DNA damage and repair is important in both mutagenesis and cancer therapy. Study of repair mechanisms is therefore important, and quick, reliable and sensitive assays are required. At the level of individual genes, Southern blotting-based methods have proved extremely valuable (1). Recently, PCR based techniques have been developed which are equally sensitive (2,3), more rapid and use less DNA compared with Southern blotting, and allow mapping of damage and repair at the sub-gene level (~500 bp) (4). PCR methods cannot, however, be used to measure strand-specific damage and repair. Due to the importance of transcription coupled repair this is a serious drawback.

We describe a new method, strand-specific-QPCR (ss-QPCR), which allows the strand-specific measurement of DNA damage and repair in mammalian cells. The method is outlined in Figure 1 (as set up to measure lesions on the transcribed strand). DNA extracted from drug treated and untreated cells is subjected to a first round ‘linear’ PCR using a single biotinylated primer (1-tB), complementary to the transcribed strand. This PCR generates a family of single-stranded molecules some of which will be truncated due to the presence of a blocking lesion on the transcribed strand of the template DNA. All are captured on streptavidin coated paramagnetic beads and washed with NaOH to remove genomic DNA including any hybridised to the PCR products. After neutralisation, the single-stranded molecules, while still attached to the beads, serve as templates in a second, exponential, PCR. In the exponential amplification the downstream primer (primer 2) is complementary to the transcribed strand and is nested with respect to primer 1. The upstream primer (primer 3) is complementary to the non-transcribed strand and its binding site determines the length of the gene region in which damage is to be measured. In this PCR only those DNA molecules which were extended past the site of primer 3, i.e. those which were not blocked by lesions on the genomic DNA, will be exponentially amplified. Thus, provided the PCR remains in the exponential phase when stopped, the amount of product (quantified by TCA precipitation and scintillation counting) will be directly proportional to the amount of undamaged template present in the region under study of the original genomic DNA.

Experiments were performed using ss-QPCR to measure damage and repair in a 350 bp region, comprising intron 1 of the human N-ras gene, after treatment of cells with the anti-cancer drug cisplatin. The primers used (Genosys, UK) were:

1-tB (5′-Biotinylated): 5′ CAG CAA GAA CCT GTT GGA AAC CAG
1-ntB (5′-Biotinylated): 5′ GGT CCT TCC ATT TGG TGC CTA CG

Initial experiments determined the PCR conditions required to ensure that a single specific product of the correct size was produced. Treatment of K562 cells and extraction of DNA was exactly as described previously (4). DNA from 100 000 cells was used per reaction in the first round linear PCR. This was carried out in a volume of 50 µl of the following composition: 0.6 pmol biotinylated primer 1-tB, 1 U Taq polymerase, 120 µM each dATP, dCTP, dGTP and dTTP, 2.5 mM MgCl2, 20 mM (NH4)2SO4, 75 mM Tris–HCl pH 9.0, 0.01% (w/v) Tween 20. PCR was performed using an MJ Research PTC-100 ‘Hot Bonnet’ cycler without oil overlay. Conditions were the same for either strand and were as follows: an initial denaturation step of 2 min at 94°C and then 20 cycles of 94°C for 1 min, 60°C for 1 min and 72°C for 1 min. After PCR the products were captured by adding the PCR mix to 1.5 ml microfuge tubes containing 5 µl washed streptavidin coated paramagnetic beads (Dynal UK) and incubating at room temperature for 30 min with occasional agitation. The beads were then washed three times with 200 µl freshly prepared 0.4 M NaOH and then once with 200 µl TE (10 mM Tris–HCl pH 7.6, EDTA). The beads were then resuspended in 40 µl H2O and transferred to 0.5 ml PCR tubes. Other components of the PCR were added and the final composition (in a volume of 100 µl) was as for the first PCR except: 50 pmol each primers 2 and 3, 2 U Taq polymerase, 2 µCi dATP. Cycling conditions were the same for both strands and were: an initial denaturation step of 2 min at 94°C and then 26 cycles of 94°C for 1 min, 60°C for 1 min and 72°C for 1 min with a final incubation of 4 min at 72°C at the end of cycling. The PCR product was then quantified by scintillation counting after trichloroacetic acid
precipitation as previously described (4). The overall conditions (quantity of genomic DNA in the first “linear” PCR and cycle number in both reactions) had been previously determined empirically to ensure that the second PCR was in the exponential phase when stopped. A control sample containing one-third and two-third amounts of (undamaged) genomic DNA was routinely used to confirm this. It is also important to control the efficiency of the washing of the beads after the first round PCR since any genomic DNA carried over would serve as a template in the second PCR and be exponentially amplified leading to false results. Therefore in the first round PCR samples were included which contained all components except Taq polymerase. In the second PCR they were treated as for the test samples, i.e. with Taq polymerase. Values above background with these samples would indicate genomic DNA carry over and invalidate the assay.

The results of measurement of DNA damage are shown in Figure 2. Agarose gel electrophoresis and autoradiography show that the product of the reaction is a single band of the expected size (Fig. 2A). In cisplatin treated cells the extent of the damage on the transcribed strand was consistently less than on the non-transcribed strand (Fig. 2B). The difference, 10–15%, was maintained over a wide range of drug concentrations and was similar in both cell lines.
Table 1. K562 or U937 cells were treated for 2 h with cisplatin (350 and 300 µM respectively) and allowed to repair for 24 h

<table>
<thead>
<tr>
<th></th>
<th>K562 (n = 3)</th>
<th>U937 (n = 3)</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>NTS</td>
<td>TS</td>
</tr>
<tr>
<td>0 h repair (L/S)*</td>
<td>0.87 (±0.020)</td>
<td>0.80 (±0.018)</td>
</tr>
<tr>
<td>24 h repair (L/S)</td>
<td>0.13 (±0.004)</td>
<td>0.13 (±0.007)</td>
</tr>
<tr>
<td>% lesions repaired</td>
<td>85.0</td>
<td>83.8</td>
</tr>
</tbody>
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(L/S) = lesions per strand; NTS = non-transcribed strand; TS = transcribed strand.

*Lesions per strand were calculated according to the Poisson equation:

\[ \text{L/S} = -\ln \left( \frac{\text{Ad}}{\text{A}} \right) \]

where A is the amount of PCR product from undamaged template and Ad that from damaged template.

In repair studies initial damage was again greater on the non-transcribed strand but was outside the 10–15% difference seen in damage studies (Table 1). This is probably due to the kinetics of binding of cisplatin since the repair studies included an exposure to drug of only 2 h rather than 16 h. The results show that both cell lines were capable of repairing damage but there was no difference in the extent of repair of either strand in this gene segment although the extent of repair was greater in the K562 cells.

Ss-QPCR showed damage to be consistently greater on the non-transcribed strand of the region of the \( N-ras \) gene under study. This agrees with previous work using single-strand ligation PCR, a method that allows DNA damage to be mapped at individual bases in single copy genes in cells (5), and confirms the strand specificity of the assay.

No preferential repair of the transcribed strand was seen in the region under study. It may be that the transformed cells used in these experiments are not capable of transcription coupled repair. Another possibility is that transcription coupled repair is heterogeneous within an active gene. Transcription coupled repair has been mainly studied at the level of the whole gene and its rate may not be uniform over the whole sequence, which would not be detected with Southern blotting. It is known that the repair of individual nucleotides occurs at different rates (6,7), ss-QPCR will be a useful technique to investigate this apparent heterogeneity of repair within a gene.

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REFERENCES