Direct in situ nucleic acid amplification: control of artefact and use of labelled primers

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Abstract

Aims—To evaluate factors which ameliorate false positive artefacts with direct in situ PCR using labelled dNTPs; to investigate the use of labelled primers to overcome this artefact whilst maintaining sensitivity.

Methods—Sections of measles (RNA virus) infected Vero cells with cytoplasmic signal or cytomegalovirus (DNA virus) infected fibroblasts with nuclear signal were collected. In situ PCR (or in situ RT-PCR) was carried out by methods permitting evaporation. Reagents or conditions which may control false positive artefacts using labelled dNTPs were investigated systematically. Labelled primers were tested to overcome artefacts, with adjuncts which improve sensitivity.

Results—No reagent nor condition investigated was able to control the artefact with labelled dNTPs. Excessive digestion and incomplete DNase treatments exacerbated the artefact, whereas novobiocin decreased both specific signal and artefact. However, the artefact was controlled by labelled primers, albeit with relatively low sensitivity. Sensitivity using labelled primers could be increased using alcohol fixation, albumin or Perfectmatch.

Conclusions—A repair process is implicated for the artefact using labelled dNTPs. Excessive digestion or DNase treatment may exacerbate DNA damage by disrupting histones or the DNA, respectively. Labelled primers control this artefact, albeit with reduced sensitivity, which may be improved by precipitation fixatives (alcohol) and reagents which enhance specific reaction.

(J Clin Pathol: Mol Pathol 1996;49:M345-M350)

Keywords: in situ nucleic acid amplification, artefacts, labelled primers.

In situ PCR was first described by Haase *et al* in 1990.¹ During in situ PCR labelled dNTPs are incorporated directly into amplicons. Alternatively, labelled primers may be incorporated into amplicons, although sensitivity may be poor as a result of the presence of one label at the 5' end of each primer. Direct in situ PCR obviates the need for detection of amplicons by in situ hybridisation (ISH) (indirect in situ PCR), thereby simplifying the procedure and avoiding artefacts of in situ hybridisation.

However, we² and others^{3,4} have observed a false positive nuclear artefact with direct in situ

PCR using labelled dNTPs when primers are omitted, the said artefact occurs unpredictably and may be due to DNA repair or internal priming, or both. Before direct in situ PCR can be used for research or routine diagnosis, the technique requires considerable modification in order to eliminate or control this artefact. The factors which ameliorate (or precipitate) the artefact have not been adequately studied. In addition, methods for preventing the occurrence of artefacts using labelled primers, and for increasing sensitivity of labelled primers, are poorly understood. The aim of the present study was to experiment using in situ PCR technologies which permit evaporation in order to reduce the effects of bubble artefact in systems where evaporation is prevented.

Methods

Cytomegalovirus (CMV) was chosen as a DNA target with a mainly nuclear signal (in situ PCR), and measles virus was chosen as a RNA target with a predominantly cytoplasmic signal for in situ reverse transcription (RT) PCR. Results were compared with ISH and immunohistochemistry.

OLIGONUCLEOTIDE PRIMERS

Previously described oligonucleotides were used as primers for PCR reactions (table 1).

CELL AND TISSUE PREPARATIONS

Vero cells (green monkey kidney) were cultured in 75 cm² flasks either alone (uninfected), or in the presence of Edmonston strain measles virus (10⁷ plaque forming units). MRC-5 fibroblasts were cultured likewise, in 75 cm² flasks either alone or in the presence of CMV (Townes strain) as described previously⁷; 5–10⁵ cells, measured using a haemocytometer (Marathon Laboratories, UK) after scraping into suspension, were centrifuged in 1.5 ml Eppendorf tubes at 200 × *g* for one to three minutes, and the supernatant discarded. Cells were resuspended in 200 µl phosphate buffered saline (PBS) and centrifuged at 200 × *g* for one to three minutes to a pellet, transferred to a 0.5 ml Eppendorf tube, and gently resuspended in either 10% buffered formalin or in 95% alcohol fixative for five minutes. Fixed, uninfected, infected or mixed samples containing both infected and uninfected cells were resuspended in PBS, centrifuged at 200 × *g* for three minutes into a cell block and embedded in paraffin wax.

SOLUTION PHASE PCR (OR RT-PCR)

Total RNA was extracted from measles virus infected and uninfected Vero cell cultures by the guanidinium isothiocyanate method and

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Accepted for publication
3 September 1996

Table 1 Oligonucleotide primers

Virus	Sequence (5'-3')	Comments
Measles (Taylor <i>et al</i> ⁶)	(MV1) AGCATCTGAACTCGGTATCAC (MV2) AGCTCTCGCATCACTTGCTCT	Amplified product 252 bp; MV1 was labelled or unlabelled at the 5' end
	TTAGGGCAAGAGATGGTAAGG GTTCTTCCGAGATTCTGCCA	Outer flanking primers used for first round of nested PCR
CMV (Cranage <i>et al</i> ⁶)	(CMV1) ACCACCGCACTGAGGAATGTCAG (CMV2) TCAATCATGCGTTTGAAGAGGTA	Amplified product 100 bp; CMV2 was labelled (or unlabelled) at the 5' end with digoxigenin

Table 2 Effect of different reaction conditions and reagents in producing false nuclear signal with formalin fixed Vero cells. Alterations in signal with specific and irrelevant primers were similar

Reagent	Conditions	Results
Proteinase K predigestion for 15 minutes	None, 0.1, 1.0, 10, 100, 1000 µg/ml	Progressive increase in nuclear signal with digestion until 10 µg/ml. The 1000 µg/ml digestion resulted in reduced signal and poor morphology
Novobiocin (0.06 g/100 µl) added to in situ PCR reaction mixture	0, 0.1, 1.0, 10 µl	Progressive reduction of signal with increasing volumes
Digoxigenin-11-dUTP (1 mM)	0.02, 0.2, 2.0 µl	Increased signal with raised volumes
Unlabelled dNTPs (10 mM)	0.01, 0.1, 1.0, 10 µl	No signal at 0.01 µl, then progressive increase in signal
Standard reaction mixture with specific primers on normal brain	10, 20, 30 PCR cycles	Intense signal throughout
Detergent W1 with standard protease digestion	1 µl	Decreased specific and irregular signal with W1 detergent
Random hexamers (50 mM)	0.05, 0.5, 5.0 µl	Increased signal with raised volume
Denhardt's solution	10 µl	Very impressive artefact signal

recovered by ethanol precipitation. Total RNA was reverse transcribed using methods adapted from Sambrook *et al*.⁸ DNA was extracted from CMV infected and uninfected MRC-5 fibroblast cells using standard techniques.⁹ Viral cDNA or DNA was amplified by nested PCR for 30 cycles using either digoxigenin labelled or unlabelled primers as presented in table 1. In experiments using unlabelled primers, digoxigenin-11-dUTP was included in the reaction mixture. Cycling conditions and reac-

Table 3 Controls for reactions with labelled primers

Category	Test	Results
General	Infected, uninfected, mixed infected/uninfected cells	Infected cells gave a positive signal (mainly cytoplasmic for measles virus; mainly nuclear for CMV); uninfected cells gave no signal
Intracellular reverse transcription (in situ RT) (measles virus)	RNAse predigestion	Reduced signal for measles virus
	No enzyme (reverse transcriptase) No random hexamers No downstream primers	No reaction for measles virus
Intracellular PCR (in situ PCR)	DNAse predigestion	No convincing reaction for measles virus
		Reduced signal
	No enzyme (Taq polymerase)	No reaction
	No primers/irrelevant primers	No reaction
	Back diffusion effect (cycling with preformed labelled amplified product)	Rim pattern around cells

tion mixtures were identical with those used for in situ PCR (see later).

SIGNAL DISTRIBUTION BY ISH AND IMMUNOCYTOCHEMISTRY

In order to examine and compare signal distribution of viral sequences using in situ RT-PCR, ISH was carried out on serial sections of measles virus or CMV infected cell samples and uninfected controls. The measles virus specific riboprobe¹⁰ or the CMV specific DNA probe (Cambridge Bioscience, Cambridge, UK), was used as described previously.⁷ Omission of the probe on serial sections was used as a negative control.

SIGNAL DISTRIBUTION BY IMMUNOHISTOCHEMISTRY

Immunohistochemistry for measles virus was carried out using a monoclonal antibody directed against the measles virus nucleocapsid protein (Serolab, Crawley, UK). The procedure used has been described elsewhere.¹¹ For CMV, immunohistochemistry was done according to the manufacturer's specific recommendations (Cambridge Bioscience). Omissions of primary antibody on infected cells and immunohistochemistry on uninfected samples were used as negative controls. Horseradish peroxidase was used as a label, and hydrogen peroxide and diaminobenzidine in the colouration step.

PRETREATMENTS FOR FORMALIN FIXED, PARAFFIN WAX SECTIONS FOR IN SITU NUCLEIC ACID REACTIONS

Sections were dewaxed in xylene (10 minutes) and rehydrated through a graded alcohol series to distilled water. The tissues were rinsed in PBS and digested in 10 µg/ml proteinase K (Sigma, Poole, Dorset, UK) in PBS/EDTA for intervals of 0-35 minutes at 37°C. Digestion was stopped by rinsing sections with 0.2% glycine/PBS for five minutes. Sections were then washed in PBS and air dried. Optimal proteinase K pretreatment conditions were as described previously.⁷ Digestion was not necessary for alcohol fixed samples.²

IN SITU REVERSE TRANSCRIPTION ON SLIDES

Measles virus infected or uninfected, or both, Vero cells were surrounded by a layer of gum (Cowgum, Cow Proofings, Slough, UK), creating a well. The gum was then allowed to set for 15 minutes until it became firm, when a thinner second layer was applied. The reverse transcription mixture was added and the well covered with a heat resistant, heat conducting plastic coverslip (Techne Hi Temp Microlids, Techne Ltd, Cambridge, UK). The slides were then placed on the flat heating block of a thermal cycler (Genee, Techne Ltd, UK). The temperature parameters (identical with those used for extracted RNA) were: 42°C for two hours, 95°C for five minutes, and 8°C for five minutes. Following reverse transcription, the coverslips and gum were removed, the slides washed in PBS, dried in air, and stored at -20°C or used directly for in situ PCR.

The in situ reverse transcription reaction mixture was comprised of the following per sample: 10 µl 25 mM MgCl₂; 10 µl 5× reverse transcription buffer (Boehringer Mannheim, Mannheim, Germany); 5 µl of each dNTP (10 mM); 5 µl distilled, deionised water; 5 µl (50 units/µl) reverse transcriptase (MMLV); 5 µl (20 units/µl) RNase inhibitor; and 5 µl (50 mM) random hexamers. Controls for in situ reverse transcription included RNase predigestion, omission of reverse transcriptase or random hexamers.

IN SITU PCR ON SLIDES

A gum well was prepared as described earlier, large enough to envelop the paraffin wax section. The PCR reaction mixture for either CMV DNA or measles cDNA contained the following: 20 µl MgCl₂ (25 mM); 40 µl 10× PCR buffer (500 mM KCl, 100 mM Tris-HCl, pH 8.3); 10 µl of each dNTP (10 mM); 4 µl (15 µM) of each primer pair (labelled or unlabelled); 328 µl distilled, deionised water; and 1.5 µl (5 units/µl) DNA polymerase. In experiments where labelled primers were used for direct in situ PCR, 4 µl (15 µM) of each digoxigenin labelled specific primer pair was used for each viral target; irrelevant labelled primers were used as controls in each experiment and consisted of measles virus primers for CMV and vice versa. For reactions involving incorporation of labelled dNTP into amplification products, 10 µl dTTP was replaced by 5 µl dTTP and 2 µl/mM digoxigenin-11-dUTP and unlabelled primers were used for the reaction.

Cycling PCR conditions were: 94°C for 10 minutes, 94°C for one minute, 58°C for two minutes, and 74°C for 90 seconds for 30 cycles; and 74°C for 10 minutes. In some experiments the reagents were added to the sample at the annealing temperature (hot start). In other experiments 1 µl 50% (w/v) dextran sulphate, 1 µl 10 mM novobiocin and serial dilutions thereof, 1 µl 50% (v/v) formamide (Stratagene), 1 µl 100% (v/v) dimethylsulphoxide (DMSO) (Stratagene), 1 µl bovine serum albumin (Stratagene), 1 µl 100% (v/v) glycerol, or 1 µl Perfectmatch (Stratagene) was added to the reaction mixture.

Controls for in situ PCR included DNase predigestion, omission of Taq polymerase or specific primers, and inclusion of irrelevant primers (as described earlier).

IMMUNODETECTION OF DIGOXIGENIN LABELLED AMPLICONS AND HYBRIDS

Sections were quenched in Tris buffered saline (TBS) containing 10% normal rabbit serum (Dako, High Wycombe, UK) and 3% bovine serum albumin. Monoclonal anti-digoxin (Sigma code D-8156) diluted 1 in 10 000 in TBS was applied for one hour at room temperature. Following three rinses in TBS, rabbit anti-mouse IgG (Dako), diluted 1 in 25 in normal human serum, in TBS was applied for 20 minutes. Slides were then rinsed again in TBS and alkaline phosphatase anti-alkaline phosphatase (APAAP; Dako), diluted 1 in 50 in TBS, was applied for 30 minutes. The

detection sensitivity was enhanced further by repeating the rabbit anti-mouse APAAP layers before rinsing in TBS. Alkaline phosphatase was developed using Vector Red alkaline phosphatase substrate kit (SK 5100, Vector Laboratories, Peterborough, UK). The colour reaction was monitored microscopically before rinsing in TBS, tap water and counterstaining in Carazzi's haematoxylin, dehydrating through graded alcohols and xylene, and mounting in DPX (BDH, UK).

Results were compared in blinded studies by two experienced histopathologists.

Amplified products were sequenced as described previously.²

Reagents that might ameliorate false positive signal by using unlabelled primers and digoxigenin-11-dUTP were investigated (table 2). Reactions and controls using labelled primers were analysed (table 3).

Results

DIRECT IN SITU RT-PCR FOR MEASLES VIRUS WITH dUTP INCORPORATION

The primers used to amplify extracted target sequences produced products of expected band sizes. Similar product bands were found when amplicons were extracted from infected tissue sections after in situ RT-PCR (results not shown). The specificity of amplified products was confirmed by sequencing studies. In measles virus infected Vero cells an almost exclusively cytoplasmic signal distribution was found for measles specific immunocytochemistry and ISH. With regard to CMV, a nuclear and focal deposits of cytoplasmic signal were found by immunocytochemistry and ISH.

No signal was found in uninfected cells, or where the relevant probe or antibody was omitted (results not shown).

As measles virus infected Vero cells produced a cytoplasmic signal by immunocytochemistry or ISH, conditions that resulted in a false positive nuclear signal (DNA) with omission of primers were investigated (table 2). Measles virus infected Vero cells produced a cytoplasmic signal with in situ RT-PCR using labelled primers of similar distribution to ISH or immunocytochemistry, when no false positive signal was found in controls (table 3). However, in over three quarters of our experiments, reactions lacking primers (or with irrelevant primers) on measles virus infected Vero cell sections (fig 1A), or reactions with specific primers on uninfected Vero cell sections (fig 1B), gave a mainly nuclear signal. This false signal occurred unpredictably. When the false signal was present in controls, a nuclear signal was similarly present within infected Vero cells when specific primers were used and the expected cytoplasmic signal was decreased in intensity. By contrast, the use of specific labelled primers showed the expected cytoplasmic signal (fig 1C) and no false signal was found when irrelevant labelled primers were used (fig 1D). Indeed, a false signal was found after only 10 PCR cycles, but was not present after 30 cycles when irrelevant labelled primers were used. When labelled dNTPs were used for

Figure 1 *In situ* PCR with measles virus specific primers. Comparison of labelled dNTPs and labelled primers. (A) Measles virus infected Vero cells using labelled dNTPs and measles virus specific primers. Predominantly false positive nuclear signal and some cytoplasmic signal. (Original magnification $\times 400$.) (B) Measles virus uninfected Vero cells using labelled dNTPs and measles virus specific primers. False positive nuclear signal. (Original magnification $\times 400$.) (C) Measles virus infected Vero cells using measles virus specific labelled primers. Cytoplasmic signal. (Original magnification $\times 400$.) (D) Measles virus infected Vero cells using irrelevant CMV specific primers. No signal. (Original magnification $\times 400$.)

the reaction, nested *in situ* PCR, substitution of Stoffel fragment for Taq polymerase, or use of the hot start method did not prevent the artefact occurring. Finally, the artefact was seen after a single cycle with a two hour extension, using either Taq polymerase or Klenow fragment (data not shown).

Increasing the concentration of proteinase K (100 $\mu\text{g/ml}$) or the digestion times (>20 minutes) increased the intensity of the false nuclear signal and tended to compromise morphology when labelled dNTPs were used. Intensity of signal in areas of artefact cellular damage similarly increased when digestion concentrations rose (data not shown). Decreasing proteinase K concentrations (<10 $\mu\text{g/ml}$) reduced or eliminated all cellular signal. Occasionally, light diffuse precipitation of signal (both intracellular and extracellular) was seen in the absence of added enzyme; this tended to be mainly at the cell periphery or with high proteinase K concentrations (10 000 $\mu\text{g/ml}$) when morphology was severely compromised.

Increasing novobiocin or decreasing digoxigenin-11-dUTP concentrations (in those experiments where labelled dNTPs were used) decreased or eliminated all cellular signal—both specific and false positive nuclear signal to the same degree. Addition of albumin to the reaction mixture improved signal intensity, but did not prevent detection of false positive nuclear artefact. Omission of random hexamers at the *in situ* reverse transcription step, or

addition of Denhardt's solution similarly had no effect on the artefact.

DIRECT IN SITU RT-PCR (FOR MEASLES VIRUS) OR IN SITU PCR (FOR CMV) WITH LABELLED PRIMERS
The use of 5'-digoxigenin labelled measles virus primers resulted in a cytoplasmic signal within infected Vero cells, of identical distribution to findings with immunocytochemistry and ISH, but no signal was present when irrelevant labelled CMV primers were used (fig 1D). For CMV infected MRC-5 fibroblasts, a predominantly nuclear signal was found with labelled CMV specific primers (fig 2A), but not with irrelevant measles specific labelled primers (fig 2B). When labelled primers were used, the signal was weaker than in experiments using digoxigenin-11-dUTP (in experiments where no artefact was present). Signal intensity was increased with alcohol fixation, addition of albumin or Perfectmatch (Stratagene) or glycerol in the amounts indicated earlier. Proteinase K gave best results at 10 $\mu\text{g/ml}$ for 15 minutes. Surprisingly, dextran sulphate inhibited the reaction. Addition of DMSO or formamide gave inconsistent results. The hot start method had little or no effect on reaction sensitivity or specificity in our hands.

Discussion

In situ PCR, with or without prior intracellular reverse transcription, offers great potential in the study of infectious disease, oncology and

Figure 2 In situ PCR using labelled primers on CMV infected MRC-5 fibroblasts using: (A) Specific (CMV) primers. Predominantly specific nuclear signal. (Original magnification $\times 250$.) (B) Irrelevant (measles virus) primers. No signal. (Original magnification $\times 250$.)

genetics. Amplicons within cells on slides may be detected using ISH (indirect in situ PCR) or using label incorporated directly into the amplicons (direct in situ PCR). Labelling with direct in situ PCR may be through reporter groups attached directly to a dNTP or to primers.

Direct in situ PCR on slides may suffer from artefacts, including misannealing of a primer to a non-specific template sequence (mispriming), misincorporation of dNTPs into the amplicon during specific primer mediated reaction and repair processes of damaged DNA. The first two respective artefacts are found in solution phase PCR and broadly represent the accuracy of specific replication (or fidelity). Repair, however, presents a problem particularly relevant to in situ PCR on slides, the significance of which is controversial.

The presence of artefacts on slides incubated with digoxigenin-11-dUTP, but lacking primers, after a single PCR cycle, with a two hour extension, is strongly suggestive of a repair mediated mechanism. Predisposing factors to damage repair on slides include loss of the three dimensional DNA configuration,¹² the predigestion steps required for permeabilisation, which disrupt nuclear histones (thereby

predisposing to damage), alteration of reaction components during thermal cycling, heat transfer kinetics, and, presumably, the quality of the templates—for example, the presence of necrosis or heterochromatin in tumours (Helen Fidler, personal communication).

The mechanisms of damage include, single or double strand DNA breaks, damaged bases, and DNA coagulated protein cross links or bulky lesions which may make oligomers, which can act as internal primers for non-specific reactions.^{13 14} The predominant (nuclear) artefact in the present study occurred with labelled dNTP reaction solution lacking primers. Some factors exacerbated the intensity and frequency of the artefact. With measles virus infected Vero cells the artefact was present after as few as 10 cycles of in situ PCR, indicating the relative efficiency of the process, and in no instance did the reaction proceed without producing the artefact. This artefact was clearly exacerbated by DNase pretreatment (even after 24 hours' incubation), suggesting that difficulty of nuclease penetration across membranes or poor nuclease action on DNA stuck to the slide causes incomplete DNA digestion and consequent DNA breaks or fragmentation. Similarly, increased concentrations of proteinase K or longer digestion times may destroy DNA histones, increasing susceptibility to heat mediated damage. DNA fragments may then be repaired by Taq polymerase, and labelled dNTP incorporated into the repair site. Alternatively, DNA fragments in vast excess may prime false reactions. Conversely, when the proteinase K concentration was lowered, the intensity of the artefact was reduced and specific signal was more obvious. DNA damage was also present in areas of necrosis or cell damage, where the artefact was obvious. A similar phenomenon may occur in the presence of abundant amounts of heterochromatin. Finally, strong protease digestion compromises morphology and increases product leakage.

Novobiocin,¹⁵ which inhibits repair at low temperatures, inhibited specific and artefactual false positive nuclear signals at high concentrations, confirming the similarity of the reaction mechanisms between specific signal and false positive nuclear signal formation. No reagent added to the reaction mixture prevented the false positive nuclear artefact with labelled dNTP. Use of the Stoffel fragment, which, unlike Taq polymerase, has no 5' to 3' exonuclease activity, did not prevent the artefact. Artefactual signal was present after a single PCR cycle with a two hour extension phase, indicating that misprimed amplification by itself is unlikely to be the mechanism generating the artefact, and that a repair process is occurring. This artefact was present with both Taq polymerase and Klenow fragment (which lacks endonuclease or 5' exonuclease activity). Enzyme nuclease activity is thus unlikely to be a major mechanism producing this false positive nuclear artefact, although the polymerase enzyme itself is involved in producing it.

No false positive artefactual nuclear signal was observed when digoxigenin-11-dUTP was replaced by labelled primers, only one of which was 5' end labelled with digoxigenin, and extending PCR to 30 cycles. However, it is unlikely that the mechanisms by which DNA is repaired were absent. The signal produced with labelled primers was relatively weak, presumably as a result of the presence of only one label per primer. The signal intensity was improved by the addition of albumin. This may be for a number of reasons: a reduction in evaporation by increasing reaction solution osmotic pressure; the stabilising effect of albumin on the enzyme; or possibly a more even distribution of reagents on the tissue surface. Although denaturation temperatures are required for PCR, formamide (which reduces denaturation temperatures) had no consistent effect at low concentrations, although high formamide concentrations inhibited PCR. DMSO is essential for enhancing amplification of some sequences (for example, retinoblastoma gene). Dextran improves ISH signals via a 'wetting' action, but inhibited in situ PCR. Glycerol improved signal intensity, perhaps by stabilising the enzyme. Although many authors claim that the hot start method improves specificity, it was of no advantage in our system and did not prevent the artefact occurring. However, Perfectmatch, which stabilises mismatched primer/template sequences, improved signal intensity.

The presence of specific signal and virtual elimination of the false positive nuclear artefact with labelled primers, suggests that aberrant thermal conductivity per se is not the cause of the artefact, as proposed by some workers.¹⁶ Superior signal intensity with alcohol compared with formalin fixation may be because of residual cross-linking by formalin. It is of particular interest that primers, only one of which is 5' end labelled, can be used for in situ PCR, although sensitivity is relatively poor.

It is important to stress that although labelled primers prevent detection of artefact, they may not prevent repair. Studies are underway to examine ways of reducing DNA repair during in situ PCR.

In conclusion, we suggest that labelled primers offer considerable advantages over labelled dNTP for in situ PCR. We deliberately chose high copy cell culture stock to evaluate the effect of damage. More work is necessary to test the use of labelled primers for low copy targets and for tumours with abundant heterochromatin.

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