for detection. The volume of the primer/probe mix that will be added to a 1X reaction is 2.5 µL. As with SYBR Green-based QPCR, the final concentration of the primer in the reaction should be 166 n*M,* so the concentration of the probe in the primer probe mix should be 1 pmol/µL. The probe should be at a final concentration of 166 n*M* in the reaction, so a concentration of 1 µ*M* should be achieved when making up the primer/probe mix.
2. Once the primer/probe mix is created, a master mix can be made to aliquot for each sample. A 1X master mix contains 2.5 µL of the primer probe mix and 7.5 µL of the 2X TaqMan PCR mix (Applied Biosystems). The volume of master mix required depends on the number of reactions that are going to be run. Create a volume sufficient for $n + 1$ reactions, where n equals the number of reactions you wish to run.
3. Once it is created, the master mix should be aliquoted into the reaction plate at a volume of 10 µL/reaction.
4. The sample can then be added to each reaction at a volume of 5 µL/reaction to yield a total reaction volume of 15 µL.

3.5.2. Cycling Conditions

The universal cycling conditions for SYBR Green-based QPCR are also applicable to primer/probe-based QPCR and are listed in **Subheading 3.4.2.** The conditions are also shown in **Fig. 3** (*see* **Note 9**).

3.6. Multiplex (Multiple Probe) QPCR

The setup and cycling of a QPCR reaction using multiple probes within a single reaction are covered in **subheadings 3.6.1.** and **3.6.2.**

3.6.1. Setup of Multiplex QPCR

The most complex aspect of setting up multiplex QPCR is the creation of the primer/probe mix. This mix should contain all primers and probes that are to be used in the reaction. The concentration of all primers within the mix should be at 1 pmol/µL to yield a final concentration of 166 n*M* in the final reaction. The concentration of all the probes within the mix should be 1 µ*M,* so as to yield a final concentration of 166 n*M* within the final reaction. It is important that all primers and probes for the reaction be diluted together within the same tube so that only 2.5 µL of the primer/probe mix is needed to yield the proper concentrations of all primers and probes in the final reaction.

One of the complications of multiplex PCR lies in the fact that the more abundant message may plateau before the less abundant mRNA and consume all reagents. If the relative abundance of both targets in the reaction is known, the primer concentration for the more abundant mRNA should be rate-limiting

(typically 1/5 to 1/10 of the less abundant mRNA) and has to be determined empirically. For the detection of viral mRNAs, we found that cellular housekeeping mRNAs are 10–1000-fold more abundant, and we typically use those as internal standards, rather than trying to measure two different viral transcripts in the same reaction.

The second step is to create a master mix containing 2.5 µL of the primer/probe mix and 7.5 µL of the TaqMan PCR mix. Once the primer/probe mix is created, the setup of multiplex QPCR is identical to primer/probe-based PCR. Determine the number of reactions needed, and create a sufficient amount of master mix for $n + 1$ reactions, where n equals the number of reactions needed. The master mix is then aliquoted into the reaction plate at a volume of 10 µL/reaction. The sample can then subsequently be added to the reaction at a volume of 5 µL/reaction to yield a total reaction volume of 15 µL.

3.6.2. Cycling Conditions

The universal cycling conditions for SYBR Green-based QPCR are also applicable to multiplex QPCR and are listed in **Subheading 3.4.2.** The conditions are also shown in **Fig. 1.**

3.7. Real-Time (Multiple Primer) QPCR Arrays

Once one has become comfortable with setting up real-time QPCR for one mRNA of interest, there is no *a priori* reason to set up a real-time QPCR for multiple mRNAs. We previously developed a set of 96 primers that query every single ORF of the KSHV genome ***(5,12)***.

3.7.1. Design of Real-Time QPCR Arrays

Real-time PCR primers are designed using the same criteria and software as before, either by hand, extracting one ORF at a time from Genbank, or by feeding the entire genomic sequence into the PrimeTime program. The design criteria stay the same. When predicted ORFs overlap, primers are selected outside the region of overlap. Unless a complete transcript map for the virus is made, one cannot exclude the possibility that some primers are located in regions in which 3′-UTR or 5′-UTR segments of two different genes overlap.

3.7.2. Setup of the Reaction

Here it is essential to conduct everything in a 96-well format **(Fig. 4)**. It is important that forward primers be synthesized on a separate plate, and then the reverse primers. We use a MerMade 96-well synthesizer (BioAutomation, Plano, TX) and store the primers in dH_20 at –80°C at a concentration of 100 pmol/µL (100 *M*). Primer length and purity are verified using a P/ACE MDQ Capillary Electrophoresis System (Beckman Coulter, Fullerton, CA). This serv-

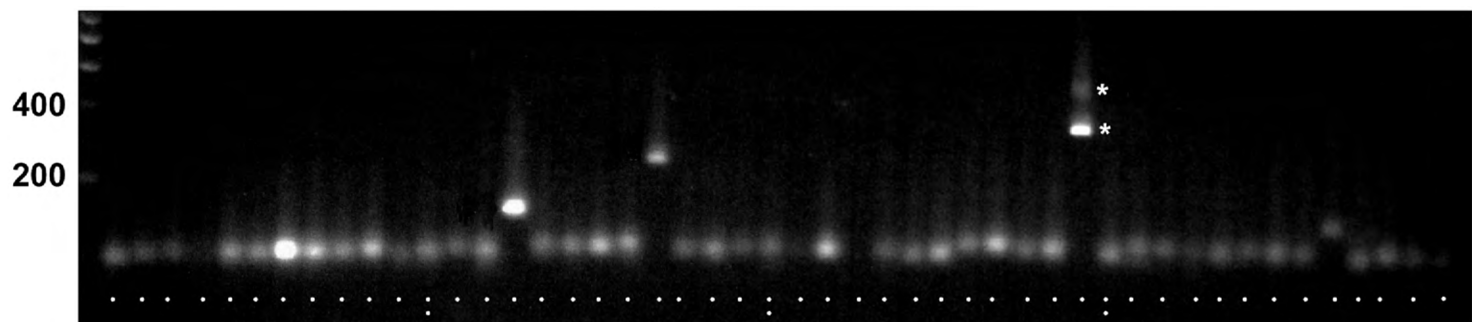


Fig. 4. Ethidium bromide-stained 2% agarose gel of the PCR products for each KSHV ORF after 40 cycles. The template was reverse-transcribed poly(A) mRNA from BCBL-1 cells 48 h after 12-0-tetradecanoyl-phorbol-13-acetate (TPA) induction. Molecular weight markers are shown on the left. Most amplicons are of the same size; exceptions are either housekeeping genes or primers against splice variants (*).

Fig. 5. The CAS-1200 pipeting robot (Corbett Research, Australia). The CAS-1200 is designed specifically for pipeting PCR reactions. The operating system for the robot is straightforward, easy to use, and runs in Windows XP. PCR reactions can be set up in 0.2-mL thin-walled PCR tubes with a 96-well or 384-well format.

ice is available in most institutional core facilities or from any number of commercial oligo provider companies (e.g., MWG). It is important to minimize freeze–thaw cycles of the master primers. Hence, we transfer 50 µL forward and 50 L reverse primer into a new 96-well plate once a month, which yields enough primer for 100 × 96 arrays. The reaction is set up essentially as listed in **Subheading 3.4.,** except that a 20-L multipipetor can be used to add primer mix to the reaction plate and later 5 L of sample per well. Mastermix is added to each well using a repeat pipetor. Alternatively, a robot **(Fig. 5)** can be used in this step.

3.7.3. Cycling Conditions

The universal cycling conditions for SYBR Green-based QPCR are also applicable to multiplex QPCR and are listed in **Subheading 3.4.2.** The conditions are also shown in **Fig. 1.**

3.7. Analysis of Real-Time QPCR and Real-Time QPCR Arrays

3.7.1. Theoretical Considerations

Prior to reaching saturation (owing to exhaustion of primers and nucleotides, loss of polymerase activity, and so on), PCR amplification proceeds exponentially and can be described by $N_i = N_0 \times (1 + k)^i$, where N_0 represents the number of molecules in the original sample and N_i the number of mRNA molecules at cycle i (i = 0.40). During the exponential phase, the amplification efficiency k ($0 \leq k \leq 1$) of a given primer pair is constant. Before real-time PCR, it was not easy to identify the eponential phase of the reaction. Either the same reaction was run for different cycle numbers (20, 22, 24, and so on) and the product quantified by gel electrophoresis using the same amount of sample in each case, or different dilutions of sample were used in multiple PCR reactions for the same cycle number. During real-time QPCR the amount of product at each cycle is quantified ***(3)***. Fluorescence intensity Rn has a logarithmic dependence on fluorophor (the PCR product) concentration, yielding $Rn = \log(N_i) = \log[N_0 \times (1 + k)^i]$. Real-time quantitative PCR compares two samples with target concentrations N_a and N_b by recording the cycle numbers (C_T) for a and b at which the amplification product yields enough fluorescence to cross an operator-determined threshold T (set at five times the SD of the nontemplate control [NTC]). Consequently, $Rn_a = Rn_b$ and $\log[N_a \times (1 + k)^a] = \log[N_b \times (1 + k)^b]$ or $\log(N_a) - \log(N_b) = \log_{(1+k)} b - \log_{(1+k)} a = \log_{(1+k)} b - a$ (for i = 0, $N_{i=0} = N_0 \times (1 + k)^0$, i.e., $N_{i=0} = N_0$). Ideally, $k = 1$ and $(1 + k) = 2$, i.e., at each cycle two reactions products are produced per target molecule. This leads to $N_i = N_0 \times (1 + 1)^i = N_0 \times 2^i$. Assuming $\log = \log_2$, $N_a/N_b = 2^{b-a}$, where N_a/N_b represents the fold difference in mRNA levels of two samples with $C_T = a$ and $C_T = b$.

Hence, it is possible to extract the relative ratio of abundance in two samples based on this calculation. Interestingly, hybridization-based DNA arrays have similar characteristics, since the color intensity ratio in a fluorescent Cy3/Cy5 DNA array exhibits a logarithmic dependence on the amount of hybridized probe ***(13)***. Analogous to the amplification efficiency k for PCR, a hybridization-efficiency K_0 applies to DNA arrays, which is a function of the length and base composition of the particular cDNA fragment at a given hybridization temperature.

3.7.2. Absolute Quantification for One Primer Pair on Multiple Samples

To quantify the abundance of a single mRNA and/or viral species in diagnostic applications, a standard curve is generated that plots the C_T number in

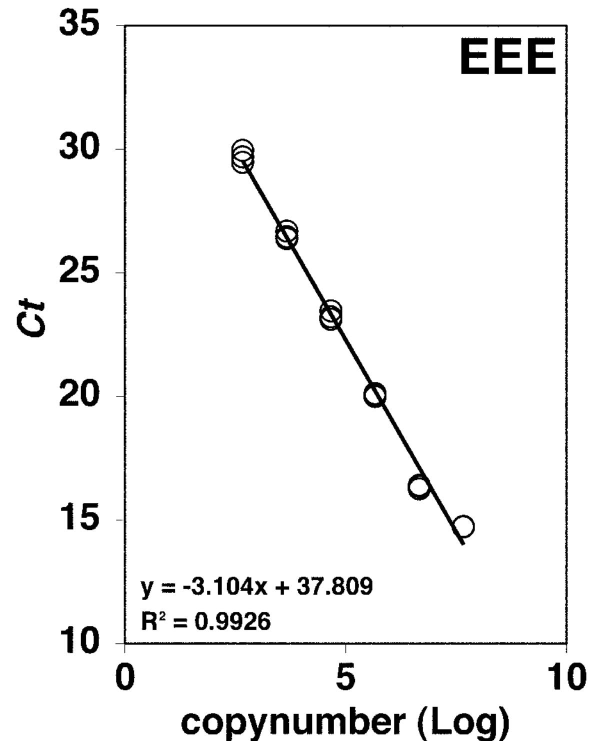


Fig. 6. Linear regression of a real-time QPCR primer pair for eastern equine encephalitis virus (EEE). Plotted on the *X*-axis is the log of the copy number against the C_T achieved for each dilution on the *Y*-axis. The slope of the line is listed in the form of $y = mx + b$, and R^2 = the regression coefficient. Each dilution was amplified in triplicate.

relation to the copy number per unit, for instance, copy number per 10^6 cells or per 1 g DNA ***(14)***. Actual values are interpolated by linear regression analysis **(Fig. 6)**. The slope of the dilution curve defines the amplification efficiency *k*. A decision is made based on the interpolated copy numbers, and the significance of the observation is established by multiple measurements per sample. Mean, standard deviation (SD), and/or confidence intervals (CVs) can be calculated from the interpolated copy number per sample, and goodness of fit of the standard curve can be gauged by its regression coefficient R^2 (reviewed in **ref. *15***). Calculations are performed using Excel or more advanced statistical software such as SPSS (SPSS Science, Chicago, IL). A standard curve will also reveal the linear range of real-time QPCR for a particular primer pair, such as in viral load assays. This type of validation is ideally suited for the quantification of multiple samples with a single primer pair. However, it is very cumber-

some, and in order to maintain perfect accuracy, a standard curve has to be included with each amplification group (96-well plate) and for each primer pair (*see* **Note 10**).

3.7.3. Absolute Quantification of Two or More Different Primer Pairs

Standard curves are generated for each primer pairs as in **Subheading 3.7.2.,** and actual copy numbers are interpolated for each target. Copy numbers can then be compared for each target over multiple samples using conventional statistics as outlined in **Subheading 3.7.2.** Furthermore, copy numbers for the two (or more) different targets can be compared with each other. For example, the relative mRNA levels for two different mRNAs in the same tissue can be recorded. Calculations are performed using Excel or more advanced statistical software such as SPSS.

3.7.4. Relative Quantification for One Primer Pair

Often transcriptional profiling is concerned only with relative differences between two samples, *a* and *b,* which are expressed in unit less fold change. Hence raw C_T numbers can be used directly. Relative quantification eliminates the intermediate use of a standard curve and allows for the direct comparison of the fold differences between two target populations. This only requires the data for each sample, not a standard curve ***(16–19)***. By applying rank-based statistics (Wilcoxon's sum rank test) or a simple *t*-test, we can determine, for instance, whether one of the tissues or treatment yields to a relative (and statistically significant) change in mRNA levels between different samples.

3.7.5. Relative Quantification for Multiple Primer Pairs

The unmanipulated C_T data for multiple primer pairs and multiple samples can also be used to extrapolate the relative expression pattern for many genes. To do so, we need to apply hierarchical clustering, as previously described ***(20)***. Importantly, the same clustering algorithms that are in use for hybridization array analysis can be used to analyze real-time QPCR arrays **(Fig. 7)**. Instead of feeding in the individual spot intensities as recorded inhybridization arrays ***(21)*** as a gene by experiment table into the program, individual C_T values in the format of a PCR primer by experiment table are used as input.

In order for relative quantification to be valid between different primer pairs, three constraints are placed on the amplification efficiency for each primer pair *k:*

1. The amplification efficiency k or $E = (1 + k)$ must not change with increasing cycle number. This assumption is valid only during exponential amplification, when neither primers, nor polymerase activity, nor nucleotides limit the reaction. Setting the threshold appropriately guarantees that this key assumption is not violated.

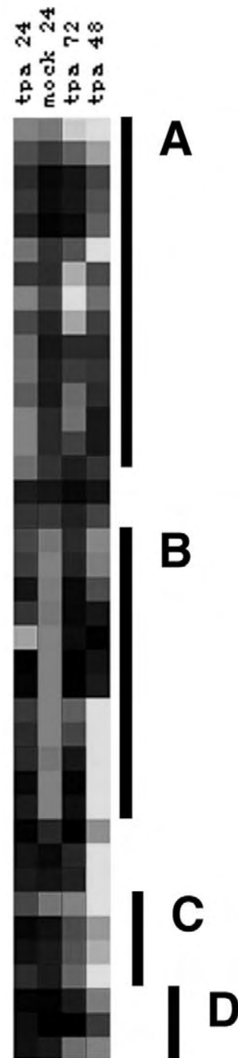


Fig. 7. Representation of hierarchical cluster analysis. Shades of gray indicate transcription level with lighter shades representing increased transcription. Groups of genes clustering together are shown by the thick black lines next to the clustogram, and each group is labeled with a letter. A total of four clusters are found in this figure (A, B, C, and D).

2. The amplification efficiency *k* is constant over a wide range of concentrations (typical five orders of magnitude for real-time QPCR, which determines the linear range of the assay) but may not be accurate for comparing very low or high levels of target DNA. In contrast to conventional end-point or gel-based PCR methods,

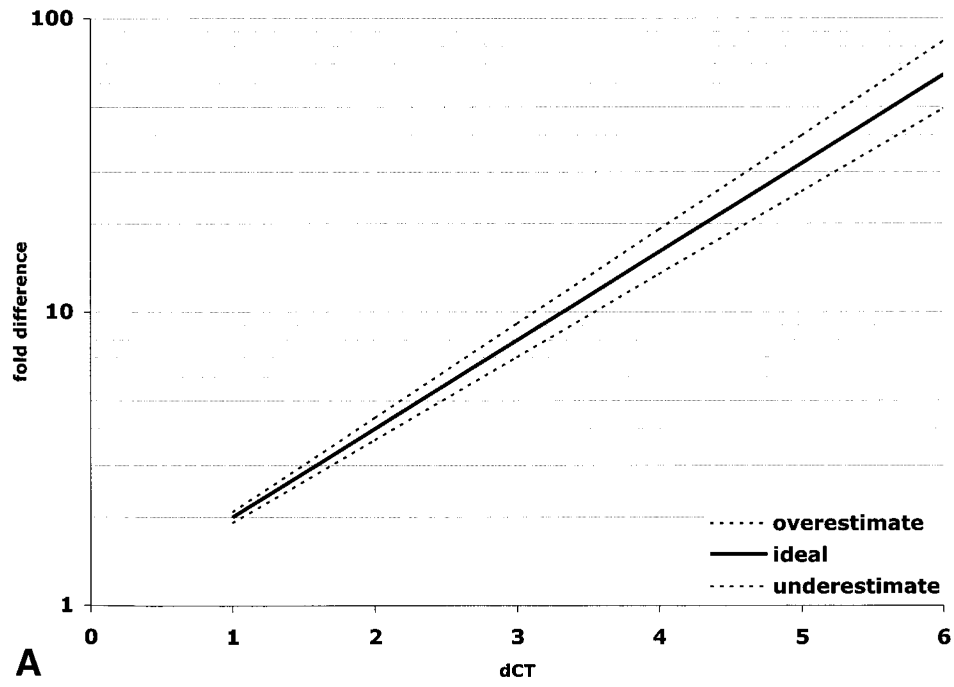
real-time QPCR instruments and fluorescent chemistry record the entire amount of product at each cycle and thus allow for the direct visual observation of constraints (1) and (2) for each data point.

3. The amplification efficiency k determines the spread, i.e., into how many fold target-level–difference a given C_T difference translates. Under ideal amplification conditions, exactly two molecules are produced per parent at each cycle. This assumption leads to the widely used shortcut to convert C_T differences into fold differences: fold difference $(a - b) = 2^{(C_T a - C_T b)}$.

Is this a reasonable supposition? **Figure 8A** visualizes the effect of changes in k by plotting relative fold difference for various amplification efficiencies $E = k + 1$. Assuming ideal amplification ($k = 1$, i.e., $E = 2$), a C_T difference of five cycles between two samples C_{Ti} and C_{Tj} translates into a 32-fold difference in input levels. However, if the amplification reaction proceed with 20% less efficiency than ideal ($k = 0.8$, i.e., $E = 1.8$), $C_{Ti} - C_{Tj} = 5$ represents only a 19-fold difference. If the PCR efficiency drops below $k = 0.6$, even a 10-cycle difference in C_T does not yield an appreciable fold difference. Since most PCR reactions do not proceed under ideal conditions, assuming $k = 1$ ($E = k + 1 = 2$) almost always overestimates the true difference in target levels. This explains some of the outrageous discrepancies in fold induction/suppression, observed when DNA hybridization array data were verified by real-time QPCR.

Multiple-primer real-time QPCR arrays compound this problem, since the aim is to compare many different primer pairs with each other. This is strictly possible only under than additional constraint, that the amplification efficiencies E_a and E_b for any two primer pairs a and b in the array do not differ from each other. It makes a comparison between different primer pairs (measuring the transcription profile of different mRNAs) impossible, without first determining the standard curves for each primer pair j, $j = 1$ m, and then comparing fold differences obtained after absolute quantification. Surprisingly, however, most primer pairs have very similar amplification efficiencies. We typically calculate the amplification efficiency by dilution once for each primer pair and exclude primers that fail to amplify with $E < 1.8$ **(Fig. 8B)**. According to the considerations in **Fig. 2A,** the maximal error introduced by different amplification efficiencies is twofold or one C_T unit. This is less than the experimental error in most cases. More elaborate schemes have been and are still being developed to compare multiple primers ***(16,17)***. For transcription profiling, however, in which addition to a change between any two samples, a pattern develops (e.g., increase over time), relative clustering of the C_T values will easily discern different response classes (*see* **Note 11**).

Finally, it is important to realize that by and large the error for each primer pair is dependent on the experimental error and handling error only, but not on the amplification efficiency or the amount of input sample **(Fig. 8C)**.


100
10
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ideal
underestimate
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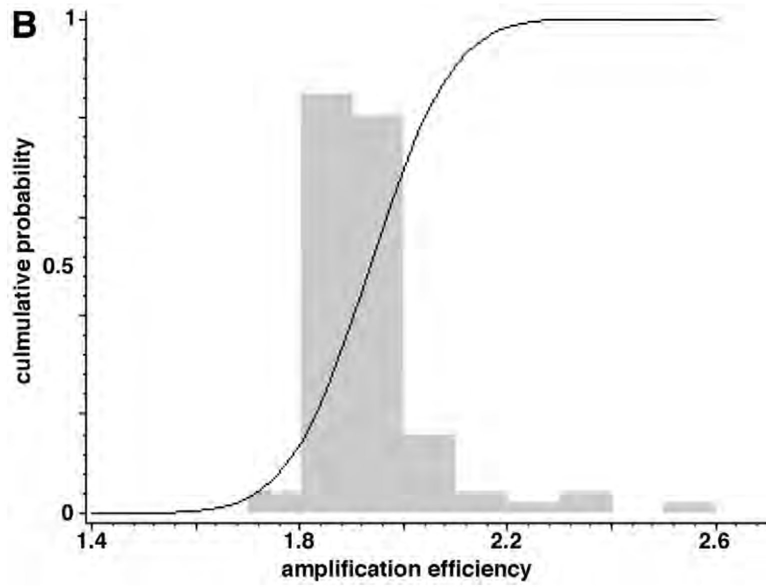
B
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0.5
0
cumulative probability
1.4
1.8
2.2
2.6
amplification efficiency

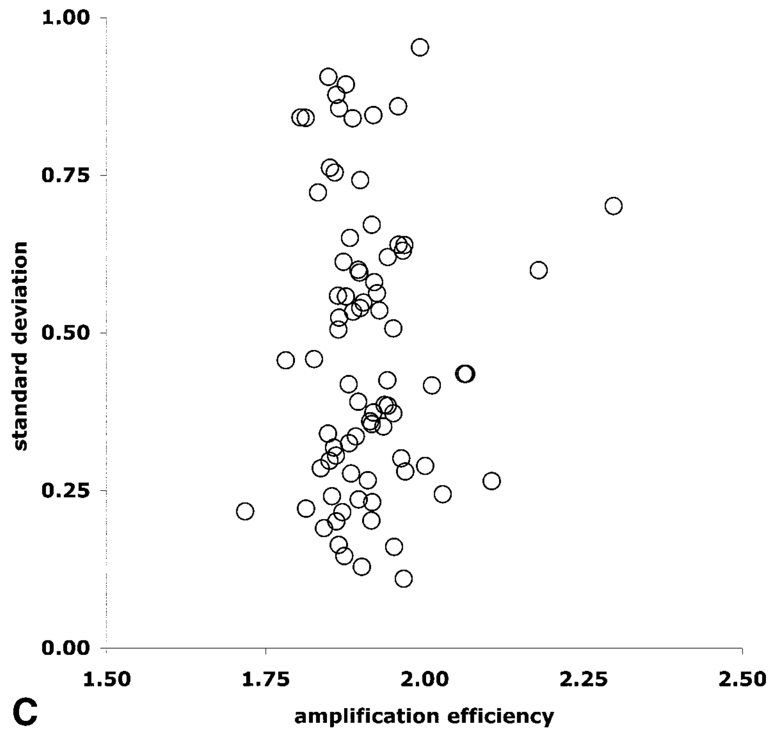

Fig. 8. Amplification efficiency of real-time QPCR. **(A)** Visualization of the effect of changes in amplification efficiency when comparing fold differences between primers. Theoretical normalized C_T values are plotted on the *x*-axis against relative fold difference of the *y*-axis. Ideal amplification is represented by the solid black line; over- and underestimates are shown with dotted black lines. **(B)** Demonstration of amplification efficiency during a real-time QPCR reaction. The shaded area represents amplification efficiency. Note that the highest efficiency is achieved during the logarithmic phase of amplification. **(C)** Error is independent of amplification efficiency. This is shown by plotting amplification efficiency (*x*-axis) against the standard deviation (*y*-axis) for each primer in the KSHV 96-primer array.

3.7.6. Normalization

For real-time QPCR, types of normalization can be applied: type I normalization relative to a reference sample t_0 or median for each gene yielded dCT, and type II normalization relative to the reference gene, e.g., GAPDH, yielded DCT. The latter eliminates differences caused by variation in the overall input cDNA concentration. Using experimental samples (e.g., response to a particular drug in culture), one should set up the experiment and normalize the input

Table 1
Normalization Possibilities for Two Genes (*A* and *B*)

	Time						
Gene	0	1	2	3	Median	Mean	SD
Raw data *A*	10	12	14	16	13	13	2.58
B	25	25	25	25	25	25	0.00
GAPDH	20	21	20	19	20	20	0.82
Normalization							
Type I (*T*0)							
A	0	2	4	6	3	3	2.58
B	0	0	0	0	0	0	0.00
GAPDH	0	1	0	–1	0	0	0.82
Type II							
A	–10	–9	–6	–3	–7.5	–7	3.16
B	5	4	5	6	5	5	0.82
GAPDH	0	0	0	0	0	0.00	
Type II followed by type I							
A	0	1	4	7	2.5	3	3.16
B	0	–1	0	1	0	0	0.82
GAPDH	0	0	0	0	0	0	0.00

material (e.g., same number of cells) such that the variation in the reference gene is $-1 \times C_T$ unit.

During type I normalization, only C_T values of a single primer pair are compared with each other. Hence amplification efficiency differences between primer pairs do not enter the calculation. In contrast, type II normalization compares two different primers pairs, such as for gene *A* and gene GAPDH, with associated, possibly different, amplification efficiencies k_A and k_{GAPDH}. After both normalizations were applied successively, we obtained dDCT. (unfortunately, the current literature uses DCT for type II and DDCT to denote the outcome after both normalizations (ABI user bulletin P/N4303859), which masks the different properties of the two operations). For relative analysis, clustering is performed in log-space (C_T values) rather than interpolated mRNA levels, and only a linear term is subtracted during normalization, which does not impact on the rank order between samples (*see* **Note 12**).

A simple time-course experiment can exemplify the analysis **(Table 1)**. It shows imaginary C_T values at different times after treatment for a set of three mRNAs named *A, B,* and GAPDH. At this point, the biological interpretation is clear: gene *A* mRNA levels decrease over time (as evidenced by increasing C_T values), whereas gene *B* and gene GAPDH mRNA levels remain constant. This

is often the only conclusion that an investigator needs to draw from a particular inquiry: which mRNAs are induced and which are suppressed relative to each other at any given time (or tumor sample or treatment modality).

In its easiest incarnation, we analyze the data as follows (*see* **Table 1**):

1. Transfer all C_T values into Excel.
2. Set up an $m \times n$ chart: the columns for condition, here *t*0–*t*3, and the rows for each primer pair, here gene *A,* gene *B,* and gene GAPDH (*see* the raw data group in **Table 1**).
3. Calculate the mean, median, and SD.
4. Apply type I normalization by subtracting the $C_T(t0)$ for each gene from all subsequent samples (*see* the type I normalization group in **Table 1**) to yield dCT. Instead of any particular sample, either the mean or median may be used as well. Note that the SD did not change.
5. Sort the data (in Excel) according to the SD. This will identify genes that are up- and downregulated over time, and the response will be monotonous. Since GAPDH has an SD of 0.82 (or ~1 C_T U), any gene that exhibits an SD great than 2X the SD of GAPDH is thought to respond to treatment.
6. Import the Excel spreadsheet into cluster from Eisen et al. ***(20)*** and http://genome-www5.stanford.edu/MicroArray/SMD/restech.html, cluster the array, and generate an image with Treeview. Note that the red–green scale in the cluster program operates on a range of –1 to +6. Graduations in color correspond to change with treatment. An mRNA that changes from 200 to 500 copies will cluster next to an mRNA that changes from 20,000 to 50,000 copies.
7. Type I normalization highlights the change with treatment and eliminates any differences in the basal levels (all genes are 0 at *t*0).
8. Generate a standard curve (simply cloning the PCR product will yield a target), and perform absolute quantification.

To accommodate samples of different experiments or different amounts of cDNA pools, we add type II normalization (*see* **Table 1**):

1. Transfer all C_T values into Excel.
2. Set up an $m \times n$ chart: the columns for condition, here *t*0 *t*3, and the rows for each primer pair, here gene *A,* gene *B,* and gene GAPDH (*see* the raw data group in **Table 1**).
3. Calculate the mean, median, and SD.
4. Apply type II normalization by subtracting the C_T for GAPDH from each gene for all samples (*see* the type II normalization group in **Table 1**) to yield DCT. Instead of any particular sample, either the mean or median may be used as well.
5. Sort the data (in Excel) according to the SD. This will identify genes that are up- and downregulated over time, and the response will be monotonous. Since GAPDH has an SD of 0.82 (or ~1 C_T U), any gene that exhibits an SD great than 2X the SD of GAPDH is thought to respond to treatment. Note that the SD of GAPDH is now zero, and the SD for all other genes has changed and reflects the combined SD for GAPDH and the gene of interest.

6. In addition, we can obtain an impression of the relative levels: assuming ideal amplification $E = 2$, at *t*3 gene *A* has a DCT of 7 [=6 – (–1)] or is expressed at approx 2^{-7} or 0.8% the level of GAPDH.
7. Import the Excel spreadsheet into cluster from Eisen et al. ***(20)*** and (http://genome-www5.stanford.edu/MicroArray/SMD/restech.html, cluster the array, and generate an image with Treeview. Note that the red–green scale in the cluster program operates on a range of –10 to +6. Graduations in color correspond to the overall level of mRNA rather than the change with treatment.
8. Generate a standard curve (simply cloning the PCR product will yield a target), and perform absolute quantification.

Finally, we can combine both normalization procedures as follows **(Table 1)**:

1. Transfer all C_T values into Excel.
2. Set up an $m \times n$ chart: the columns for condition, here *t*0 *t*3, and the rows for each primer pair, here gene *A,* gene *B,* and gene GAPDH (*see* the raw data group in **Table 1**).
3. Calculate the mean, median, and SD.
4. Apply type II normalization and type I normalization to each data point (*see* the type II followed by type I normalization group in **Table 1**) to yield dDCT. Instead of any particular sample, either the mean or median may be used as well.
5. Sort the data (in Excel) according to the SD. This will identify genes that are up- and downregulated over time, and the response will be monotonous. Since GAPDH has an SD of 0.82 (or ~1 C_T U), any gene that exhibits an SD great than 2X the SD of GAPDH is thought to respond to treatment.
6. We still obtain an impression of the relative levels for all data points except at *t*0; assuming ideal amplification $E = 2$, at *t*3 gene *A* has a DCT of 7 (=7 – 0) or is expressed at approx 2^{-7} or 0.8% the level of GAPDH.
7. Import the Excel spreadsheet into cluster from Eisen et al. ***(20)*** and (http://genome-www5.stanford.edu/MicroArray/SMD/restech.html, cluster the array, and generate an image with Treeview.
8. Generate a standard curve (simply cloning the PCR product will yield a target), and perform absolute quantification.

4. Notes

1. The following web sites offer free programs and further discussion:
   Cluster analysis: shareware and publications
   http://genome-www5.stanford.edu/MicroArray/SMD/restech.html.
   http://www.biochem.ucl.ac.uk/bsm/virus_database/vgbg.html.
   http://lymphochip.nih.gov/index2.html.
   http://www.gene-regulation.com/pub/databases.html.
   http://srs.ebi.ac.uk/srsbin/cgi-bin/wgetz?-page+srsq2+-noSession.
   http://llmpp.nih.gov/.
   http://nciarray.nci.nih.gov/.
   http://srs.embl-heidelberg.de:8000/srs5/.

http://www.embl-heidelberg.de/ chenna/clustal/darwin/.
Real-time QPCR: shareware and resources
http://www.wzw.tum.de/gene-quantification/.
http://medgen31.rug.ac.be/primerdatabase/links_menu.php.
Commercial sites
http://www.optimaldesign.com/index.html.
http://www.silicongenetics.com/cgi/SiG.cgi/Products/GeneSpring/index.smf.
http://www.affymetrix.com/community/index.affx.
http://www.panomics.com/.
http://www.gene-regulation.com/pub/databases.html.

2. Although the stipulations for primer design do not state that the primer T_m should be 60°C, it is best if the primers are as close to this temperature as possible. The reason for this strict adherence is because 60°C is the temperature used in the universal cycling conditions. If the primers are designed to work at this temperature, then the cycling conditions do not need to be altered for the primers to amplify. It better to design your primers around the conditions than to alter the conditions to the primer pair, especially in the comparison of multiple primer pairs, as occurs when normalizing to cellular housekeeping genes.
3. Whole Genome Primer Design using PrimeTime *PrimeTime* (Vahrson and Dittmer) is a software program for large-scale primer design. It automates the procedures of designing PCR primers, making it feasible to generate hundreds of primers for a whole viral genome in a few minutes. It is written in the Ruby programming language ***(22)*** and is built on top of *EMBOSS* ***(10)*** and *Primer3* ***(7)***. It extends the capabilities of the underlying programs by allowing one to position primers relative to the start or end of an ORF, by a fail-soft option that retries a primer search with slightly varied parameters when the original request failed, and by employing basic quality-control procedures to ensure the uniqueness of a primer combination in the genome. *PrimeTime* is invoked with the name of the file containing the sequence as parameter. The file must be in EMBL database format ***(23)***: *PrimeTime* writes the results of the primer search into file *sequence-file.primetime.* For each primer pair, it reports the positions, sequences, GC ontents, T_M, and lengths for both primers, as well as the length of the amplification product. The file is in a tab-delimited format that can easily be read into a spreadsheet program like Excel.
4. A general problem in genome-wide primer design is the presence of repeats in the template sequence, which may lead to ambiguous amplification products. *PrimeTime* checks the resulting primers in a way that is independent of the original primer search, as performed by *Primer3.* It identifies and flags problematic regions, which then need to be inspected manually.
5. The use of SYBR Green as a detection method does not allow for the recognition of a specific amplicon, as with a labeled probe. This is because SYBR Green binds to all dsDNA in the reaction including primer dimers. This problem is overcome by two solutions. The first is that the concentration of the primer is very low (166 nm), and therefore the primer will preferably bind to the sample, reducing the risk of primer dimer formation. The second comes with the application of melting curve

analysis. By measuring the fluorescence of the sample over a range of temperatures (e.g., 60°C–92°C) after completion of the reaction, one can determine the melting point of the amplicon by the fluorescence emitted. Only a single, sharp peak should be in evidence. The T_m of the amplicon can then be compared with that of the positive control to determine that the correct sequence has been amplified.

6. It is important to choose the proper real-time QPCR equipment. Different molecular beacons (e.g., FAM and JOE) as well as SYBR Green emit fluorescence at different wavelengths, and not all thermocyclers are compatible with all chemistries. Some models can only read a within a certain wavelength, only allowing the detection of a single chemistry, and therefore are not compatible for multiplex QPCR. Other machines, such as the ABI Prizm 7700, can read all available chemistries and would therefore be a better choice for running multiplex over a single detection machine.
7. All samples need to be analyzed in triplicate. In our hands the combined pipeting and instrument error was less than 6%. The ABI7900HT can distinguish twofold differences (between 5000 and 10,000 copies) with a 97.7% confidence level. Most biologically relevant changes in viral transcription should exhibit a much higher level of variation.
8. When using TaqMan probes to identify amplified products, we have not experienced any problems with nonspecific signals ***(14)***. However, should such problems be encountered, a number of recent developments that have increased the specificity of real-time quantitative PCR can be used. These are (1) substitution of the conventional, fluorescent quencher TAMRA with a nonfluorescent (dark) quencher, (2) incorporation of a minor-groove binder (MGB) ***(27,28)*** or (3) incorporation of 5-propyne-2′-deoxyuridine into the probe ***(29,30)***. These should solve any and all specificity problems that might arise. It is also a good idea to monitor the web site 7700taqman@listserv.acns.nwu.edu to keep abreast of the latest improvements in quantitative real-time PCR technology.
9. What are the sensitivity and specificity of PCR and RT-PCR, respectively? We previously used 2.5 μg total DNA (corresponding to ~5×10^5 cells) and were able to detect 1000 copies of KSHV in the sample. Although this sensitivity proved sufficient for our studies in animals, clearly better sensitivity can be achieved, for instance, by extending the PCR to 50 cycles, and DNA isolation can be improved using a QiAmp DNA isolation kit instead of the traditional proteinase K digestion. This sensitivity equals published reports that demonstrate a linear range of TaqMan-based quantification of 10^2–10^6 copies of KSHV per 10^6 peripheral blood mononuclear cells with a CV of 10% ***(24,25)***. In the case of influenzavirus, real-time quantitative PCR was shown to be as sensitive as nested PCR, with less nonspecific amplification ***(26)***.
10. Normalization is a recurring problem in comparative mRNA analysis. We now include five TaqMan amplicons (gapdh, actin, actin-2, c-myc, and hprt) that are specific for human housekeeping genes and use iterative geometric averaging ***(18)*** to determine the most appropriate control for a given data series. Adding a synthetic mRNA of known copy number (coding for the bacterial gene for neomycinR

and β-galactosidase), prior to reverse transcription, may be used to control for enzyme efficiency of both the reverse transcriptase and the *Taq* polymerase.

11. In conventional PCR, different primers perform with different amplification efficiencies and require different annealing temperatures. In contrast, real-time QPCR primers are designed to fit very narrow performance criteria (ABI Bulletin #P/N4303859). We found no need to compute initial mRNA levels or to use an external standard curve for purely comparative analysis, since the average amplification efficiency E for each primer pair in our published viral real-time QPCR array was 1.94–0.12 (n = 91), and the associated standard error across these primers directed against the same target (purified viral linear genomic DNA) was 0.06-fold ***(6)***. By contrast, cellular mRNA levels typically change several fold in response to specific stimuli. We estimate that for any target in the array, the biological variation associated with clinical specimens is well above the experimental error for this technology. By including a defined copy number of an exogenous, synthetic RNA prior to reverse transcription, another layer of standardization may be added. This yields a truly quantitative assay with a linear range of six orders of magnitude.
12. The primary achievement of real-time QPCR is that for the first time PCR delivers reliable quantitative information without the need for dilution series, internal competitors, and so on. The quantitative information can be extracted because the PCR reaction is monitored in real time, i.e., the reaction product is quantified at every cycle, and only data points during exponential amplification are used to compute the target concentration ***(3)***. In adapting real-time QPCR to comparative transcription profiling ***(5,6)***, we realized that we could use the real-time QPCR output (the so-called C_T value) for all primers in the array directly in existing cluster analysis programs such as those developed by Eisen et al. ***(20)***. In fact, the initial step in hybridization-based analysis is to compute the logarithm of the signal intensity, in order to improve statistical performance ***(21)***, whereas the real-time QPCR output (CT) already represents a logarithmic measure of the target concentration and can therefore be used directly for robust analysis.

References

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