# Chapter 11

# One-Step RT-LATE-PCR for mRNA and Viral RNA Detection and Quantification

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## Abstract

LATE-PCR is an optimized form of asymmetric PCR that efficiently generates high levels of single-stranded DNA amplicons. Single-stranded amplicons are advantageous because, as shown in this chapter, they can be probed at low temperature(s) with one or more probes. Based on its properties, LATE-PCR is also useful for constructing multiplex assays.

Viral RNA or RNA present in cells can be detected and quantified using LATE-PCR preceded by a reverse transcription (RT) step that converts RNA into cDNA. According to the conventional two-step approach, RT is primed using nonspecific random oligonucleotides prior to performing PCR amplification in a separate step. Recently, however, the so-called "one-step" RT-PCR strategy has gained increasing popularity because all reagents needed for both reactions are added together in a single mix, thus reducing the possibility of contamination, a consideration particularly relevant to viral samples analysis. We describe below two protocols using RT-LATE-PCR and provide specific guidelines for the general application of this technique. The first protocol is devised to quantify mRNA from mouse embryos by real-time PCR; the second protocol employs end-point analysis to detect a number of different viral-like sequences in a multiplex reaction.

**Key words:** LATE-PCR, One-step RT-PCR, qRT-PCR, RNA virus assay, mRNA assay, End-point quantification, Single-stranded amplicon, Low-temperature probe, Multiplex PCR

## 1. Introduction

### *1.1. The Logic of LATE-PCR*

Symmetric PCR is currently the predominant method used to amplify relatively short segments of DNA. Each amplicon is generated using two primers having equal melting temperatures ($T_m$s) and equal concentrations ($T_m$ is the temperature at which half of the primer molecules are hybridized to their targets). The resulting reactions are reproducible during the phase of exponential amplification, until a point when they become inefficient and

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eventually plateau. Thus, real-time analysis (fluorescent measurement of total product after each cycle) of these reactions reveals a characteristic, sigmoidal curve and shows that the levels of double-stranded amplicon accumulated at the end are not correlated to the number of targets present at the beginning of the reaction. In addition, exit from exponential amplification in symmetric PCR is stochastic and generates highly variable levels of double-stranded DNA among replicates. For the above reasons, symmetric PCR reactions must be analyzed in real-time to quantify the starting numbers of target molecules using measurements of the threshold cycle or $C_T$. The $C_T$ is the cycle at which the fluorescence resulting from the accumulated product reaches a predefined threshold and is generally close to the end of exponential amplification.

Asymmetric PCR potentially allows to correlate end-point fluorescence with template numbers and to solve the problem of scatter at end-point. This is achieved by replacing equimolar primers with an excess primer (XP) at higher concentration and a limiting primer (LP) at lower concentration. As a result, amplification switches from exponential production of double-stranded DNA (ds DNA) to linear production of single-stranded DNA (ss DNA) when the limiting primer is used up (1). However, the timing of the transition from ds DNA to ss DNA synthesis in traditional asymmetric PCR is primer-pair specific, making it difficult to construct multiplex reactions using this method.

Our laboratory has discovered that simply diluting one member of a pair of symmetric primers does not take into account the fact that lowering a primer's concentration also lowers its $T_m$, resulting in less efficient amplification. We found that the transition from ds DNA to ss DNA can be made predictable and uniform for all asymmetric reactions by designing primers with appropriate length and composition to insure that the actual melting temperature of each LP at its initial concentration, $T_{m\,[0]}^{L}$, is as high or higher than that of the excess primer at its respective initial concentration, $T_{m\,[0]}^{X}$. Thus, the primary axiom of LATE-PCR (see Note 1) is $T_{m\,[0]}^{L} - T_{m\,[0]}^{X} \geq 0$ (2, 3). As a result, LATE-PCR assays carry out efficient exponential amplification during Phase I and then reliably switch to linear amplification in Phase II, when the LP is exhausted (Fig. 1). In real-time, probe-based LATE-PCR assays, the LP runs out shortly before single-strand production is first detected, i.e., at the $C_T$. Single-stranded XP-strand molecules accumulate linearly thereafter. As a result, LATE-PCR fluorescence end-points with sequence-specific probes are reproducible for a given amount of target. If LATE-PCR reactions are stopped after 20–30 cycles of linear amplification, the amount of ss DNA at end-point quantitatively reflects the amount of target present at the start of the reaction just as the $C_T$ values do (see Fig. 2, right panel). However, LATE-PCR reactions keep on generating more

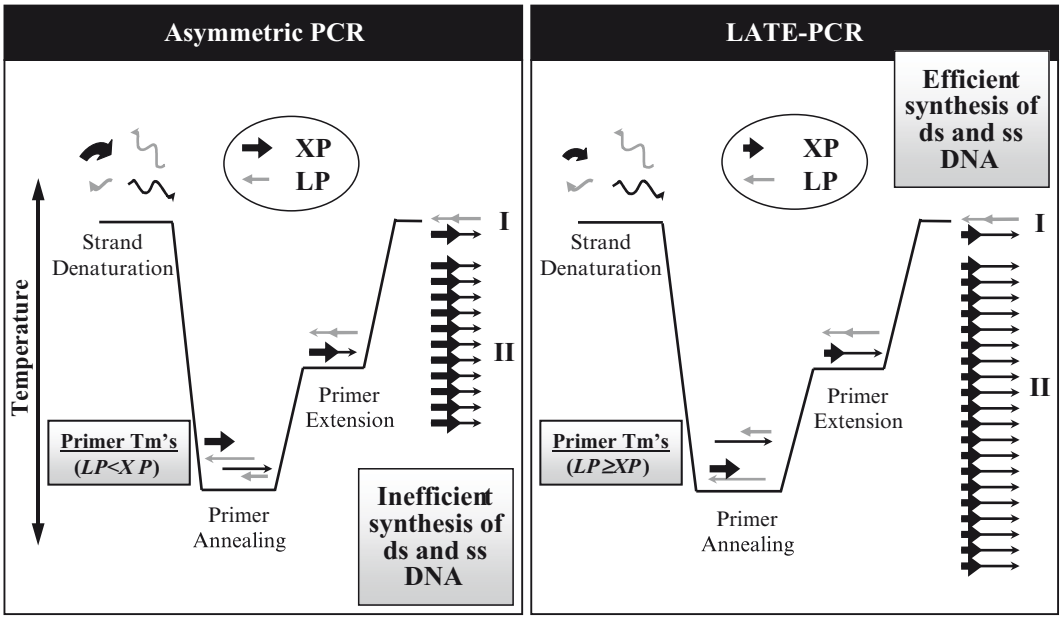


Fig. 1. Comparison of asymmetric PCR and LATE-PCR. In asymmetric PCR, the primers are designed to have equal $T_m$s, but the $T_m$ of the LP is actually lower than that of the XP because its concentration is lower. This leads to inefficient priming. In LATE-PCR, this problem is corrected by designing the LP to have a concentration-corrected $T_m$ equal or higher than that of the XP. Using a 50–100 nM LP concentration in LATE-PCR assays, real-time reactions switch from exponential to linear at the $C_T$ which can be used for template quantification

and more single-stranded molecules for at least 100 cycles. Thus, even reactions initiated with very few molecules (including single molecules) eventually generate detectable levels of ss DNA. Moreover, very abundant and very scarce templates can be amplified in the same LATE-PCR reaction (see Fig. 3) because unlike double-stranded amplicons, accumulating ss DNA does not bind the DNA polymerase (4, 5). Phase I ds-DNA amplicons of a LATE-PCR can be detected in real-time by using nonspecific intercalating fluorescent dyes such as SYBR Green, while detection of Phase II ss-DNA amplicons requires the use of fluorescently tagged hybridization probes (Fig. 2). Both types of LATE-PCR products can also be analyzed by agarose gel electrophoresis. Double-stranded amplicons are strongly stained by ethidium bromide, while single-stranded amplicons stain less intensely because ethidium bromide binds only to hairpin regions in which the ss DNA is folded on itself (GC-rich regions, see Fig. 4). In addition, single-stranded amplicons migrate more slowly than the corresponding double-stranded products due to lower linear charge density (6).

### 1.2. Properties of One-Step RT-LATE-PCR

In one-step RT-PCR, the same set of primers drive both RT and PCR, which actually take place sequentially in the same closed tube (Fig. 5). RT is primed by the primer antisense to the RNA

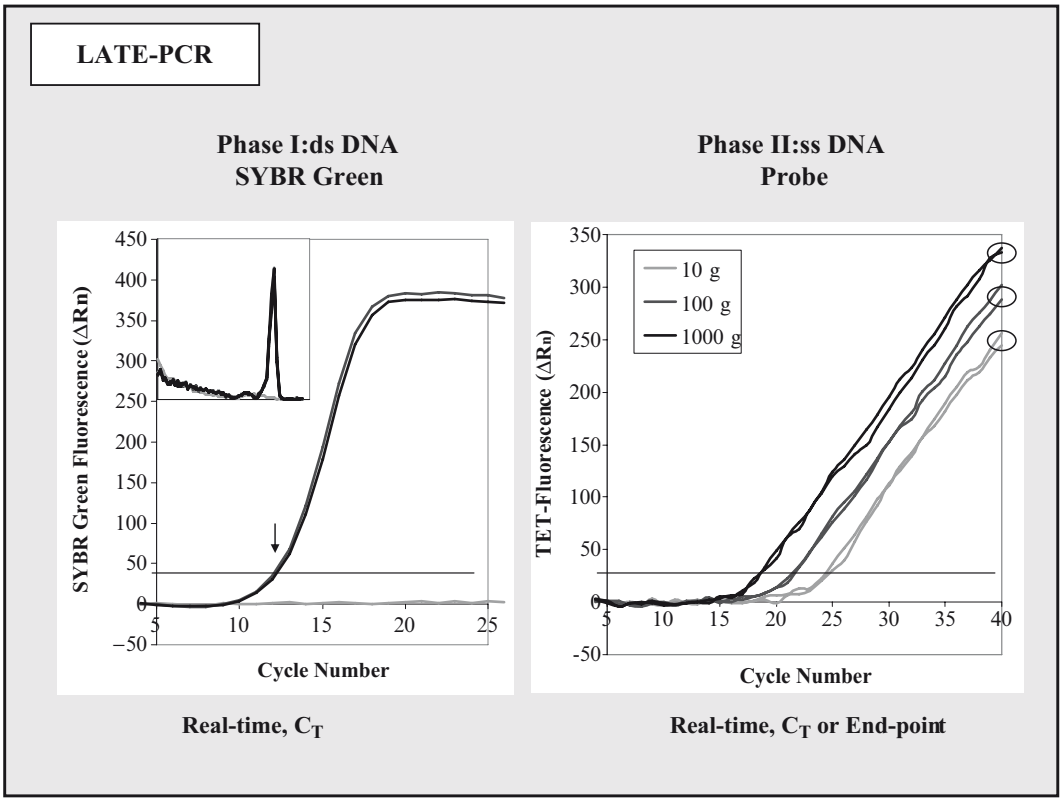


Fig. 2. The two phases of LATE-PCR. During Phase I, double-stranded amplicons are produced exponentially until the LP is exhausted. Real-time LATE-PCR in the presence of nonspecific intercalating dyes such as SYBR Green generates sigmoidal curves that visualize this first phase of the reaction. Template quantification can be achieved using the $C_T$ values from these curves but not using end-point fluorescence because all curves reach a plateau. The purity of the product can be monitored by melt peak analysis (inset of left panel). During Phase II of LATE-PCR, only the XP is still available and is elongated into single-stranded product, thus requiring sequence-specific probes for visualization. The reaction switches to linear and, within a certain number of cycles, end-point fluorescence is proportional to template number and can be used for quantification as much as the $C_T$ values

target (which is in almost all cases single-stranded), and PCR is primed by both primers. In RT-LATE-PCR, either the XP or the LP can function as the RT primer. In both protocols presented in this chapter, the XP was chosen to prime RT in one-step RT-LATE-PCR reactions. Also in both protocols, we took advantage of the fact that LATE-PCR amplicons can be probed at low temperature because they are single-stranded, and the probe is not out-competed by an amplicon's complementary strand. Low-temperature probing was achieved by adding data acquisition steps at the end of PCR amplification, either at every cycle in real-time (first protocol, Subheading 3.1.2) or after all cycles at end-point (second protocol, Subheading 3.2.4).

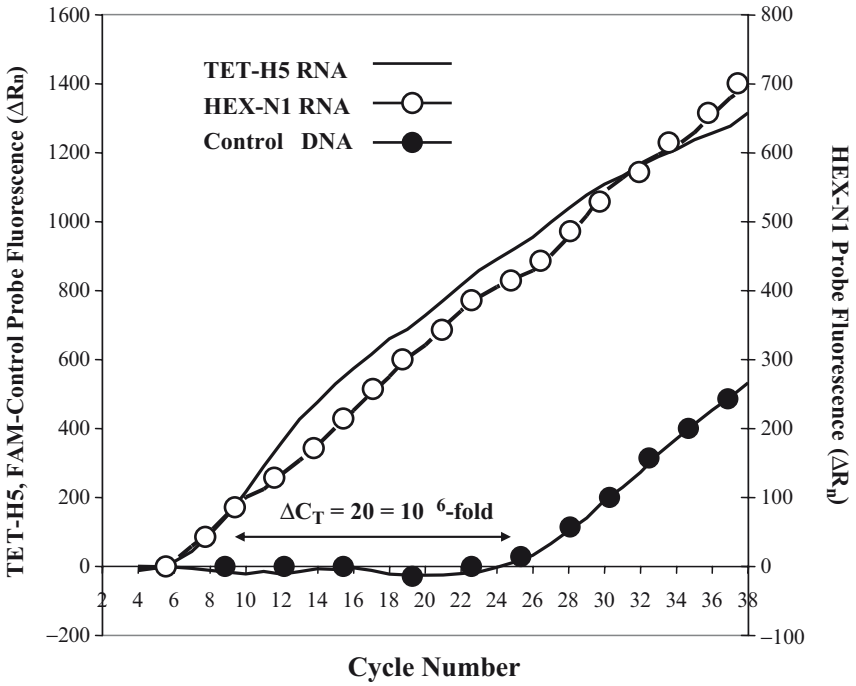


Fig. 3. Triplex RT-LATE-PCR single-stranded amplification of templates of different abundance. This real-time reaction contained $10^3$ copies of a control DNA and two viral-like RNAs at $10^6$-fold higher concentration. Three probes with different fluors were used to monitor individual amplicon's generation in an ABI 7700 thermocycler

## 2. Materials

All instrumentations and reagents listed in this chapter can be replaced by different products having equivalent characteristics. Specific identification of the products used in our laboratory is provided below for reference purpose only, in consideration of the wide variety of instruments and RT-PCR reagents commercially available and having different properties.

### 2.1. Instrumentation and Materials

1. Set of micropipettes
2. Sterile filter pipette tips
3. Vortex mixer
4. Microcentrifuge (capable of reaching at least 14,000 × *g*)
5. Optical 200-µl PCR tubes with optical caps
6. Sterile 0.5-mL Eppendorf tubes.
7. Thermocyclers: iQ™5 Real-Time PCR Detection System (BioRad), or instrument with equivalent capabilities, and (optional) ABI Prism 7700 Sequence Detector (Applied Biosystems) or equivalent (see Note 2)

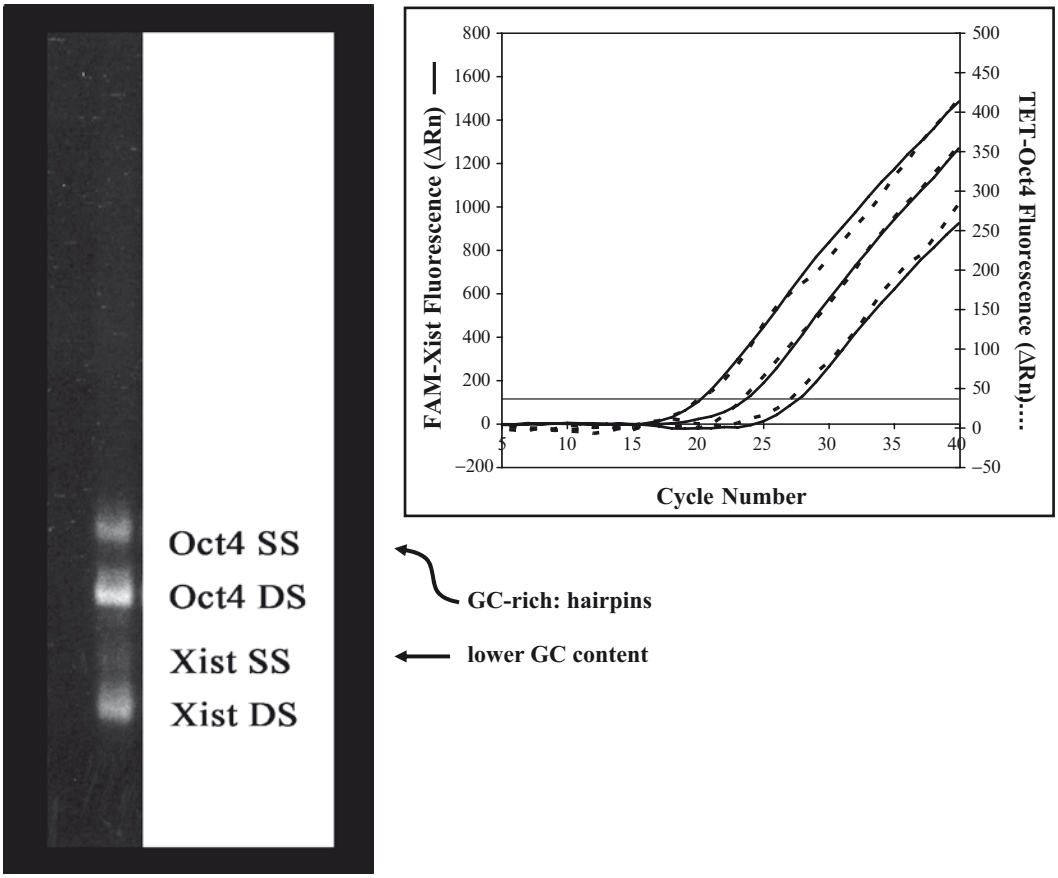


Fig. 4. Visualization of LATE-PCR amplicons by gel electrophoresis. The products of a duplex LATE-PCR assay were analyzed on agarose gel and stained with ethidium bromide (*left panel*). Both templates (sequences from the Oct4 and Xist mouse genes) generated ds DNA amplicons during the first phase of LATE-PCR, as shown by the presence of strongly stained bands of the expected size. In contrast, the single-stranded amplicons of LATE-PCR's second phase show differential staining, much stronger for the GC-rich Oct4 than for the AT-rich Xist. However, the real-time curves of this assay demonstrate that both Oct4 and Xist single-stranded amplicons were efficiently produced at template concentrations ranging from 10 to 1,000 copies (*right panel*)

8. Freezers (–20°C and –70°C)
9. Heating block or water bath
10. Sterile disposable gloves
11. Bleach solution (10%) for decontamination

### 2.2. Oct4 RT-PCR

All reagents need to be DNase- and RNase-free, molecular biology grade.

1. LATE-PCR primers (see Note 3)
2. FAM-conjugated probe with Black Hole Quencher 1 (see Note 4)

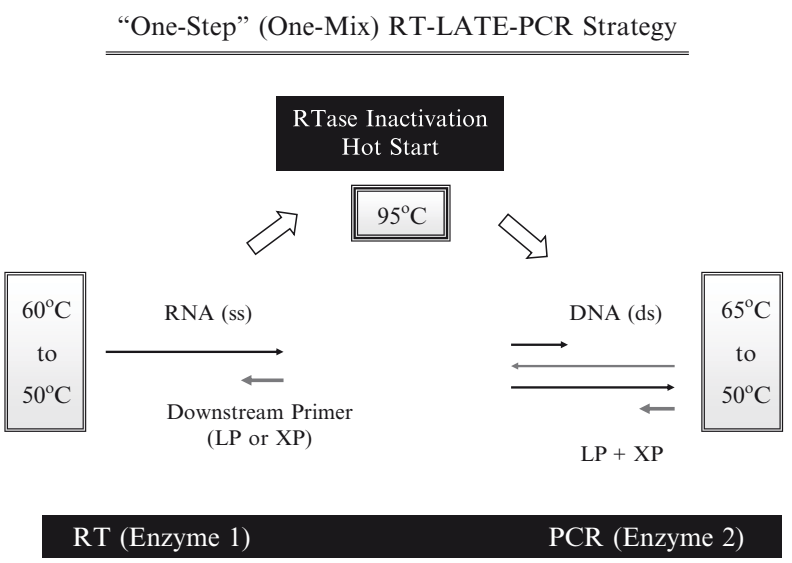


Fig. 5. Diagram of the two reactions within one-step RT-LATE-PCR. All reagents are present together in one mix. The reaction is started when the primer antisense to the RNA strand anneals to target and primes RT, which is mediated by a reverse transcriptase (*left*). RT temperature usually ranges between 50°C and 60°C. At this stage, PCR is blocked by a DNA polymerase-specific antibody. (The use of a DNA polymerase-specific antibody is highly recommended in one-step RT-PCR to prevent nonspecific PCR activity during RT.) RT is terminated by increasing the temperature to 95°C, which denatures the reverse transcriptase and the antibody, thus freeing the DNA polymerase ("hot start", middle). The polymerase is now able to perform PCR amplification by elongating both primers; annealing temperatures range in most cases between 50°C and 65°C (*right*)

3. PrimeSafe 043 or PrimeSafe 002 (7), available at biodetection@smithsdetection.com, or other mispriming preventing compound (see Note 5)
4. Purified mouse genomic DNA (see Note 6)
5. RNase-degrading product to treat surfaces and instrumentation (see Note 7)
6. SuperScript™ III Platinum® One-Step Quantitative RT-PCR System (see Note 8)
7. Tris–Cl, 10 mM, pH 8.3
8. Platinum® Taq DNA polymerase, also containing 10× PCR Buffer and 50 mM $MgCl_2$ (see Note 8)
9. dNTPs, 10 mM
10. Nuclease-free water
11. RNaseOUT, 40 U/μl (see Note 8) or other ribonuclease inhibitor (optional)

### *2.3. Viral Multiplex RT-PCR (Additional/ Alternative Reagents)*

1. LATE-PCR primers (see Note 3)
2. Tris–EDTA Buffer Solution, pH 8.0: 10 mM Tris–Cl, 1 mM disodium EDTA, pH 8.0

3. SYBR® Green (any brand)
4. SuperScript™ III Reverse Transcriptase (see Note 8)
5. Platinum® Tfi Exo(-) DNA polymerase, also containing 5× Platinum® Tfi Reaction Buffer and 50 mM $MgCl_2$ (see Note 8)
6. FAM, CalOrange 560, CalRed 610 and Quasar 670-conjugated probes with the appropriate Black Hole Quencher (see Note 4)
7. PrimeSafe 001 (7) (see Note 5)
8. Armored RNA (Asuragen, (8)) or other RNA control (see Note 9)

***2.4. Software for Nucleic Acid Design, Folding and Analysis***

Visual OMP (DNA Software, Inc.) (see Note 10)

***2.5. In Vitro Transcription (Additional Solutions, Kits, and Reagents)***

1. Expression plasmids containing a transcription promoter and the target sequence inserts (see Note 11)
2. *Eco*R V, also containing 10X REACT®2 Buffer (see Notes 8 and 11) or another appropriate restriction enzyme
3. Riboprobe® in vitro Transcription System (Promega) or other appropriate IVT kit (see Note 12)
4. Citrate-saturated phenol (pH 4.7):chloroform:isoamyl alcohol (125:24:1)
5. Chloroform:isoamyl alcohol (24:1)
6. Ammonium acetate solution for molecular biology, 7.5 M
7. EtOH, absolute, 200 proof, for molecular biology
8. Yeast transfer RNA or equivalent nucleic acid co-precipitant (see Note 13)

## 3. Methods

*Important:* Perform all assays in a dedicated, "clean" lab space. Treat all surfaces, micropipettes, and small equipment with an RNase-degrading product; rinse well prior to use. Use only DNase- and RNase-free reagents and plastic ware. Wear gloves at all times and change them frequently. At the end of the PCR, seal the tubes in a disposable container and discard them in a bin outside the lab. Do not reopen them and do not autoclave them (tubes may pop open during this process), as this could lead to amplicon contamination of the working space (see Note 14).

### 3.1. Quantification of Oct4 mRNA from Mouse Embryos by RT-Real-Time-LATE-PCR

#### 3.1.1. Primer and Probe Design and Analysis for RT-LATE-PCR

1. Design one LP and one XP according to LATE-PCR guidelines (see Introduction under Subheading 1.1 and (2, 3)). Design primers within an exon of the gene of interest, so that cDNA templates derived from mRNA after RT are identical to the corresponding genomic sequence (5, 9). This strategy allows the use of commercially available mouse DNA to build quantification standards having the same amplification efficiency as the cDNA from the embryos (see Subheading 3.1.2.) (see Note 15).
2. Establish which primer will prime RT (primer antisense to mRNA).
3. Design a probe. The probe is comprised by a linear portion complementary to the target plus a "2-nucleotide (nt) stem." This stem is formed by adding two nucleotides at the 5′ end and two complementary nucleotides at the 3′ end. In the example below, the FAM fluor is conjugated at the 5′ end and the quencher (Black Hole Quencher 1) at the 3′ end.

   RT-LATE-PCR primer and probe sequences for Oct4 mRNA quantification:

| | | |
|---|---|---|
| LP: | 5′-TGGCTGGACACCTGGCTTC AGACT-3′ | $T_m = 71°C^a$ |
| XP (RT primer): | 5′-CAACTTGGGGGACTAGGC-3′ | $T_m = 67°C^a$ |
| Probe: | 5′-FAM-CTGGGATGGCATACTG TGAG-BHQ1-3′ | $T_m = 60°C^a$ |

[a]$T_m$s are calculated for each primer/probe at its starting concentration, at annealing temperature and in the presence of a ds DNA target

4. Analyze the interactions and $T_m$s of your primers and probe in silico under both RT and PCR conditions, as illustrated in Fig. 6. Visual OMP analysis calculates "Effective $T_m$" values that take into account all the reaction's components and template folding. As shown in Fig. 6, the Effective $T_m$ of Oct4 XP during the annealing step of PCR is only slightly lower than its calculated $T_m$ (66°C rather than 67°C). Prior to PCR, however, this primer also needs to prime RT. When the template is ss RNA (rather than ds DNA), the Effective $T_m$ of Oct4 XP drops to 53°C, although the RT temperature was set to be the same as the PCR annealing temperature (55°C). At 55°C, about half of the Oct4 XP molecules bind to the RNA target, providing efficient priming (the XP is present at high concentration). Above this temperature, however, priming would become inefficient. Lowering the RT temperature below 55°C leads to an increase in ss RNA secondary structure, further decreasing Oct4 XP's Effective $T_m$ (see Note 16).

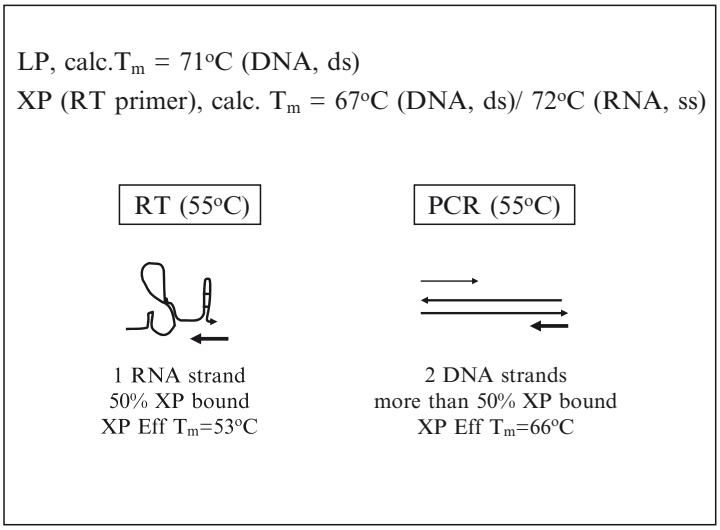


Fig. 6. Visual OMP analysis of the Oct4 RT-LATE-PCR primers. The $T_m$s of Oct4 LP and Oct4 XP, calculated based on the primer and target composition and salt concentration, are shown at the top for DNA and RNA targets. Oct4 XP functions as a primer during both RT and PCR. Its Effective $T_m$s, which also take in account the secondary structure of the target at different temperatures, are shown at the bottom of the figure. The secondary structure of ss RNA results in decreased binding efficiency of Oct4 XP during RT when compared to the PCR annealing step, although both reactions are simulated to take place at 55°C

5. Based on the above analysis, determine the best RT temperature. For this protocol, we chose an RT temperature of 55°C because Oct4 XP primes RT very poorly above or below 55°C due to its effective $T_m$ in the presence of ss RNA (Fig. 7).
6. Choose a reverse transcriptase highly active and thermostable at the set RT temperature. Keep in mind that SuperScript™ III, used in this protocol, works well at 55°C but has a half-life of only 2.5 min at 60°C ((10) and Fig. 7).

*3.1.2. One-Step RT-LATE-PCR Protocol*

Prepare three sets of assays, with each containing in replicate: "+RT", "No RT" and No Template Controls (NTCs). Also prepare a DNA standard (see step 5 below).

1. For "±RT" and "No RT" samples: Preincubate the RT primer with the RNA template for 5 min at 65°C in the following mix (volumes given per reaction):

| | |
|---|---|
| Tris–Cl, 10 mM, pH 8.3 | (to 10 µl) |
| Oct4 XP[a], 100 µM (RT primer) | 0.5 µl |
| RNA from mouse embryo (see Note 17) | approx. 6 µl |
| | 10 µl |

[a]2 µM in the final RT-PCR assay, 5 µM during preincubation

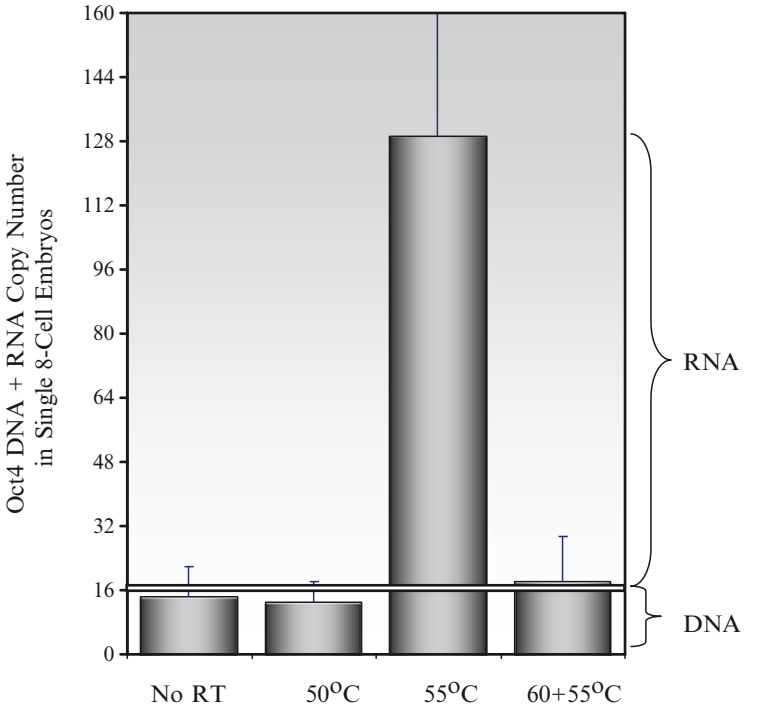


Fig. 7. Thermo-dependency of RT efficiency in one-step RT-LATE-PCR. Single mouse embryos at the 8-cell stage were processed with the PurAmp procedure, so that samples contained both genomic DNA and mRNA (5, 9). The Oct4 RNA + DNA content of each embryo was measured with one-step RT-LATE-PCR, varying the RT temperature as shown on the abscissa; Oct4 DNA copy numbers were obtained from "No RT" samples and amounted to about 16 copies/embryo as expected (two copies of the Oct4 gene/cell). Columns are the average of several embryos; the portion of each column above the horizontal bar gives a measure of Oct4 RNA in the samples. It is evident that Oct4 RNA (present in all embryos) is primed efficiently during RT only when the reaction is carried out at 55°C. When the 55°C incubation is preceded by 10 min at 60°C (column on the far right), RT fails because the enzyme is denatured

2. Cool down and add to the RT-PCR Mix below (10 µl from preincubation + 15 µl of RT-PCR Mix = *25 µl final volume of RT-PCR assay*) or to the "No RT" PCR Mix described below (step 3):

   *RT-PCR Mix* (per reaction):

| | **Final concentration** | |
|---|---|---|
| 10× PCR Buffer[a] (see Note 18) | 1× | 2.5 µl |
| $MgCl_2$, 50 mM | 3 mM | 1.5 µl |
| dNTPs, 10 mM | 0.25 mM | 0.625 µl |
| Oct4 LP, 10 µM | *100 nM* | 0.25 µl |
| FAM-Oct4 probe, 100 µM (see Note 19) | 500 nM | 0.125 µl |

| | Final concentration | |
|---|---|---|
| PrimeSafe 043, 10 µM (see Note 20) | 300 nM | 0.75 µl |
| RNaseOUT, 40 U/µl (optional) | 0.8 U/µl | 1 µl |
| SuperScript™ III RT/Platinum® Taq Mix[b] | (1 U Taq) | 0.5 µl |
| Nuclease-free $H_2O$ | | 7.75 µl |
| | | 15 µl |

[a]From Platinum® Taq kit: 200 mM Tris–HCl, pH 8.4, and 500 mM KCl
[b]From SuperScript™ III Platinum® One-Step Quantitative RT-PCR System

3. Prepare a "No RT" PCR Mix by substituting the SuperScript™ III RT/ Platinum® Taq Mix with 1 U of Platinum® Taq DNA polymerase.
4. For NTCs: Follow the same procedure as for the "+RT" samples, but do not add RNA to the preincubation mix.
5. Prepare a DNA standard using genomic DNA (5, 9):

   Prepare serial dilutions of mouse genomic DNA in "No RT" PCR Mix, so that tubes (in triplicate) will contain 10, 100, and 1,000 genomes. (The size of a diploid mouse genome is 6 pg (11)).
6. Run all assays ("+RT", "No RT", NTCs, genomic standard dilutions) with the following thermal profile (optimized for Prism 7700 Sequence Detector; it can also be used on iQ5 thermocycler, see Note 19).

| | | | |
|---|---|---|---|
| 55°C, 30 min (RT) → | 95°C, 5 min→ | 95°C, 10 s→ | 95°C, 15 s→ |
| | | 63°C, 20 s | 55°C, 25 s |
| | | 72°C, 30 s | 72°C, 35 s |
| | | | 45°C, 30 s[a] |
| 1× | 1× | 15× | 40× |

[a]Data acquisition (see Note 21)

Plot the data from the genomic standard, $C_T$ values vs template copy number. Keep in mind that each genome contains two copies of the autosomal Oct4 gene. Quantify Oct4 RNA copy numbers in the unknown samples using the standard's regression line (9).

*3.1.3. PrimeSafe Titration*

Prior to performing the RNA assays, it is recommended to titrate PrimeSafe on the DNA standard, as some batch-to-batch variability is possible (see Note 22). The slope of LATE-PCR reactions, which affects end-point fluorescence, is a very sensitive tool to monitor efficient amplification, in addition to $C_T$ value analysis (see Fig. 8 and (5)).

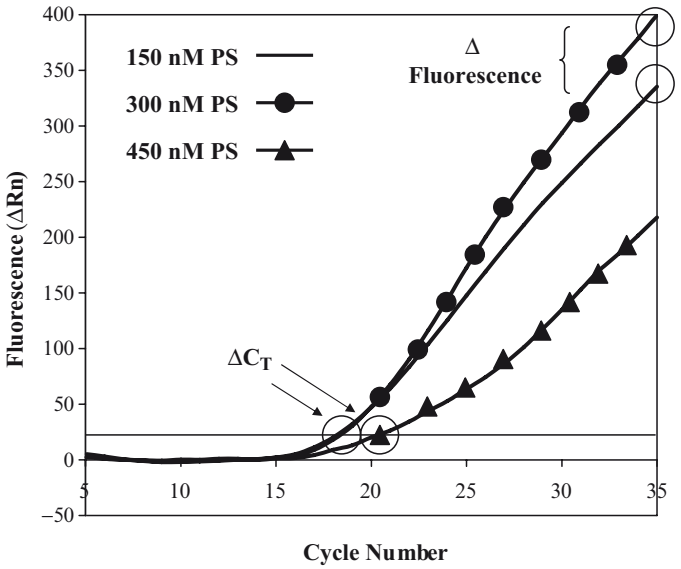


Fig. 8. Effect of PrimeSafe concentration on Oct4 LATE-PCR. All real-time reactions were carried out in the presence of 2,000 copies of genomic DNA and different concentrations of PrimeSafe 043. The LATE-PCR curves show that a concentration of 300 nM PrimeSafe is optimal because 450 nM results in excessive $C_T$ delay and low final fluorescence (overall PCR inhibition), while 150 nM does not improve the $C_T$ value but end-point fluorescence is still lower than with 300 nM. In this second case, a lower-than-optimal end-point fluorescence suggests that nonspecific products are generated as PCR progresses, not "seen" by the probe, because the PrimeSafe concentration is too low

*3.1.4. Duplex RT-LATE-PCR and Assay Optimization by Slope Analysis*

Two different types of transcript can be simultaneously measured in a sample as small as a single cell using RT-LATE-PCR, independent from their abundance (5, 12). For one-step reactions, use the protocol described above with the following modifications:

1. Design primers and a probe for the second mRNA, all having $T_m$s as close as possible to those of the Oct4 primers and probes.
2. Select primers so that the XP for the second target will also prime RT, as shown above for Oct4 XP.
3. The probe for the second mRNA needs to have a different fluor. The fluor choice for the two probes depends on the PCR cycler used. We use FAM and TET with a Prism 7700 cycler, and CalOrange 560 and Quasar 670 with an iQ5 cycler.
4. Use the second pair of primers and the second probe at the same concentrations used for the Oct4 primers and probe. (Adjust if necessary.)
5. Increase dNTPs concentration to 0.4 mM.

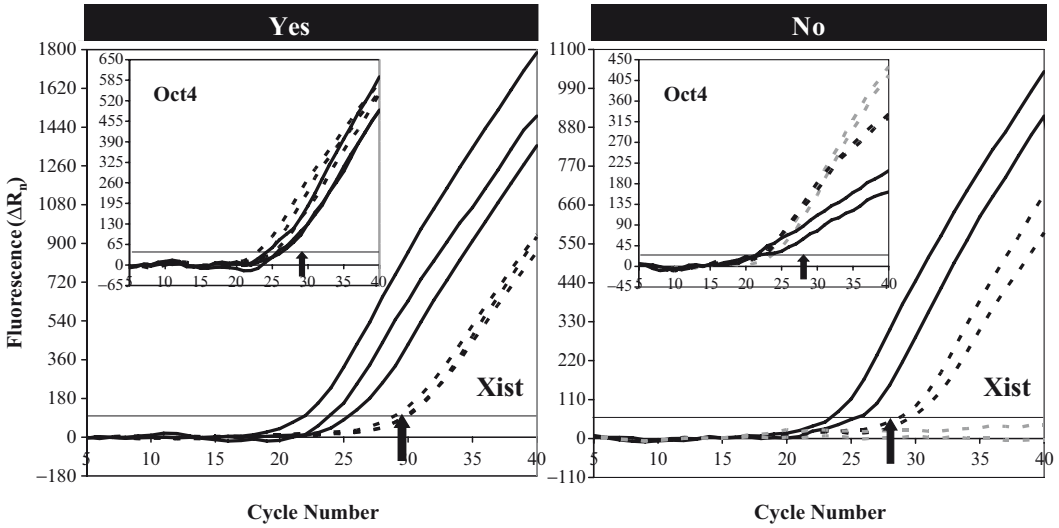


Fig. 9. Optimization of duplex RT-LATE-PCR by slope analysis. The linear phase of LATE-PCR allows assay optimization by slope analysis. Two templates, Xist and Oct4 (RNA + DNA), were simultaneously amplified from single mouse embryonic cells. As shown in the charts, male cells (*broken lines*) contain a single copy of the Xist gene and no Xist RNA while female cells also contain abundant Xist RNA (*solid lines*). Black arrows indicate the $C_T$ value expected for a single template copy based on a genomic DNA standard. Slope analysis reveals that under suboptimal conditions (*right panel*), amplification of the second template, Oct4 (inset), is much less efficient in the female cells that contain high amounts of Xist RNA than in male cells that only contain one Xist template. An optimal Oct4 slope is only obtained when Xist amplification fails (*broken grey lines*). After optimization (*left panel*), all Oct4 slopes are similar, although the Xist RNA + DNA contents of the cells analyzed span from one to several hundred copies

6. Retitrate PrimeSafe. This is very important for a duplex because the two amplicon sequences will always present differences affecting PCR efficiency and, hence, sensitivity to PrimeSafe. Again, use slope analysis to optimize the assay, as shown in Fig. 9.

### 3.2. End-Point Detection/ Quantification of Multiplex Viral Sequences by RT-LATE-PCR

#### 3.2.1 Multiplexing Scheme

Select the desired RNA sequences to be probed in the assay. Sequences can include genes from the same virus and be subtype specific. Additionally, one same, long sequence can contain the targets for two different probes (see Note 23). Sequences from different viruses are amplified in alternative outcomes, so that not all primers and probes are expected to be used during one assay although they are all present together in the reaction mix. The final number of selected probe targets depends on the available PCR cycler. The iQ5 cycler allows detection of a maximum of five different fluors at the same time; Fig. 10 shows our preferred fluor selection for this instrument: FAM, CalOrange 560, CalRed 610, and Quasar 670, the last one being the brightest (see Note 24). LATE-PCR allows probing at low temperature at the end of the reaction (rather than during annealing) because the product is single-stranded. For this reason, at least two sets

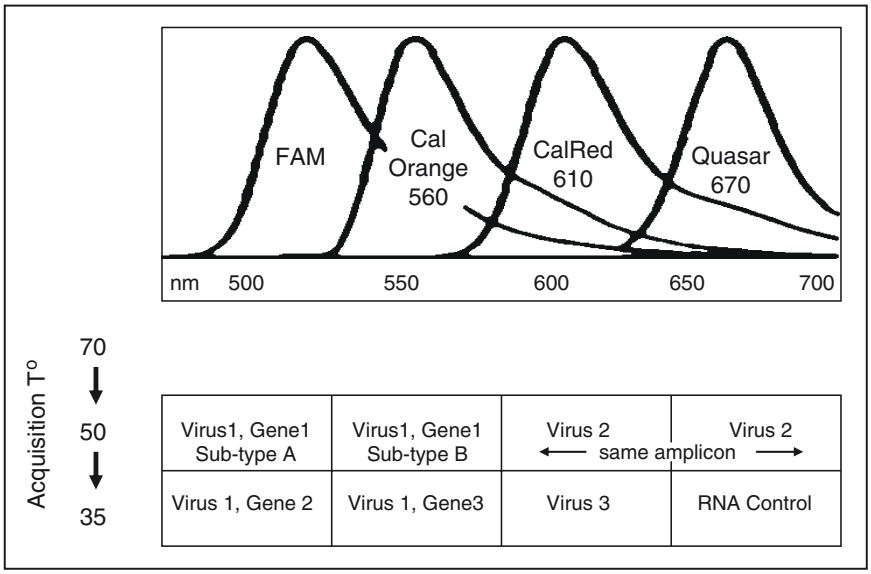


Fig. 10. Eight-target multiplex for virus detection. Four different fluors are chosen with minimally overlapping emission spectra (see Note 11; spectra in the figure are from (15)) and two sets of probes are designed to hybridize to the selected viral targets (see text), so that each fluor is used twice in the multiplex. Both sets of probes have low $T_m$s compared to traditional probes, but one set has $T_m$s higher than the other set. Fluorescence intensities are acquired at 70°C (normalization temperature, see Fig. 11), 50°C (higher Tm probes) and 35°C (lower Tm probes). In the scheme shown here, three alternative outcomes are possible: Virus 1 is detected by multiplex amplification, while Virus 3 produces a single amplicon; Virus 2 generates one amplicon probed on two segments. An external RNA control is added to all assays to verify efficient RT-LATE-PCR

of probes can be designed having the same fluor but different $T_m$s. These probes can be distinguished because they hybridize at different temperatures (35°C and 50°C, see Fig. 10). Thus, a total of eight different probe targets are chosen for this multiplex (see Note 25), one being a designated external RNA control (see Note 26).

*Caveat:* Probes that bind at 35°C will not be bound at 50°C, but probes that bind at 50°C will still be bound at 35°C (see Fig. 11). Keep this in mind when choosing each target/probe pair. In the example of Fig. 10, the presence of Virus 1 is flagged by FAM and CalOrange 560 probes binding at 35°C. If the virus is of either subtype A or B, fluorescence of either of these two fluors will also increase at 50°C (see Note 27).

#### 3.2.1.1. Development of the Alternative-Outcome Multiplex RT-LATE-PCR Assay

Keep in mind that all assay components have to work under the same conditions during both RT and PCR.

1. Design all LPs with similar $T_m$s (67–69°C).
2. Design all XPs with similar $T_m$s (63–65°C).
3. Make sure that each amplicon's $T_m$ is no more than 20°C higher than its XP's $T_m$ (see Note 28).

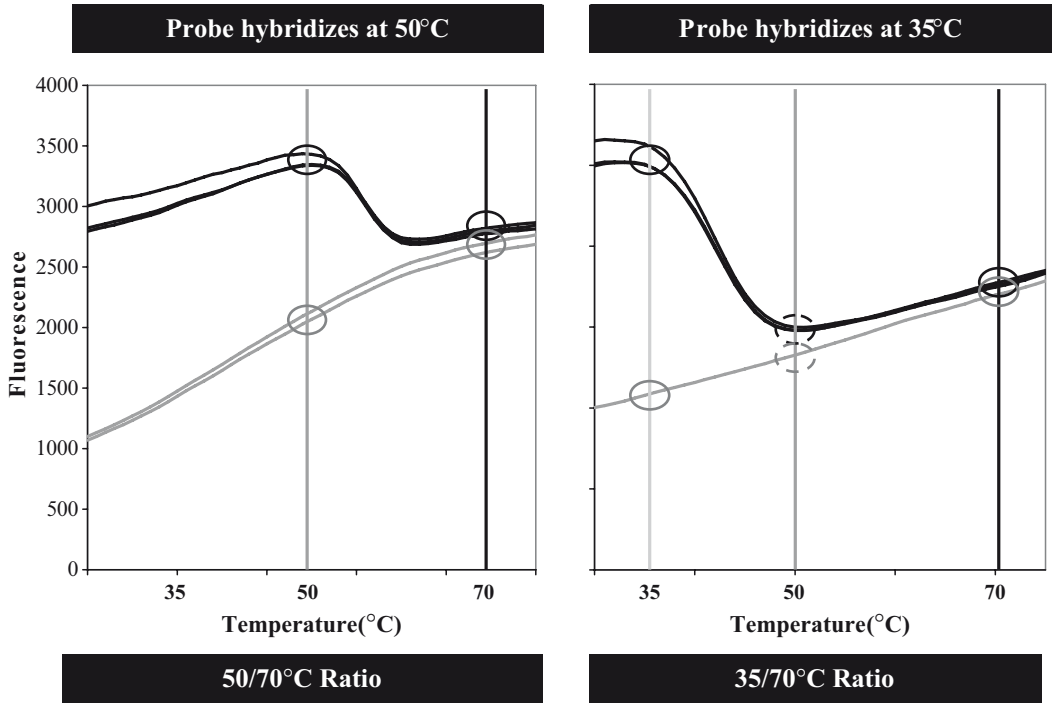


Fig. 11. Temperature-specific low-$T_m$ probes. Two sets of probes are required for a multiplex RT-LATE-PCR viral assay as shown in Fig. 10. The higher-$T_m$ probes are designed to be bound at and below 50°C. The lower-$T_m$ probes are designed to be bound at 35°C but not at 50°C. Probe/target melting curves for two such probes are shown here (*black lines*), together with the respective NTCs melting curves (*grey lines*). Left panel: higher-$T_m$ CalOrange 560-probe. *Right panel*: lower-$T_m$ CalRed 610-probe. As shown by the NTCs curves, each fluor has a characteristic temperature-dependent fluorescence profile; in addition, there are sample-to-sample variations in fluor content (curves do not overlap perfectly at 70°C, when all probes are open). The effect of both these factors is eliminated by normalizing each sample's fluorescence at probe-binding temperature (50°C and 35°C) by that same sample's fluorescence at 70°C

4. Prime RT with the same type of primer (XP) for all targets (see Note 29).
5. Design all probes expected to bind at 50°C with similar $T_m$s (58–61°C).
6. Design all probes expected to bind at 35°C with similar $T_m$s (47–50°C).
7. Make sure that the probes that bind at 35°C are not still partially bound at 50°C (see Fig. 11).
8. Analyze all the primer/probe/amplicon sequences with nucleic acid folding software. *Avoid any interaction that could lead to primer elongation or mispriming. Take into account buffer composition and salt concentration when calculating* $T_m$ *values.* In our experience, Visual OMP provides the most rigorous sequence interaction analysis and the most accurate $T_m$ estimates.

#### 3.2.1.2. Low-$T_m$ Probe Design

As for Oct4 mRNA (Subheading 3.1.1.), probes are designed to include a linear portion complementary to the target and having the appropriate $T_m$. A "2-nt stem" is included in the probe by adding two nucleotides at the 5′ end and two complementary nucleotides at the 3′ end. The fluor of choice is conjugated at the 5′ end and the appropriate Black Hole Quencher at the 3′ end. For FAM probes, the stem must include a GC base pair in order to improve fluor/quencher interaction (see Notes 30 and 31).

1. Design a set of probes hybridizing at 50°C.
2. Design a set of probes hybridizing at 35°C keeping in mind the following: (a) fluor and quencher need to be sufficiently distant once the probe is bound to target (avoid very short probes; the probe should be about 20-nt long including the stem); (b) you can introduce mismatch(es) to reach a low $T_m$ without sacrificing length; alternatively, add a few nucleotides noncomplementary to target between the target-specific sequence and the stem (avoid making the stem longer because it would not open at low temperature); (c) expect the experimentally determined $T_m$ of this type of probes to be several degrees lower than the calculated $T_m$. Visual OMP estimates are less accurate at this very low temperature.

Figure 12 shows an example of two probes having the same fluor but different $T_m$s and their use in a viral RNA multiplex assay.

### *3.2.2. Preparation of Viral RNA Sequences In Vitro (see Note 32)*

RNA sequences identical to selected viral gene sequences are generated in the lab from plasmids containing the desired sequence preceded by the T7 RNA polymerase promoter. We use Plasmid pGS-21a, custom-modified in order to eliminate any intervening sequence between the T7 promoter and the desired insert sequence (ClaI/NcoI modification) (see Note 11). With this method we have successfully generated RNA targets over 400 nt-long.

#### 3.2.2.1. Plasmid Linearization Protocol

1. Resuspend plasmid (plasmid miniprep = ~4–5 µg) in 20 µl of nuclease-free water.
2. Heat at 50°C for 15 min before taking an aliquot for linearization.
3. Prepare the *Linearization Mix:*

   8 µl plasmid (=~1.6–2 µg)

   1 µl 10X REACT 2 Buffer

   1 µl *Eco*R V stock

   10 µl total

   Final glycerol concentration = 5% (*Important*: higher glycerol concentrations affect enzyme's specificity)

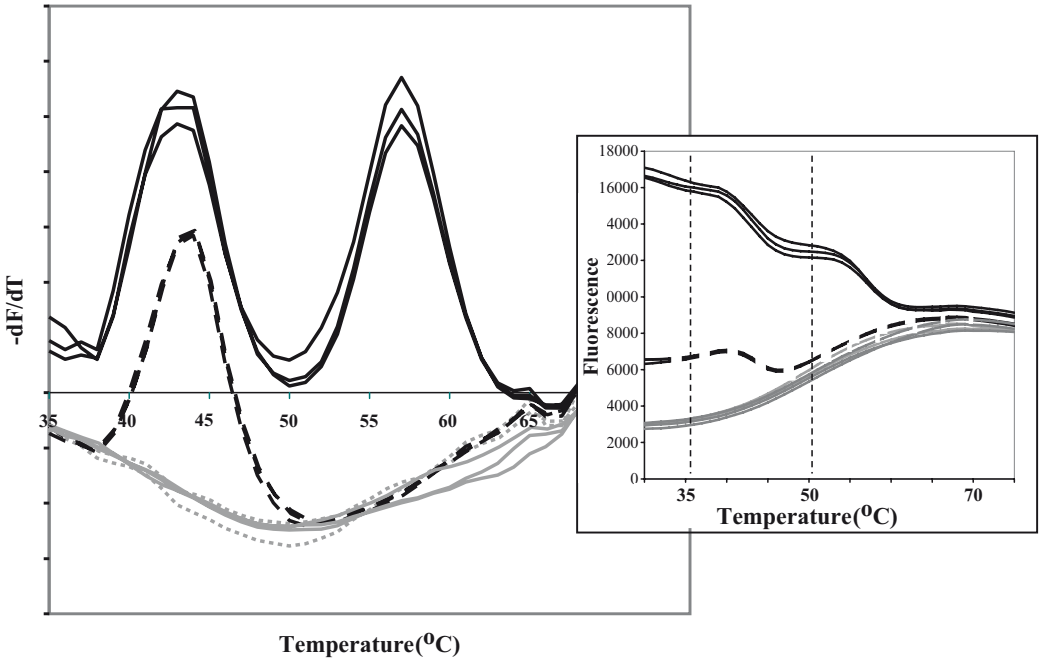


Fig. 12. Probes with the same fluor and different $T_m$s in a multiplex RT-LATE-PCR assay. Two Quasar 670-probes were designed with different target-specificity and different $T_m$s, as detailed in Fig. 11.11. The target for the lower-$T_m$ probe is an external RNA control that is added to all assays; the target for the higher-$T_m$ probe is a sequence from the H5N1 avian influenza virus (see also Fig. 11.15). RT is required for target amplification. (Neither probe hybridizes in "No RT" assays, *grey solid lines*; *grey broken lines* = NTCs.) The lower-$T_m$ probe binds to target in all RT-assays, because the RNA control is always present. The higher-$T_m$ probe binds to target only when the viral RNA sequence is added to the assay: in this case, both probes hybridize to target (bimodal *black solid line*; broken *black line*: only RNA control present in the assay). Main chart: negative derivatives of the probe-to-target melting curves (in the inset)

Final *Eco*R V concentration = 10 U/10 µl

4. Incubate at 37°C for 1 h, then inactivate enzyme by heating at 75°C for 10 min.
5. Store at –20°C.

3.2.2.2. In Vitro Transcription (IVT)

Use the Riboprobe in vitro Transcription System.

1. *Have all reagents at room temperature* (except for enzymes and rNTPs) to avoid RNA precipitation in the spermidine-containing buffer.
2. Prepare the IVT Mix:

| | |
|---|---|
| Transcription Opt. 5× Buffer | 4 µl |
| DTT, 100 mM | 2 µl |
| RNasin, 40 U/µl | 1 µl |

| | |
|---|---|
| rNTPs mix, 2.5 mM each[a] | 4 µl |
| T7 RNA polymerase, 20 U/µl | 1 µl |
| Nuclease-free water | 4 µl |
| Linearized plasmid[b] | 4 µl |
| | 20 µl total |

[a]Freshly prepared by adding 1:1:1:1 each of the 10-mM stock solutions
[b]Added last to avoid contamination; 4 µl = ~0.6–0.8 µg

3. Incubate at 37-to-39°C for 1 h.

3.2.2.3. DNase Digestion and RNA Purification

1. Add 0.8 U DNase from the same IVT kit to the sample (0.8 µl from 1 U/µl stock): ~1 U DNase/µg of DNA.
2. Incubate at 37°C for 15 min.
3. Without delay, perform phenol–chloroform extraction as explained on p.13 of the kit manual.
4. In the last step (ammonium acetate/EtOH), add a coprecipitant: 1 µl (10 µg) of yeast transfer RNA.
5. Allow to precipitate at –70°C O/N (longer is fine)
6. Centrifuge in a microcentrifuge for 1 h at max speed (at least 14,000 × *g*).
7. Discard supernatant and wash pellet with 0.5 ml of 70% EtOH (prepared with nuclease-free water).
8. Centrifuge in a microcentrifuge for 10 min at max speed (at least 14,000 × *g*).
9. Remove supernatant and allow pellet to dry for a few minutes, but not completely (complete drying of the RNA pellet hinders its resuspension).
10. Resuspend pellet in 100 µl of Tris–EDTA buffer.
11. Divide in 10 µl-aliquots and store at –70°C (see Notes 33 and 34).

*3.2.3. Test of Selected Primers' Ability to Prime RT on Individual RNA Targets*

Prior to multiplexing, test each RNA prep with its own RT primer, in order to establish efficient cDNA generation (see Figs. 6 and 7, Note 16 for possible problems due to RNA secondary structure). Prepare three sets of assays, each in duplicate: "+RT", "No RT" and No Template Controls (NTCs).

1. For "±RT" and "No RT" samples: Preincubate the RT primer with the RNA template for 5 min at 65°C, in the following mix (volumes given per reaction):

| | |
|---|---|
| Tris–Cl, 10 mM, pH 8.3 | 8.75 µl |
| XP[a], 100 µM (RT primer) | 0.25 µl |
| RNA prep diluted 1:500 in Tris–EDTA buffer | 1 µl |
| | 10 µl |

[a]1 µM in the final RT-PCR assay, 2.5 µM during preincubation

2. Cool down and add to the RT-PCR Mix below (10 µl from preincubation + 15 µl of RT-PCR Mix = *25 µl final volume of RT-PCR assay*) or to the "No RT" PCR Mix described in Step 3 below:

*RT-PCR Mix* (per reaction):

| | **Final concentration** | |
|---|---|---|
| 5X Platinum® Tfi reaction buffer (Platinum® Tfi DNA polymerase kit) | 1× | 5 µl |
| $MgCl_2$, 50 mM (Platinum® Tfi DNA polymerase kit) | 3 mM | 1.5 µl |
| dNTPs, 10 mM | 0.25 mM | 0.625 µl |
| LP, 10 µM | 100 nM | 0.25 µl |
| SYBR Green, 10X (see Note 35) | 0.25× | 0.625 µl |
| PrimeSafe 001, 10 µM | 100 nM | 0.25 µl |
| Platinum® Tfi Exo(-) (see Note 36) | 2 U | 0.4 µl |
| SuperScript™ III (200U/µl), | 200 U | 1 µl |
| Nuclease-free $H_2O$ | | 5.35 µl |
| | | 15 µl |

3. Prepare a "No RT" PCR Mix by substituting SuperScript™ III with water.
4. For NTCs: Follow the same procedure as for the "+RT" samples, but do not add RNA in the preincubation mix.
5. Run all assays ("+RT", "No RT", NTCs) with the following thermal profile (optimized for iQ5 cycler; use the Persistent Well Factor option because the Experimental Well Factor option is not available for SYBR Green):

| **Cycle** | **Repeats** | **Step** | **Dwell time** | **Setpoint (°C)** | **Data acquisition** |
|---|---|---|---|---|---|
| 1 | 1 | 1 | 10 min | 55 | |
| | | 2 | 5 min | 95 | |
| 2 | 50 | 1 | 5 s | 95 | |
| | | 2 | 10 s | 58 | |

| Cycle | Repeats | Step | Dwell time | Setpoint (°C) | Data acquisition |
|---|---|---|---|---|---|
| | | 3 | 20 s | 68 | Real-time |
| 3 | 121 | 1 | 15 sec | 35–95 (temp. change 0.5°C) | Melt Curve |

6. Data analysis. Compare "+RT" (RNA + DNA) and "No RT" (DNA only) amplification curves. "No RT" curves reveal the number of the plasmid-derived DNA templates still present in the RNA preparation after DNase digestion. This number is usually very low, typically 0.1% of total (RNA + DNA) template copies or less, see Fig. 13. A wide delta between "+RT" and "No RT" curves confirms efficient RT priming. Flat NTCs lines are desirable because they indicate that no primer-dimers are formed during PCR. The negative derivative of the melt curves is used to confirm that only one product, with the correct $T_m$, is generated during RT-PCR (see inset in Fig. 13).

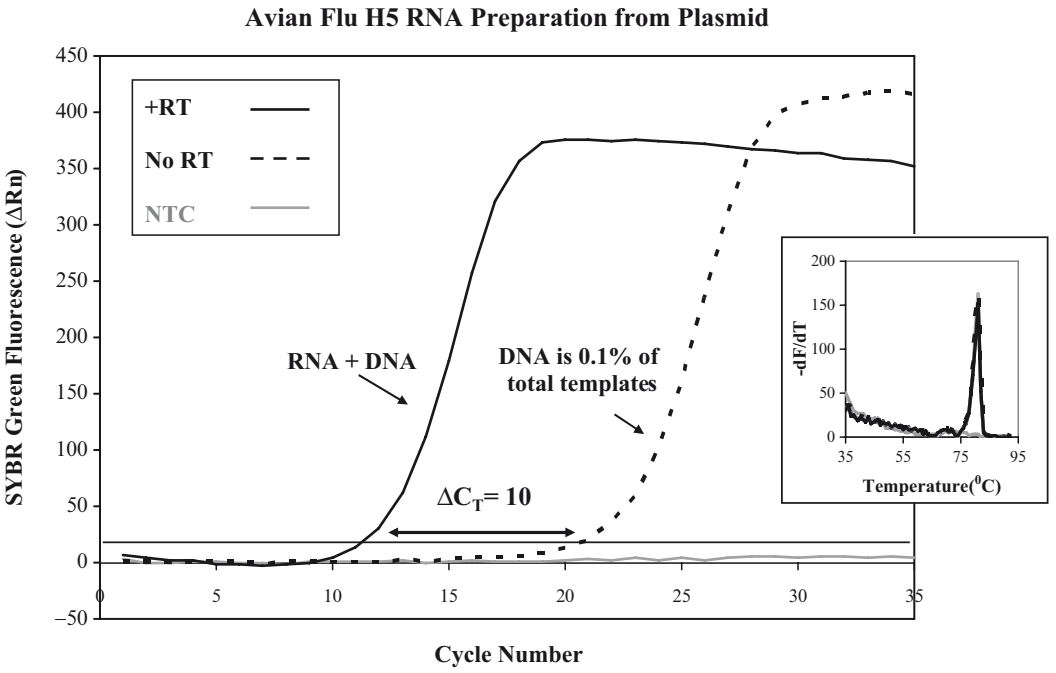


Fig. 13. RNA and DNA content of an IVT-RNA preparation from plasmid. IVT was carried out on a viral-like RNA sequence inserted in a plasmid, as described in the text. The preparation was tested by real-time RT-LATE-PCR in the presence of SYBR Green and the DNA content (carry-over from the plasmid after DNase digestion and RNA purification) was determined based on a set of "No RT" assays. The data show that RNA is 1,000-fold more abundant than DNA in this preparation ($\Delta C_T = 10$ cycles). The RNA and DNA templates are identical and clean, as indicated by the melt peaks in the inset

### 3.2.4. Multiplex RT-LATE-PCR Mix and Protocol

1. *Templates:* The assay described here is a model for a diagnostic assay. Depending on the presence of different viruses in each diagnostic sample, not all possible targets would be amplified at the same time. (Only the RNA control will be amplified every time.) We mimic these alternative outcomes by grouping sets of IVT-derived RNAs as they would be found in a viral sample. The RNAs in each set are added at equimolar concentration based on the $C_T$ values obtained from real-time qPCR of the individual RNA preps (see Subheading 3.2.3. and Fig. 13). Referring to Fig. 10, the presence of Virus 1, Subtype A is modeled by mixing three RNA preps (probed with FAM at 50°C and 35°C and with CalOrange 560 at 35°C). The presence of Virus 1, Subtype B is also modeled by mixing three RNA preps (two of them also used for Subtype A) and probed with FAM at 35°C and with CalOrange 560 at 50°C and 35°C). The presence of Virus 2 is mimicked by one RNA prep and probed with CalRed 610 and Quasar 670 at 50°C, and the presence of Virus 3 is mimicked by yet another RNA prep and probed with CalRed 610 at 35°C. Fifty-thousand copies of Armored RNA (ARNA, external control) are added in all the assays to ensure that RT-LATE-PCR did not fail.
2. *Armor Lysis:* For control RNA preparation. Heat ARNA (50,000 copies/assay) at 75°C for 3 min, as recommended by the manufacturer (see Note 37).
3. RT-LATE-PCR: The general strategy is similar to the one detailed in Subheading 3.2.3. Begin by preincubating the RT primers (XPs) with the RNA templates for 5 min at 65°C, in the following mix (volumes given per reaction):

| | |
|---|---|
| Tris–Cl, 10 mM, pH 8.3 | (to 10 µl) |
| XP for each RNA prep[a], 100 µM (RT primer) | 0.25 µl each |
| XP for ARNA, 100 µM (RT primer) | 0.25 µl |
| Lysed ARNA | 2 µl |
| Set of RNA preps diluted in Tris–EDTA buffer | 3 µl |
| | 10 µl |

[a]1 µM each in the final RT-PCR assay, 2.5 µM during preincubation

4. Cool down and add to the RT-PCR Mix below (10 µl from preincubation + 15 µl of RT-PCR Mix = *25 µl final volume of RT-PCR assay*) or to the "No RT" PCR Mix described in Step 5 below:

*RT-PCR Mix* (per reaction):

| | Final concentration | |
|---|---|---|
| 5× Platinum® Tfi Reaction Buffer(Platinum® Tfi DNA Polymerase kit) | 1× | 5 µl |
| $MgCl_2$, 50 mM(Platinum® Tfi DNA Polymerase kit) | 3 mM | 1.5 µl |
| dNTPs, 10 mM, | 0.4 mM | 1 µl |
| *LP for each RNA prep*, 10 µM | 100 nM | 0.25 µl each |
| LP for ARNA, 10 µM | 50 nM | 0.125 µl |
| FAM probe (50), 100 µM | 200 nM | 0.05 µl |
| FAM probe (35), 100 µM | 200 nM | 0.05 µl |
| CalOrange560 probe (50), 100 µM | 200 nM | 0.05 µl |
| CalOrange560 probe (35), 100 µM | 200 nM | 0.05 µl |
| CalRed610 probe (50), 100 µM | 200 nM | 0.05 µl |
| CalRed610 probe (35), 100 µM | 200 nM | 0.05 µl |
| Quasar670 probe (50), 100 µM | 200 nM | 0.05 µl |
| Quasar670-ARA probe (35), 100 µM | 200 nM | 0.05 µl |
| PrimeSafe 001, 10 µM | 100 nM | 0.25 µl |
| Platinum® Tfi Exo(-) | 2 U | 0.4 µl |
| SuperScript™ III (200U/µl), | 200 U | 1 µl |
| Nuclease-free $H_2O$ | | (to 15 µl) |
| | | 15 µl |

5. Prepare a "No RT" PCR Mix by substituting SuperScript™ III with water.
6. For NTCs: Follow the same procedure as for the "+RT" samples, but do not add RNA in the preincubation mix.
7. Run all assays ("+RT", "No RT", NTCs) with the following thermal profile (optimized for iQ5 cycler; use the Experimental Well Factor option):

| Cycle | Repeats | Step | Dwell Time | Setpoint (°C) | Data Acquisition |
|---|---|---|---|---|---|
| 1 | 1 | 1 | 10 min | 55 | |
| | | 2 | 1:30 min | 89 (see Note 38) | |
| 2 | 50 | 1 | 5 s | 95 | |
| | | 2 | 10 s | 58 | |
| | | 3 | 20 s | 68 | |

| Cycle | Repeats | Step | Dwell Time | Setpoint (°C) | Data Acquisition |
|---|---|---|---|---|---|
| 3 | 2 | 1 | 2 min | 71–70 (temp.change –1°C) | Melt C. (see Note 39) |
| 4 | 2 | 1 | 2 min | 51–50 (temp. change –1°C) | Melt C. |
| 5 | 2 | 1 | 5 min | 36–35 (temp. change –1°C) | Melt C. (see Note 40) |
| 6 | 1 | 1 | 3 min | 95 | |
| 7 | 71 | 1 | 30 s | 95–25 (temp. change –1°C) | Melt C. (see Note 41) |

*3.2.5. Data Analysis*

Positive or negative results (virus present or absent) are scored based on ratios of end-point fluorescence readings (see Note 42). Fluorescence is first acquired at 70°C (all probes are open at this temperature) and these values are used to normalize each assay for its probe content, eliminating assay-to-assay variations, as follows:

1. Divide data acquired at 50°C by the corresponding readings at 70°C (50/70°C ratios).
2. Divide data acquired at 35°C by the corresponding readings at 70°C (35/70°C ratios).
3. The 50/70°C and 35/70°C ratios of the NTCs are the threshold for a "Negative outcome." These ratios are different for different fluors (see Fig. 11 and Note 43).
4. Plot all assays' 50/70°C and 35/70°C ratios versus the NTCs' 50/70°C and 35/70°C ratios and determine a positive/negative result for each of the probes in the assay (see example in Fig. 10).
5. Analyze the melt curves and their derivatives (see Fig. 12) to establish that the probes worked as expected (see Note 44).

*3.2.6. End-Point Quantification with Temperature-Specific Probes*

A qualitative result is usually sufficient when investigating the presence of viruses in a sample, but quantitative data are also useful. To test the efficiency of end-point quantification for a single LATE-PCR amplicon or in a multiplex, it is necessary to know the amount of target added in the reaction:

1. For target quantification, use the $C_T$ values obtained during real-time amplification of each individual target in the presence of SYBR Green (see Subheading 3.2.3. and Fig. 13).
2. Prepare dilutions of the targets.
3. Run RT-LATE-PCR (if RNA targets are used) or LATE-PCR (if DNA targets are used) assays in the presence of sequence-specific probes for all the targets being tested and different target concentrations.
4. Calculate probe fluorescence ratios (50/70°C and 35/70°C) and plot them as a function of the calculated copy numbers

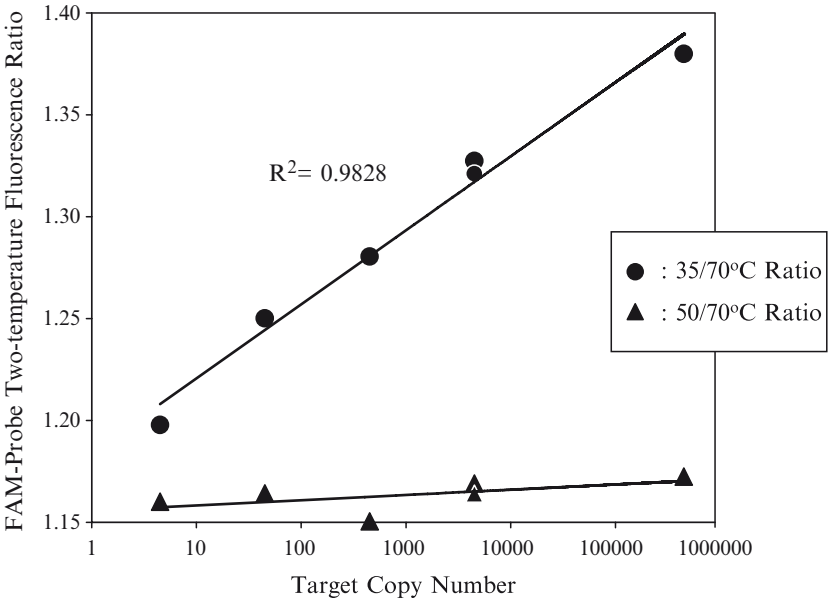


Fig. 14. LATE-PCR end-point quantification with a temperature-specific low-$T_m$ probe. A FAM-conjugated probe was designed with a $T_m$ of 49°C (experimentally-determined $T_m$ = 46°C), so as to be bound to target at 35°C but not at 50°C. DNA oligonucleotides at known concentrations were used as the probe target (*abscissa*) and fluorescence intensity was acquired at 70°C, 50°C, and 35°C. The 35/70°C (*circles*) and 50/70°C (*triangles*) fluorescence ratios (*ordinate*) were calculated and plotted vs the target copy numbers (*abscissa*). The results demonstrate that the probe is temperature-specific as desired and that end-point quantification is linear from 4 to 400,000 target copies

on a log scale (Fig. 14) or of the SYBR Green $C_T$ values on a linear scale (Fig. 15). Figure 14 illustrates the results for a single target probed by a temperature-discriminating probe. Figure 15 shows the results obtained from one of the amplicons in a triplex, probed in two different regions.

## 4. Notes

1. US Pat No. 7,198,897 issued April 3, 2007, European Patent: 02 795963.4 granted May 7, 2008.
2. Considerable differences exist between thermocyclers regarding the approach used to measure and analyze fluorescence emitted from multiple fluors. In addition, excitation and emission filter sets vary, in some instances, limiting the spectrum of fluors that can be used in the same assay. Finally, not all thermocyclers can reach low temperatures such as 35°C. (This is the case in particular for air-cooled cyclers, which are sensitive to ambient temperatures [see Subheading 2.1].)

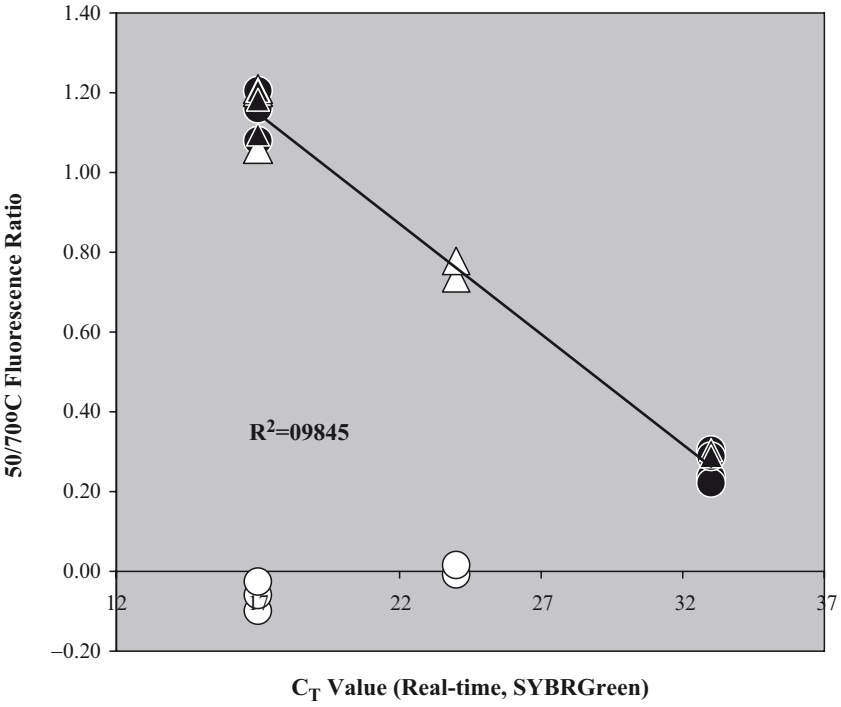


Fig. 15. LATE-PCR end-point quantification using two probes on the same amplicon in a triplex. Three viral-like RNAs were coamplified in sets of RT-LATE-PCR assays mimicking the presence of different substrains of the avian influenza H5N1 virus. This figure summarizes results obtained from the longest RNA in the triplex (~400 nt), which contains the targets for two probes (Quasar 670-probe, *triangles*, and CalOrange 560-probe, *circles*). Both probes bind to target at 50°C and their 50/70°C ratios (*ordinate*) were calculated as a function of target abundance. (A correction was introduced to account for the different fluorescence intensities of the two fluors.) Target abundance was determined using the $C_T$ values of SYBR Green real-time LATE-PCR assays of the individual amplicons comprising the triplex (*abscissa*). The target for the Quasar 670-probe is conserved in both low- and high-pathogenic substrains of H5N1 (*black triangles* and *white triangles*, respectively); the target for the CalOrange 560 probe is found in the low-pathogenic H5N1 substrains (*black circles*) but not in the high-pathogenic substrains (*white circles*). The data demonstrate target-specificity of the two probes and good correlation between end-point quantification and target abundance

All these factors need to be kept in mind when selecting a thermocycler different from those indicated in the Materials section. The commercial names of those thermocyclers are provided to enable perspective users with an accurate term of comparison when selecting an alternative instrument.

3. We have observed that the quality of primers supplied by different manufacturers is not always consistent. It may be worthy to switch supplier if the PCR products are not as expected.
4. A wide array of fluors is available from different manufacturers, with different properties. (Keep in mind brightness, background levels, and fluorescence change at increasing temperatures.) Careful consideration should be given to the

choice of multiple fluors for a single assay, with the aim of minimizing emission spectra overlaps. The choice of the quencher is also important, as quenchers affect background fluorescence levels (when the stem of the probe is closed). When using more than one probe with the same fluor in a single assay, it is essential to lower as much background fluorescence as possible by selecting the best quencher. We had the best results with the the CAL Fluor® dyes conjugated to Black Hole Quenchers®, both proprietary of Biosearch Technologies, Inc., but the final choice of fluors also depends on the thermocycler used for the assay (see Note 2).

5. The PrimeSafe series was developed in our laboratory specifically for use with LATE-PCR. We have not tested other mispriming preventing compounds in our assays.
6. Mouse DNA is commercially available or can be prepared and purified in-house. Attention: if the DNA is sheared in short fragments during the preparation, PCR may fail or may underestimate the number of targets actually present. In fact, the sequences targeted by both primers need to be present on the same DNA segment in order to generate the full PCR product.
7. We used RNase ZAP®, but other similar products are available. Keep in mind that bleach is not sufficient to degrade all RNases.
8. All of these products are available from Invitrogen and contain specific, proprietary enzymes and buffers that determine the experimental conditions and protocols described in this chapter. In addition, the optimal temperature for the selected reverse transcriptase was a factor in the design of the RT primers because the $T_m$ of this type of primer must allow its efficient hybridization to the RNA target during RT. For the above reasons, we believe that it is necessary to specifically identify the enzymes and buffers that we chose after testing numerous other reverse transcriptases and DNA polymerases. Researchers experienced in RT-PCR may make alternative choices keeping in mind that these will potentially require modifications of protocols and experimental methods, including buffers, cations, primer/probe design (affecting their $T_m$s), etc.
9. Armored RNA consists of an RNA standard sequence enveloped by MS2 bacteriophage coat protein. It is produced in *Escherichia coli* by the induction of an expression plasmid that encodes the coat protein and the RNA standard sequence (8). The packaged RNA is stable and ribonuclease-resistant. The MS2 phage (containing its own native RNA) is a cheaper, if less reliable, alternative for a ribonuclease-resistant RNA standard (13).

10. Other software programs are available for designing primers and probes, but do not offer the same analysis capabilities as the one cited here, particularly regarding $T_m$s calculations under different conditions. Researchers unable to access Visual OMP may have to assess effective $T_m$ values empirically. (For instance, melt peak analysis can be used to determine probe-to-target $T_m$s.)
11. We use pGS-21a plasmids (ClaI/NcoI custom modification) from GenScript Corporation. These plasmids contain the T7 promoter directly followed by a DNA sequence which is the template for generation of the desired RNA in vitro. At the end of this sequence, there are restriction sites for an array of restriction enzymes. The *Eco*R V enzyme was selected after restriction sequence analysis owing to its compatibility with all of our targets and because it produces a blunt-ended cut in the DNA template, ensuring better results during IVT.
12. As for Note 8, in order to describe a specific protocol, it is necessary to refer to a specific product, considering the wide range of products and procedure available. Again, an expert researcher will be able to make the necessary modifications to the overall protocol when choosing an alternative IVT kit. Keep in mind that the chosen RNA polymerase needs to bind to the correspondent promoter in the expression plasmid: for instance, we use the T7 RNA polymerase which binds to the T7 promoter in our plasmids.
13. Glycogen solutions are often used as inert coprecipitants of nucleic acid; we, however, had better results with tRNA.
14. Amplicon contamination becomes apparent when the specific PCR product is generated in the absence of any template (see "No Template Controls", described later in this chapter). The best solution to this problem usually consists of cleaning thoroughly surfaces and instrumentation and in replacing all reagent aliquots with new, never used ones.
15. If the sequence between the primers includes a gene's intron or part of it, the PCR product generated from the genomic DNA target will be different from that generated from the corresponding cDNA target, because intron sequences are eliminated from mature mRNA by splicing. (If one of the primers is placed within the intron, no product will actually be generated from the cDNA template.) It cannot be assumed that two amplicons having different sequences and lengths are generated with equal efficiencies during PCR. A standard using genomic DNA templates (mouse DNA) would not, therefore, be absolutely reliable for quantification of the corresponding cDNA templates. If, however, the primer sequences are placed within an exon, the amplicons generated

from genomic DNA and cDNA targets will be exactly identical; hence, quantification of cDNA copy number can be reliably achieved by comparison with a genomic DNA standard (9).

16. RNA targets with high level of secondary structure may require moving the RT primer to a different part of the sequence in order to achieve good results.
17. Mouse embryo RNA can be prepared with any commercial method. Our lab has developed a single-tube RNA/DNA preparation-to-quantification method for small samples (single cell, single embryo) called PurAmp (9), US Pat No. 7,465,562. If using the PurAmp method, maintain an RNA volume of 6 µl, but increase the assay final volume to 50 µl by doubling the volume of all other mix components and adjusting the water volume.
18. We have found this buffer to considerably improve the reaction's efficiency over the buffer provided with the SuperScript™ III Platinum® One-Step RT-PCR Kit.
19. This FAM probe is recommended when using the ABI 7700 thermocycler. The fluor can be substituted by Quasar 670 and the quencher by Black Hole Quencher 2 when using a BioRad iQ5 or a Mx3005P Stratagene cycler. For the ABI assay, we have also successfully used a TET/Dabcyl-Oct4 molecular beacon at 1 µM concentration (5). Traditional molecular beacons with 5-to-6 nt stems (14), however, are harder to design for low temperature annealing due to their longer stem that remains closed when cooling the reaction.
20. PrimeSafe 002 (a more recent version of PrimeSafe) at 200 nM can be substituted for 300 nM PrimeSafe 043 in this assay.
21. Data acquisition starts after the initial 15 cycles in order to limit possible mispriming when the intended amplicon is first generated. Mispriming may take place at the low temperature necessary for probe hybridization.
22. A concentration range between 100 and 300 nM works in most cases. If primers are designed with $T_m$s lower than those shown in this protocol (see Subheading 3.1.2.), we recommend the use of PrimeSafe 001 as detailed in Subheading 3.2.
23. Unlike symmetric PCR, LATE-PCR allows generation of hundreds-of-nucleotides long amplicons. Two probes on the same sequence are useful to detect mutations that often occur in viral strains. The first probe is located on a conserved region and signals the presence of the virus; the second probe is located on a variable region and only hybridizes when major

mutations have not occurred. (See for instance the insertion of a "cleavage region" responsible for increasing the pathogenicity of influenza strains.)

24. A useful list of fluors with multiplex-compatible absorption/emission spectra can be found at (15). Select your PCR instrument to update the list.
25. Design the assay to avoid the possibility of false positive, or false negative for dangerous agents: build in redundancy for most important targets.
26. If the RNA control is an armored RNA (see protocol), the control is also useful to monitor effective sample preparation. The BioSeeq®-Vet PCR System, currently being developed by Smiths Detection Diagnostics, Watford, UK, includes a field-portable instrument capable of viral sample preparation-to-detection by RT-LATE-PCR in a single, disposable cartridge.
27. An alternative strategy is to detect different viruses with probes hybridizing at different temperatures, see the CalRed 610-probes for Virus 2 and Virus 3 in Fig. 10.
28. A greater difference will decrease amplification efficiency. Ideally, all amplicons should be generated with the same efficiency. If not, there is the risk that the least efficient amplicons will "drop out" if targets are present at low copy number. In practice, however, it is not possible to have all amplicons with the same $T_m$ and length (ideally around 100 nt): in some cases, it is desirable to fit two probes in the same, long amplicon; in other cases, conserved regions (ideal for priming) are separated by long variable regions. Keep also in mind that long sequences are particularly problematic during RT due to the high level of secondary structure of ss RNA.
29. LP can also be used for RT priming, but it may require higher RT temperature because of its higher $T_m$.
30. Although the fluor/quencher interaction affects the $T_m$, we found more accurate results by not including this modification when calculating probe $T_m$s with Visual OMP. If the stem nucleotides are also complementary to the target, they will contribute to the probe's $T_m$.
31. The positions of fluor and quencher can be switched. When using two probes on the same amplicon, relatively close to each other, it is better to have one quencher on the 3′ and the other on the 5′, in close proximity, rather than having the quencher of the first probe close to the fluor of the second probe. It is also recommended to have the quencher at the 3′ end when using DNA polymerases that, unlike Tfi(-), display exonuclease activity, because Black Hole Quenchers prevent probe cutting.

32. If desired, the multiplex assay can be initially tested on DNA targets. In this case, keep in mind the following caveats: (a) DNA oligos longer than 100–150 nt cannot be synthesized; longer templates will need to be shortened by eliminating some of the intervening sequence and will thus be a poor model for the actual RNA target. In addition, the $T_m$s of these shorter amplicons will be lower than those of the amplicons generated from the RNA targets, affecting the PCR outcome; (b) DNA oligos do not allow testing for RT efficiency.
33. Storage of RNA at higher temperature greatly affects its stability.
34. You will generate an extremely high number of RNA molecules. Be extremely careful to avoid template contamination when doing RT-PCR after RNA preparation. Work in a different, dedicated area if possible and treat all instrumentations, surfaces, and pipettes used for IVT with bleach.
35. The use of specific probes is not necessary at this point, for single amplicons. These real-time curves are sigmoidal because they visualize generation of ds DNA during phase I of LATE-PCR (see Fig. 2).
36. Platinum® Taq polymerase (1.5 U/reaction) can be used instead of Platinum® Tfi(-), with the appropriate buffer. In this case, use the SuperScript™ III/ Platinum® Taq mix available from the manufacturer as part of a one-step kit (see Subheading 3.1.2.) and switch to PrimeSafe 002 at 300 nM concentration. Also, change annealing temperature to 62°C and extension temperature to 72°C.
37. Avoid product contamination by using a heating block, not the cycler where PCR is carried out.
38. This temperature value is necessary when using "Experimental Well Factor" in an iQ5 cycler because the instrument will collect two readings at 95°C to establish a normalizing value for each well. The readings will be taken before RT, thus denaturing the enzyme, if any of the steps in the first Cycle is above 89°C. Using the settings shown above, two readings at 95°C (1:30 min total) will be taken after RT and before PCR.
39. The iQ5 cycler does not allow data acquisition in more than one cycle, except when using the Melt Curve option. For this reason, we set all end-point collection data (at 70°C, 50°C, and 35°C) as "mini-melts."
40. A long dwell time at 35°C is useful to improve probe binding at this low temperature.
41. This is actually an Anneal Curve, from high to low temperature.

42. The switch from real-time to end-point data gathering is necessary in multiplex LATE-PCR because the probes are designed to hybridize at low $T_m$. In real-time PCR, data are acquired at every cycle and lowering the temperature at every cycle favors mispriming when multiple pairs of primers are present in the reaction. PrimeSafe can efficiently prevent this problem in duplex real-time PCR (see Subheading 3.1.4.). In triplex real-time reactions (see Fig. 3), we observed scatter at end-point among replicates, indicating the generation of nonspecific products in the background. End-point data acquisition bypasses this problem because the temperature is lowered to allow probe hybridization only at the end of PCR, after amplification.
43. The reason for this difference is that temperature-dependent change in fluorescence is fluor-specific.
44. If a probe binds with $T_m$ lower than expected when the assay is used for tests on real viruses, mutations may have occurred in the viral sequence being probed. LATE-PCR allows direct sequencing of the single-stranded product by simple dilution (7), which would be very informative in this case.

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### References

1. Gyllensten UB, Erlich HA (1988) Generation of single-stranded-DNA by the polymerase chain-reaction and its application to direct sequencing of the Hla-Dqa Locus. Proc Natl Acad Sci USA 85:7652–7656
2. Sanchez JA, Pierce KE, Rice JE, Wangh LJ (2004) Linear-after-the-exponential (LATE)-PCR: an advanced method of asymmetric PCR and its uses in quantitative real-time analysis. Proc Natl Acad Sci USA 101:1933–1938
3. Pierce KE, Sanchez JA, Rice JE, Wangh LJ (2005) Linear-After-The-Exponential (LATE)-PCR: primer design criteria for high yields of specific single-stranded DNA and improved real-time detection. Proc Natl Acad Sci USA 102:8609–8614
4. Raja S, El-Hefnawy T, Kelly LA, Chestney ML, Luketich JD, Godfrey TE (2002) Temperature-controlled primer limit for multiplexing of rapid, quantitative reverse transcription-PCR assays: application to intraoperative cancer diagnostics. Clin Chem 48:1329–1337
5. Hartshorn C, Eckert JJ, Hartung O, Wangh LJ (2007) Single-cell duplex RT-LATE-PCR reveals *Oct4* and *Xist* RNA gradients in 8-cell embryos. BMC Biotechnol 7:87
6. Chou CF, Tegenfeldt JO, Bakajin O, Chan SS, Cox EC, Darnton N, Duke T, Austin RH (2002) Electrodeless dielectrophoresis of single- and double-stranded DNA. Biophys J 83:2170–2179

7. Rice JE, Sanchez JA, Pierce KE, Reis AH Jr, Osborne A, Wangh LJ (2007) Monoplex/multiplex linear-after-the-exponential-PCR assays combined with PrimeSafe and Dilute-'N'-Go sequencing. Nat Protoc 2:2429–2438
8. Pasloske BL, Walkerpeach CR, Obermoeller RD, Winkler M, DuBois DB (1998) Armored RNA technology for production of ribonuclease-resistant viral RNA controls and standards. J Clin Microbiol 36:3590–3594
9. Hartshorn C, Anshelevich A, Wangh LJ (2005) Rapid, single-tube method for quantitative preparation and analysis of RNA and DNA in samples as small as one cell. BMC Biotechnol 5:2
10. http://www.invitrogen.com/content/sfs/ProductNotes/F_Superscript%20III%20Enzyme%20RD-MKT-TL-HL0506021.pdf?ProductNoteId=36
11. Laird CD (1971) Chromatid structure: relationship between DNA content and nucleotide sequence diversity. Chromosoma 32:378–406
12. Hartung O, Hartshorn C, Wangh LJ (2007) Cdx2 and Oct4 mRNA in pre-implantation stage mouse embryos and their single cells. Fertil Steril 88:S309–S309
13. Dreier J, Störmer M, Kleesiek K (2005) Use of bacteriophage MS2 as an internal control in viral reverse transcription-PCR assays. J Clin Microbiol 43:4551–4557
14. Tyagi S, Kramer FR (1996) Molecular beacons: probes that fluoresce upon hybridization. Nat Biotechnol 14:303–308
15. http://www.biosearchtech.com/display.aspx?pageid=53