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Address correspondence to Alexander Zhyvoloup, Cell Regulation Laboratory, Ludwig Institute for Cancer Research, 91 Riding House Street, London W1W 7BS, UK. e-mail: zhyvoloup@ludwig.ucl.ac.uk

Effectiveness and limitations of uracil-DNA glycosylases in sensitive real-time PCR assays

Kenneth E. Pierce and Lawrence J. Wangh
Brandeis University, Waltham, MA, USA

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PCR is sufficiently sensitive to detect single copy genes from single cells. This extreme sensitivity is also the basis of one of its potential problems; even a single product molecule from a previous amplification can lead to a false positive result. Substituting dUTP for dTTP during PCR and treating subsequent reactions with uracil-DNA glycosylase (UDG) prior to amplification is one strategy for limiting carryover contamination (1). However, total elimination of contaminants is not always accomplished using this technique, particularly where PCR product length is short (2), which is a common situation in real-time PCR assays. In addition, there is the possibility, particularly when the initial sample contains only one or a few target molecules, that inclusion of UDG may reduce amplification efficiency and thereby delay or prevent detection.

As a first step toward implementing a general protocol for using UDG at the level of single copy genes in single cells, we investigated conditions for preventing contamination during amplification of a 133-bp segment within the multicopy testis-specific protein gene (*TSPY*) from single male lymphocytes. Cell lysis and real-time PCR with molecular beacons were carried out as described previously (3), except that the extension step of thermal cycling was increased to 30 s and the dTTP was replaced with dUTP at a 3-fold higher concentration. The resulting PCR product contained 27 uracil residues in the sense strand and 28 uracil residues in the antisense strand.

Initial experiments compared the efficiency of amplification in the presence of dUTP or dTTP in terms of the mean detection cycle (C_T) value (i.e., the point at which the molecular beacon fluorescence intensity reaches a threshold of 200 U) and the final fluorescence

after 45 cycles. Amplification plots are shown in Figure 1A. The mean C_T values of 33.9 and 34.4 for dUTP and dTTP samples, respectively, were in the range of values previously observed using single lymphocytes, reflecting the presence of approximate 30 copies of the *TSPY* gene on the Y chromosome (3). The differences in mean C_T value and mean final fluorescence between the two sample groups were not statistically significant ($P > 0.05$). Thus, substitution of dUTP (at 3× concentration) for dTTP did not reduce amplification efficiency for this sequence. Similarly, the use of dUTP did not significantly alter amplification efficiency of a 175-bp segment of *U2*, another multicopy gene (3) (data not shown). The *U2* PCR product contained 26 uracil residues in the sense strand and 37 uracil residues in the antisense strand.

Next, we tested the effect of UDG (from Roche Applied Science, Indianapolis, IN, USA) on the efficiency of *TSPY* and *U2* amplification, since some enzyme activity remains after 10 min at 95°C (a typical inactivation step) (4). Theoretically, residual enzyme activity might degrade a fraction of the PCR product during each annealing step of the subsequent PCR and might, thereby, delay or prevent signal detection. Samples (all containing dUTP) with or without UDG were incubated at 95°C for 10 min prior to thermal cycling. Real-time detection of *TSPY* amplification is shown in Figure 1B. Mean C_T values and final fluorescence were not significantly altered by the presence of UDG ($P > 0.05$). Similarly, amplification of *U2* was not significantly affected by the presence of UDG (data not shown). It should be noted that the annealing temperature used for these amplifications was 58°C, and additional tests may be warranted

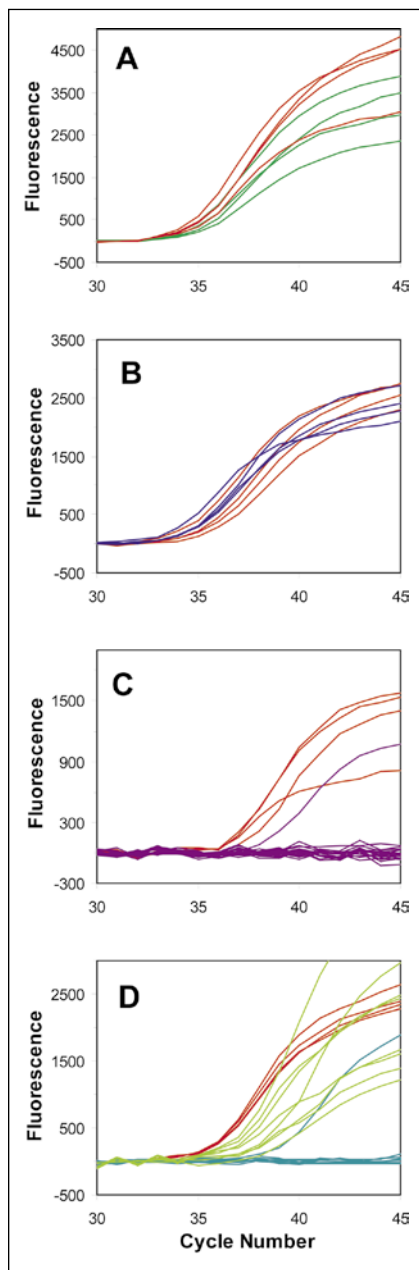


Figure 1. Molecular beacon fluorescence detection of testis-specific protein gene (*TSPY*) amplification during real-time PCR. (A) *TSPY* amplification in samples containing single male lymphocytes and either dTTP (dark green) or dUTP (red), but no uracil-DNA glycosylase (UDG). (B) *TSPY* amplification in samples containing single male lymphocytes in the presence (blue) or absence (red) of UDG. All samples contain dUTP. (C) *TSPY* amplification in samples with diluted *TSPY* PCR product (averaging about three molecules per sample) following a 10-min incubation at 37°C with UDG (purple) or without UDG (red). (D) *TSPY* amplification in samples with diluted *TSPY* PCR product (averaging about 10 molecules per sample) following a 10-min (green) or 30-min (blue-green) incubation at 30°C with heat-labile UDG or without heat-labile UDG (red).

Table 1. Ability of UDG and Heat-Labile UDG to Prevent Amplification from *TSPY* PCR Products Containing Uracil

Enzyme	Incubation Conditions		Samples		
	Temperature (°C)	Duration (min)	Total Tested (n)	No Signal (n)	(%)
UDG	25	10	8	5	(63) ^a
	37	10	24	23	(96) ^a
Heat-Labile UDG	25	10	20	6	(30) ^b
	30	10	8	0	(0)
	37	10	8	5	(63)
	30	20	8	4	(50)
Heat-Labile UDG (2× concentration)	30	30	28	23	(82) ^b
	30	10	8	2	(25)

^aStatistically significant difference, $P < 0.05$, Fisher's exact test.

^bExtremely statistically significant difference, $P < 0.001$, Fisher's exact test.

TSPY, testis-specific protein gene; UDG, uracil-DNA glycosylase.

in cases where annealing temperatures are closer to 50°C, at which UDG has maximal activity (5).

The ability of UDG to degrade PCR products prior to amplification was tested using *TSPY* PCR product molecules generated with dUTP. The goal of these experiments was to determine whether a high percentage of samples could be completely “cleared” of product molecules using various protocols with UDG. Initial conditions employing a 10-min incubation at 25°C prior to PCR, as recommended by the manufacturer, were found to be inadequate. Three of eight samples generated detectable *TSPY* product from residual target molecules (Table 1). Subsequent tests employed a 10-min incubation at 37°C. All four samples without UDG, but only 1 of 24 samples with UDG, showed detectable amplification (Figure 1C). The mean C_T value of 37.3 in samples without UDG indicates an average of about three target molecules in each of the tested samples, while the positive signal in the sample with UDG had a C_T value of 38.9 and was therefore likely due to amplification from a single undigested PCR product. Similar results were obtained using the 37°C protocol with PCR product amplified from the *U2* gene. Reamplification was observed in only 1 of 24 samples containing UDG. Thus, the treatment at the higher temperature was successful, although not absolute in preventing amplification from PCR product generated

using dUTP. Higher temperatures were not tested, but might further decrease the percentage of samples with undigested product.

The potential problem with residual UDG activity after PCR has led some investigators to use a heat-labile form of the enzyme (5,6). Incubations with heat-labile UDG (Roche Applied Science) at the manufacturer's recommended conditions of 25°C for 10 min prevented detectable amplification in only 6 of 20 samples containing the *TSPY* PCR product (Table 1). An unacceptably high percentage of samples also generated detectable product even when the enzyme concentration was doubled or the incubation temperature increased to 30° or 37°C. However, increasing the duration of a 30°C incubation to 30 min did decrease the percentage of samples generating product from the PCR product molecules. In one experiment using that protocol, only one of eight samples generated a detectable signal, and that C_T value suggested amplification from a single target (blue-green line in Figure 1D). Eight samples treated for only 10 min all generated amplification, although the range of C_T values was above that of untreated controls (green and red lines, respectively, in Figure 1D), indicating that some of the estimated 10 target molecules in each sample were degraded prior to amplification. A subsequent experiment using the 30-

min incubation on samples containing an average of only three PCR product molecules still resulted in detectable amplification in 4 of 20 samples, indicating that the starting number of molecules was not the sole reason for the poorer results with the heat-labile enzyme. In all, 23 of 28 (82%) samples incubated at 30°C for 30 min with heat-labile UDG were successfully cleared of the PCR product molecules. Long incubation at higher temperatures was not tested because of the short half-life of the enzyme, which is 2 min at 40°C according to the manufacturer.

In summary, dUTP and UDG can be used in real-time PCR applications without affecting the efficiency of amplification and thus the ability to detect and quantify targets of interest, even when those targets are few in number. It may be important to maintain relatively high annealing temperatures when UDG is present in order to avoid effects of residual enzyme activity during amplification. Incubating samples with UDG at 37°C prior to PCR greatly reduces the probability of contamination, although it does not guarantee complete removal of PCR product molecules from all samples. Since the nucleotide sequence and the choice of PCR buffer are likely to affect the degradation of products, we recommend similar tests be carried out for any assay in which avoiding contamination is critical. Such tests are definitely warranted for the use of heat-labile UDG. None of the protocols tested here with that enzyme proved satisfactory for the complete removal of PCR product molecules. While it may be possible to obtain a satisfactory protocol, in particular where those molecules are longer, it may require much higher concentrations of the heat-labile enzyme and/or long incubation times that increase expense and duration of the assay. In any case, users should always remember that either enzyme should be an adjunct to, rather than a replacement for, strict handling protocols aimed at avoiding contamination.

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Address correspondence to Kenneth Pierce, Department of Biology MS-008, Brandeis University, Waltham, MA 02454-9110, USA. e-mail: pierce@brandeis.edu