OD units attributed by laser scanning to individual DNA fragments of 200, 400 and 600 bp vary linearly with the number of cells extracted over a range of 5×10^3 to 4×10^4 cells. This linear relationship allows a quantitative estimate of the amount of apoptotic DNA in a given cell preparation based on the OD value attributed by densitometry to a single DNA band.

In Figure 2A, apoptotic DNA extracted from a graded number of human lymphocytes was hybridized with the probe synthesized from Jurkat DNA. Distinct DNA banding could be detected from as few as 2×10^4 lymphocytes, whereas a minimum of 5×10^5 cells was necessary to demonstrate the classical ladder by direct visualization after ethidium bromide staining (data not shown). As with Jurkat cells, densitometric quantitation varied linearly with increasing cell number (Figure 2B).

Because of its sensitivity, this application of the Southern blot technique allows visualization of DNA fragmentation process in a small number of apoptotic cells, where detection attempted by the standard ethidium bromide staining technique would be illusory. The random-primed DIG-labeled probe derived from total DNA can be easily obtained in large quantity from any cell preparation. This probe is stable well over a period of one year. The chemiluminescent detection can be performed in one hour. The densitometric analysis of well-individualized DNA bands provides an easy-to-perform quantitative estimate of the intensity of the apoptotic process.

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Single-Tube Chemical Cleavage of Mismatch: Successive Treatment with Hydroxylamine and Osmium Tetroxide

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Chemical cleavage of mismatch (CCM) detects and localizes mutations in heteroduplex DNA (or RNA) molecules due to the reactivity of hydroxylamine with mismatched C bases and osmium tetroxide with mismatched T bases. Piperidine cleaves the DNA at the modified bases. Fragments are ana-

lyzed by electrophoresis to determine the position of the mutation (3). The advantages of CCM compared with other mutation detection methods are that (i) it can detect almost 100% of the mismatches in pieces of DNA up to 1.7-kb-long, (ii) the position of the mutation can be determined and (iii) the nature of the base change is identified by reaction with one or both chemicals (1). The disadvantages of CCM are the use of toxic chemicals and the number of manipulations in the method.

Normally osmium tetroxide (OsO_4) and hydroxylamine reactions are performed in separate tubes. We have examined the possibility of combining the two reactions, osmium tetroxide (detecting mismatched T bases) and hydroxylamine (detecting mismatched C bases), to decrease the number of steps. Halving the number of tubes would make the test cheaper and simpler to automate.

Probes were end-labeled with ^{32}P , and known mutations were selected for the test system. Heteroduplex molecules containing a C/T mismatch were chosen as cleavage at the C mismatch on one strand, and the T mismatch on the other strand would result in fragments of different sizes. Therefore, the effects on mismatched C's and T's could be examined simultaneously. Two C/T mismatches were tested to ensure that the results were consistent in different sequence contexts. Furthermore, an example of a C/C mismatch and a T/T mismatch were also studied to ensure that the results were consistent when the osmium tetroxide and hydroxylamine were not reacting at the same position on the DNA strand.

The two chemicals were tested (i) individually, as is usual with the method, (ii) in sequence and (iii) together in the same tube. Standard reactions are as described previously (3). Variations to the reaction steps were as follows: For the experiment with the chemicals in sequence, the DNA was precipitated, washed with 70% ethanol and resuspended in 6 μL of dH_2O before addition of the second chemical.

For the experiment with the chemicals together, the total volume was 40 μL . The hydroxylamine reactions are normally incubated for 20 and 60 min and the osmium reactions for 1 and 5

Benchmarks

Table 1. Reactivity of Various Mismatches with Different Combinations of Chemicals

Chemicals Mismatch	C/T PAH		C/T β-Globin		C/C β-Globin	T/T β-Globin
	Detection with H	Detection with OT	Detection with H	Detection with OT	Detection with H	Detection with OT
OT then H	Yes	No	Yes	Yes	Yes	Yes
H then OT	Yes	Yes	Yes	Yes	Yes	Yes
OT and H together	Yes	No	Yes	No	Yes	No

H is hydroxylamine. OT is osmium tetroxide.

min. The 4% osmium tetroxide solution was diluted so that the reaction times could be increased to match the times of the hydroxylamine reactions. Two different reaction conditions were tested. The first was a 60-min reaction with 20 μL hydroxylamine solution, 15 μL OsO₄ (diluted 1:6) and 5 μL OsO₄ buffer (3). The second was a 120-min reaction with 20 μL hydroxylamine solution, 15 μL OsO₄ (diluted 1:12) and 5

μL OsO₄ buffer. The increased reaction times gave the new conditions a maximum chance to cleave at the mismatch. Appropriate controls were included for the dilution of the osmium tetroxide and the increased reaction times used. Controls, which included dH₂O instead of one of the two chemicals, were included to ensure that the increased volume did not alter the reactivity. Results for the four experiments are given in

Table 1. An example of one of the C/T mismatches is given in Figure 1.

When osmium tetroxide and hydroxylamine were in the same tube, the hydroxylamine modification was consistently detected, but the osmium tetroxide modification was not. When water replaced the hydroxylamine solution, the osmium tetroxide reaction was successful. The T reactivity of OsO₄

Figure 1. Position A indicates cleavage bands from hydroxylamine reaction, and position B indicates cleavage bands from osmium tetroxide reaction. Lanes 1–6 are controls for detection of the mutation by CCM using the standard method at 0, 1 and 5 min with OsO₄ and 0, 20 and 60 min with hydroxylamine, respectively. Lanes 7 and 8 are the OsO₄ then hydroxylamine reactions (1 min and 20 min) and (5 min and 60 min), respectively. Lanes 9 and 10 are the hydroxylamine then OsO₄ reactions (20 min and 1 min) and (60 min and 5 min), respectively. Lanes 11 and 12 are the two chemicals together in the same tube for 60 min and 120 min, respectively. Lanes 13 and 14 are diluted OsO₄, 60 min and 120 min, respectively, and lanes 15 and 16 are diluted hydroxylamine, 60 min and 120 min, respectively.

has been shown to be inhibited by CaCl_2 and KCl (4). The preparation of hydroxylamine used in CCM is hydroxylammonium hydrochloride and therefore chloride ions may be inhibiting the reaction. It may be theoretically possible to adapt the CCM method, by using another form of hydroxylamine such as hydroxylamine hydroxide, so that the OsO_4 and hydroxylamine reactions may be performed simultaneously, but this has not been investigated.

It seemed possible that the reactions could be successively performed in the same tube with a precipitation step between chemical modifications. This was tested using OsO_4 as the first chemical. The reactivity with the T mismatches varied between experiments from being not detectable to being as strong as the controls. When the osmium tetroxide was the second step, the reaction was consistently as strong as the osmium controls alone. The order of the chemicals did not affect the hydroxylamine reaction. Therefore, we suggest that the most suitable reaction condition for reliably detecting both C and T mismatches are 60 min with hy-

droxylamine followed by 5 min with OsO_4 . The single-tube chemical cleavage of the mismatch method is given in Figure 2. This protocol is based on a recent protocol of the traditional CCM method (5). Solutions are given in other protocols (3,5).

The use of hydroxylamine followed by OsO_4 in the same tube introduces one extra precipitation step but effectively halves the number of piperidine reactions and subsequent precipitations. As electrophoresis is required to detect mismatch bands, this change also halves the number of lanes required on an acrylamide gel. The reduction in the number of tubes and manipulations means that a single operator can screen more DNA fragments in a single day. This improvement also makes the CCM method more suitable for automation. The one minor drawback to this change is that the nature of the mismatched base is not identified. When cleavage is performed separately, a band in the hydroxylamine lane indicates a mismatched C and a band in the osmium lane a mismatched T. However, detected mismatch bands are normally sequenced to determine the actual base change. The use of the single-tube chemical cleavage of mismatch eliminates the problem of T/G mismatches, which are unreactive with osmium tetroxide (2). The C/A mismatch on the complementary heteroduplex will give a hydroxylamine reaction in the same position on the gel as the expected osmium tetroxide reaction.

Figure 2. The single-tube chemical cleavage of mismatch method. Boxes indicated reactions, and arrows show precipitation steps. Solutions are as in the traditional CCM methods (3,5).

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Optimized Filter Set and Viewing Conditions for the S65T Mutant of GFP in Living Cells

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Investigators are increasingly taking advantage of the autonomous fluorescence from the green fluorescent protein (GFP) to detect the cellular and subcellular location of GFP-fusion proteins within living cells (3,8-10,13,14). Work with these fusion constructs has shed new light on the kinetics underlying fundamental biological processes such as mitosis (9). In an attempt to study how a novel rat brain kinesin-related motor protein (rbKRP1) functions within living cells, we have transfected BHK and PC12 cells with a cDNA encoding the serine 65 → threonine 65 (S65T) GFP mutant (6) fused to the amino terminus of rbKRP1 (S65T GFP-rbKRP1). We use the S65T GFP mutant since it emits four times as

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