

## Demystified . . .

## In cell amplification

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The localisation of cellular DNA and RNA sequences in human tissues has greatly facilitated our understanding of disease processes. More than any other technique, in situ hybridisation (ISH) allowed this to be accomplished. However, ISH does not stand alone and must be taken in the context of other developments in cellular and molecular biology that preceded and post-dated its introduction.

Today, the repertoire of in situ hybridisation has been extended to include: DNA in situ hybridisation with double stranded DNA probes, oligo DNA probes and peptide nucleic acid probes (PNAs); RNA in situ hybridisation with riboprobes, oligoprobes and cDNA probes; and the newer techniques of in cell amplification, including DNA polymerase chain reaction ISH (DNA PCR ISH), labelled primer driven DNA in situ amplification (LPD ISA), DNA/PNA PCR ISH, RNA reverse transcription PCR ISH (RT PCR ISH), labelled primer driven in situ RNA amplification (LPD ISRA), and isothermal in situ amplification techniques including in situ isothermal replication (IS 3SR). The marriage of PCR technology and in situ hybridisation now allows single copy mammalian gene detection and extremely sensitive mRNA detection. In this review, we analyse the historical background to in cell amplification, the discovery of non-isotopic ISH (NISH), PCR, and early techniques that were used for DNA and RNA amplification. We will then outline detection sensitivity issues and review the major in cell amplification techniques. Like most new molecular biological systems, there has been an explosion of newly described modifications, many of which we will review. Finally, we will present realistic alternative techniques that give equally sensitive results with in cell PCR amplification and discuss the advantages and disadvantages of each system.

**Historical background**

In 1955, Arthur Kornberg of Stanford University, USA discovered DNA polymerase, an enzyme normally involved in DNA replication and repair and the essential powerhouse tool of the PCR.<sup>1</sup> When the PCR technique was first introduced, the Klenow fragment of DNA polymerase I from *Escherichia coli* was used. Recombinant Taq polymerases are now used extensively, and greatly facilitate the use of the PCR process.

Another important milestone for those working with ISH and in cell amplification

came in 1975, when Edwin Southern described a technique for the localisation of specific sequences within genomic DNA using electrophoretic transfer techniques. Specific sequences were subsequently detected using oligonucleotide probes (short fragments of single stranded DNA), complementary to the DNA sequences of interest; he called this technique Southern hybridisation.<sup>2</sup> Analogous techniques for RNA and protein analyses are called northern and western hybridisation, respectively.

Initially, radioactively labelled probes (<sup>32</sup>P, <sup>35</sup>S, <sup>3</sup>H) were used to detect sequences in Southern analysis, but later non-isotopic labels including biotin, digoxigenin, fluorescein, and dinitrophenol (DNP) were used.<sup>3</sup>

The technique of in situ hybridisation was first published in 1969.<sup>4,5</sup> For the first time, it allowed direct correlation between hybridisation signals and tissue morphology. Specific applications to cryostat, paraffin wax, chromosomal, and electronmicroscopic preparations were soon reported.<sup>6–9</sup>

The PCR appears to have been described initially by the Russian scientist Khorana in the early 1970s<sup>10</sup> but was named PCR in 1983 by Kary Mullis, who subsequently received the Nobel Prize for Chemistry in 1994 for his work on the PCR.

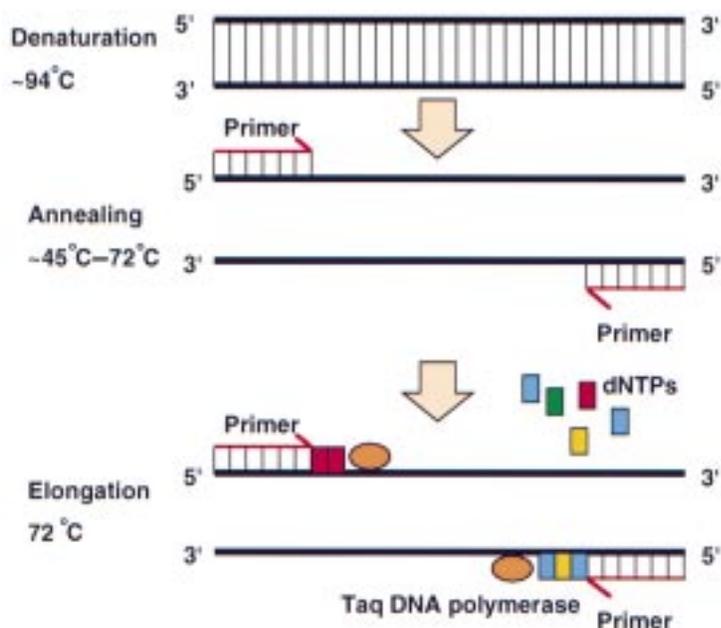
The story of the development of the PCR is an interesting one and is central to our understanding of how solution phase PCR and in cell amplification works. Initially, the PCR method used the Klenow fragment of *E coli* DNA polymerase I, which conveniently was found to amplify short DNA fragments. However, its inability to withstand high temperatures during the PCR reaction limited its effective use as a core enzyme for DNA PCR. Essentially, the PCR consists of three steps: denaturation of the DNA sample at 94°C; annealing of primers (short strands of DNA complementary to the ends of the sequence to be amplified), usually carried out at 45–72°C; and an extension phase, which allows Taq DNA polymerase to add deoxynucleotides to the end of the primers, thus creating a new DNA strand. This process is repeated 20–40 times, depending on the initial starting copy number, the amount of competing DNA sequences, the state of the DNA molecule being amplified, the efficiency of primer design, and the optimisation of the PCR kinetics. Subsequently, with the discovery of thermostable DNA polymerases such as Taq (*Thermus aquaticus*) polymerase, the PCR

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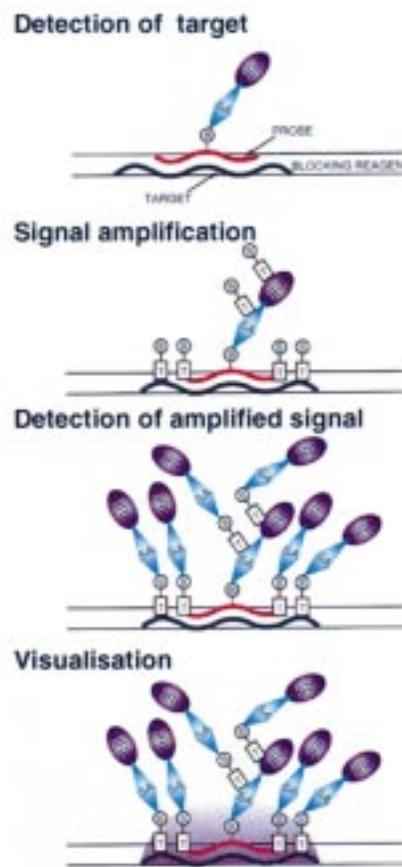


**Figure 1** Schematic representation of the PCR technique. The reaction is catalysed by Taq DNA polymerase in the presence of two short strands of DNA oligonucleotides (primers), which prime the synthesis of new DNA strands in a 5'→3' direction. Magnesium chloride drives the reaction and dNTPs act as the essential building blocks, which Taq DNA polymerase uses to create new DNA strands. This results in an exponential increase in target, following the rule of 2<sup>n</sup> accumulation, finally resulting in millions of copies of the target gene.

process became simpler. These enzymes are active at higher temperatures, thus increasing specificity and the rate of DNA synthesis.<sup>11 12</sup>

There are two different targets for nucleic acid amplification. DNA, the nucleic acid information located in the nucleus of each cell is determined by the sequence of nucleotide bases, which are linked in a phosphate backbone of double stranded DNA. The other molecule of interest is RNA, which is the precursor molecule for each protein. RNA is transcribed from DNA, with splicing out of intronic sequences, leaving an mRNA molecule composed of exonic DNA sequences only. Occasionally, some genes, such as CD 44, show evidence of intron splicing and retention, where intronic DNA and RNA sequences are represented in RNA molecules.

The PCR uses essential reagents to bring about amplification of DNA and RNA molecules. For DNA amplification, a primer pair is used, along with deoxynucleotides (dNTPs: dATP, dCTP, dGTP, dCTP, and dUTP), magnesium chloride, potassium chloride, Taq DNA polymerase, Taq DNA buffer, and DNA template. The primer pair is essentially the foundation of the reaction (fig 1), from which DNA strand specific synthesis occurs. For RNA amplification, the rationale is different. Here, the initial step is to create a complemen-



**Figure 2** Schematic representation of tyramide signal amplification (TSA). See text for details of the chemical reaction. TSA increases the sensitivity of ISH and PCR-ISH. The more recently developed dinitrophenol (DNP) tyramide avoids background problems occasionally associated with biotinylated tyramide. Courtesy of New England Sciences, Boston, USA.

tary DNA (cDNA) template from the RNA in the sample. This is achieved through the use of a reverse transcriptase (present normally in retroviruses). Again, many reverse transcriptases are now commercially available including moloney murine leukaemia virus (MMLV), avian myeloblastosis virus (AMV), superscript I and II, and the intriguing rTth DNA polymerase, which has both reverse transcription and DNA polymerase activity, making RNA amplification a simple one step procedure. The success of the PCR is determined largely by the design of the primers, optimisation of PCR conditions (such as optimising the magnesium chloride concentration for DNA amplification or the manganese acetate concentration for rTth RNA amplification), and the integrity of the DNA and RNA in the sample. In addition, some clinical samples contain Taq polymerase inhibitors, and this phenomenon is not understood clearly.<sup>13</sup>

There are now several well described modifications of the basic PCR technique including asymmetric PCR (using a molar excess of one of the PCR primers to make a single stranded PCR product), inverse PCR (which allows the investigator to look at sequences outside the region of interest), competitive PCR (for gene dosage assays), and “Taq Man” PCR (which

**Table 1** Detection sensitivities of NISH assays

Technique	Sensitivity
Two step immunocytochemical non-isotopic ISH	30–50 copies viral target <sup>17</sup>
Three step immunocytochemical non-isotopic ISH with biotinylated/digoxigenin labelled probes	5–15 copies viral target <sup>17</sup>
Five step immunocytochemical non-isotopic ISH with digoxigenin labelled probes	1–5 copies viral target <sup>17</sup>
Tyramide signal amplification system	1–5 copies viral target <sup>18</sup>

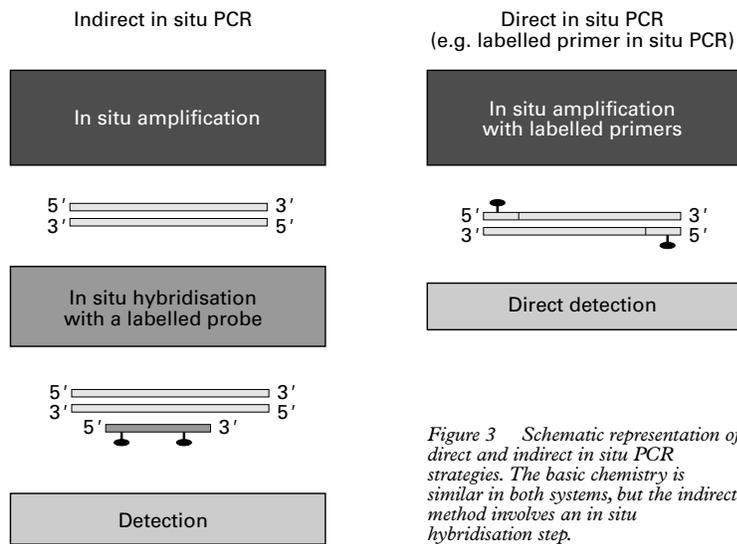


Figure 3 Schematic representation of direct and indirect in situ PCR strategies. The basic chemistry is similar in both systems, but the indirect method involves an in situ hybridisation step.

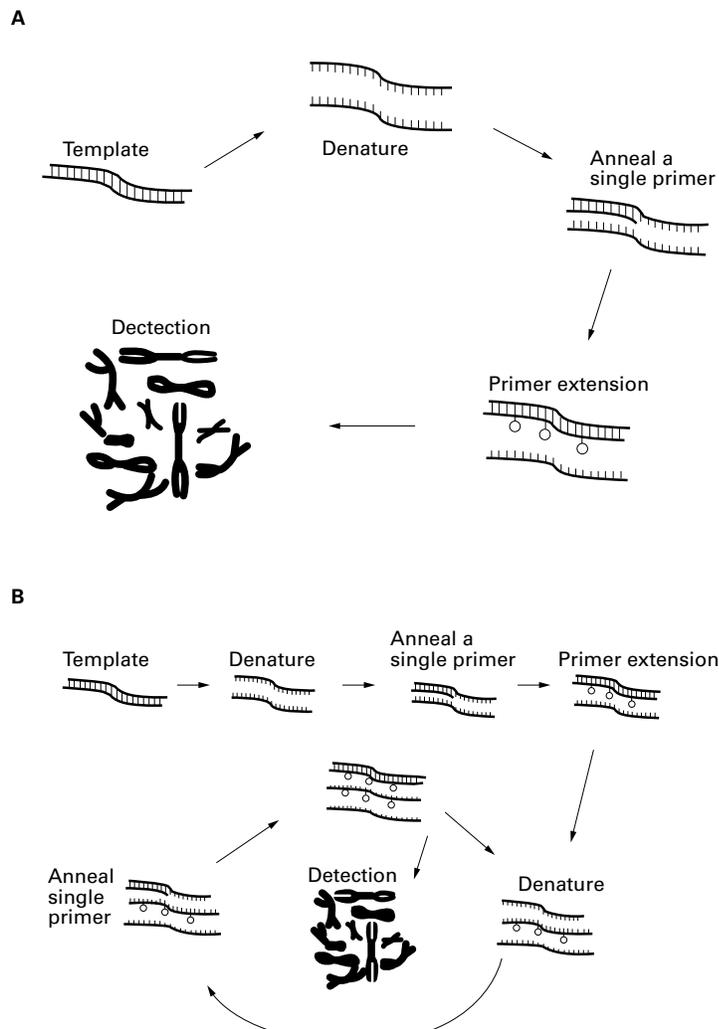


Figure 4 (A) Primed in situ synthesis (PRINS) and (B) cyclic PRINS, in which a single primer is used to catalyse a single stranded product accumulation. The technique is suitable for metaphase spreads and interphase nuclei.

uses a conventional primer pair in addition to a "Taq Man" probe), for accurate gene quantification studies. Analogous techniques such as ligase chain reaction (LCR) and isothermal replica-

tion (3SR) have also been described. These are basic modifications of the PCR, but they use different chemistries. Thus, LCR uses a thermostable ligase, which effectively joins pieces of DNA strands, and 3SR uses T7 polymerase to create cDNA templates from RNA.<sup>14 15</sup>

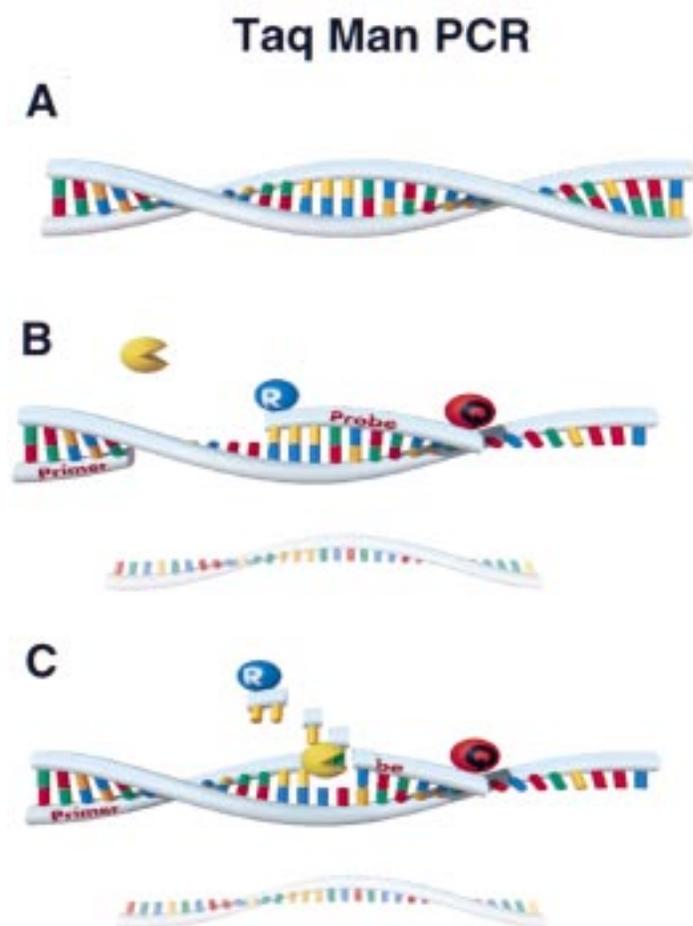
In cell amplification techniques allow the specific detection of DNA and RNA molecules by amplification of specific target sequences within fixed tissue or cells.<sup>16</sup> For Southern, northern, and solution phase PCR assays, nucleic acid extraction is required first, necessitating the destruction of tissue architecture. ISH and in cell amplification overcome this problem by allowing us to correlate DNA and RNA hybrids/amplified products directly within cells. The ability of in cell amplification to achieve single copy mammalian gene detection is an obvious advantage. The detection sensitivities of standard non-isotopic in situ hybridisation, and tyramide signal amplification (TSA) enhanced ISH are given in table 1. TSA is based on the principle of deposited biotinylated tyramide, catalysing enhanced chromogenic detection (fig 2). As can be seen in table 1, most non-complex ISH techniques allow the investigator to detect 5–15 copies of viral target, with single copy mammalian gene detection rarely achieved except when using TSA and complex five step immunocytochemical techniques. Under certain conditions of high stringency, fluorescent in situ hybridisation (FISH) does allow single copy gene detection, but this is very much dependent upon the operator and is not always reliable.

#### DNA in cell amplification techniques

Direct in cell amplification methods use labelled dNTPs or labelled primers in the PCR mix, giving a directly labelled PCR product within the cell. Indirect methods require an in situ hybridisation step to detect unlabelled product. In general, direct techniques are simpler to perform, but are fraught with difficulties, because non-specific signal generation is a common problem.

#### IN SITU PCR (DIRECT METHOD)

This refers to DNA amplification using solution phase PCR reagents placed on top of fixed and permeabilised tissue specimens or cells attached to glass slides. The reaction proceeds by incorporating labelled oligonucleotides (such as digoxigenin-11-dUTP, biotin 11dATP, or fluorescein labelled nucleotides) into newly synthesised DNA during amplification. The fixation process preserves cell morphology and proteolytic digestion facilitates access of PCR reagents to their target DNA sequences. Subsequently, the labelled amplicon (PCR product) is detected within the cell by means of standard immunocytochemical protocols, such as a one step detection with an antidigoxigenin antibody conjugated with alkaline phosphatase (Boehringer Mannheim, Mannheim, Germany). A hybridisation step is not necessary (fig 3).<sup>19-21</sup> The disadvantages of this technique will be discussed later and are reviewed by Long *et al.*<sup>22 23</sup>



**Figure 5** “Taq Man” PCR, based on the 5′→3′ exonuclease activity is an accurate and robust method of measuring gene dosage in cells and tissues. The reaction follows the basic principles of a standard PCR, except that a unique probe system is used. This probe, called a “Taq Man” probe, has a fluorescent reporter molecule (such as FAM or JOE, dyes used commonly in automated DNA sequencing chemistry) at its 5′ end and a quencher sequence TAMRA at its 3′ end. When the probe is intact, the proximity of the reporter to the quencher results in Förster-type energy transfer, with quenching of the fluorescence signal from the reporter molecule. When Taq DNA polymerase extends from the 3′ end of the primer, the reporter molecule is cleaved from the probe as a result of the exonuclease activity of Taq DNA polymerase. Therefore, for each new target strand created, one fluorescent molecule is released, making the assay directly quantitative. The assay works well for the measurement of both DNA and RNA. (A) Double stranded (ds) DNA, before amplification; (B) denatured dsDNA in single stranded (ss) DNA form. The forward primer and the “Taq Man” probe have annealed to their target sites on the DNA strand. (C) Cleavage of the reporter molecule during extension by Taq DNA polymerase.

#### LABELLED PRIMER DRIVEN IN CELL AMPLIFICATION (DIRECT METHOD)

This is a special modification of the basic in situ PCR technique using labelled primers (biotin, digoxigenin, or DNP) for in situ DNA amplification within cells and tissues (fig 3).<sup>21</sup>

**Table 2** Methods for incorporation of labelled nucleotides into probes for in situ hybridisation and PCR ISH

Method	Enzyme	Template	Vector	Type and length of sequence
Nick translation	DNase I DNA polymerase	dsDNA	Plasmid	300–800 dsDNA
Random priming	Klenow polymerase	dsDNA	Plasmid	200–300 dsDNA
Tailing	Terminal transferase	ssDNA	Nil	Depends on length of oligonucleotide
5′ unique primer	Klenow polymerase	ssDNA	M13	Vector labelled as dsDNA, attached to ss unlabelled probe
SP6, T7, T3	RNA	dsDNA	Plasmid	ssRNA

ds, double stranded; ss, single stranded.

#### PCR IN SITU HYBRIDISATION (PCR ISH) (INDIRECT METHOD)

This refers to in cell DNA amplification using the PCR.<sup>21</sup> The amplified product is detected by in situ hybridisation using a labelled internal probe, or a double stranded genomic probe. Conventionally, 5′ end labelled (such as digoxigenin, biotin, or DNP) oligoprobes are used for ISH reactions. Conveniently, when using genomic probes, nick translated double stranded probes (as used in conventional ISH) can be used. In addition, randomly primed probes can be applied (table 2).

#### DNA IMMERSION HISTO PCR

This involves the amplification of whole tissue sections and/or cell preparations attached to glass coverslips in a standard eppendorf PCR tube, with conventional PCR conditions applied. The PCR product is detected using in situ hybridisation, or a labelled primer approach.<sup>24</sup>

#### PRIMED IN SITU SYNTHESIS (PRINS)

This uses a single primer, which is annealed to chromosomal DNA sequences (interphase nuclei or metaphase spreads), and then amplified in the presence of a labelled modified oligonucleotide (such as digoxigenin 11 dUTP) by Taq DNA polymerase to generate a single stranded PCR amplicon (asymmetric PCR) (fig 4).<sup>25</sup>

#### CYCLIC PRINS (DIRECT METHOD)

This is a more sensitive detection system compared with PRINS. This technique uses several cycles of amplification to increase the concentration of amplicon present and thus increase detection sensitivity.

#### IN CELL “Taq Man” PCR

“Taq Man” PCR is an ultrasensitive, quantitative, solution phase PCR method, which enables low copy nucleic acid detection in an endpoint or real time format (fig 5).<sup>26–27</sup> “Taq Man” technology is based on the 5′→3′ nuclease activity of DNA Taq polymerase, allowing direct detection of the PCR amplicon by release of a fluorescent reporter molecule during the amplification reaction. The probe is usually a 20–30 mer in size, with a fluorescent reporter dye at the 5′ end and a rhodamine quencher molecule at the 3′ end. When the probe is intact, no fluorescence is seen. After the probe has hybridised with the target sequence (which is always between the two primer sites), the 5′→3′ nuclease activity cleaves the reporter molecule during DNA strand polymerisation in each PCR cycle. As a result, fluorescence increases because the reporter is now removed from the influence of the quencher sequence. In addition, the 3′ end of the “Taq Man” probe is phosphorylated to prevent extension by Taq DNA polymerase. “Taq Man” PCR technology facilitates the measurement of gene expression and DNA load in cells. The impact of this technology on in situ applications will be enormous because it will be possible to examine and determine the

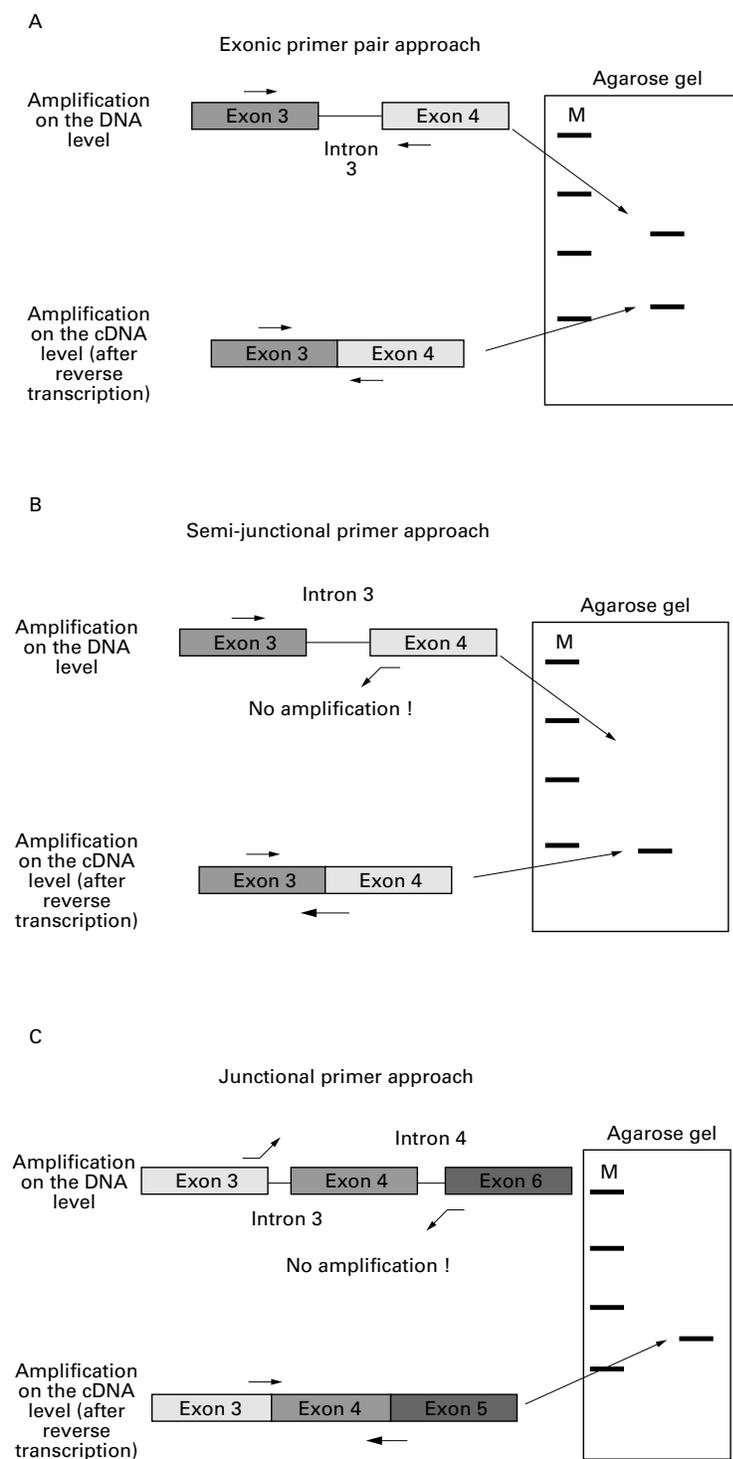


Figure 6 Primer approach for RNA amplification. (A) Exonic primer pair spanning an intron. (B) Semi-junctional primer approach, only allowing the amplification of mRNA molecules. Note, single stranded DNA is still made by the exonic primer, but is not seen on an agarose gel. (C) Junctional primer approach, where only RNA molecules are amplified.

copy number of any gene in any cell and also it will allow us to calculate the sensitivity of in situ amplification techniques.

#### IN SITU LIGASE CHAIN REACTION (IS LCR)

This is a sister technique to PCR, which uses two sets of primers A,A\* and B,B\*.<sup>14</sup> A thermostable ligase is used to ligate the primers on opposite strands. Thus, the length of the LCR product is the sum of the lengths of the A

and B and A\* and B\* primers, usually 40–60 mer in size. In addition, the ligase chain reaction can be coupled with PCR, which increases the yield of the amplicon. This procedure is called LDR (ligase detection reaction). The advantage of this technique is that mutation specific LCR primers can be used for the analysis of single point mutations or polymorphisms within the cell.

#### RNA in cell amplification techniques

To amplify RNA sequences within the cell, we can adopt three basic primer approaches: (1) exonic primers, (2) semi-junctional primers, and (3) junctional primers (fig 6). There are several techniques used to amplify RNA.

#### IN SITU REVERSE TRANSCRIPTION PCR (IS RT PCR) (DIRECT METHOD)

This refers to mRNA amplification in tissue specimens and in cells by initially creating a cDNA template, using a reverse transcriptase enzyme, such as MMLV or AMV. This cDNA is then the target for Taq DNA polymerase catalysed PCR amplification. Alternatively, a simple one step method can be applied using rTth polymerase, which possesses both reverse transcriptase and DNA polymerase activities. Labelled dNTPs can easily be incorporated during the PCR amplification process.

#### RT TRANSCRIPTION PCR IN SITU HYBRIDISATION (RT PCR ISH) (INDIRECT METHOD)

This refers to mRNA amplification in tissue specimens by first creating a cDNA template and then amplifying this cDNA template, as in IS RT PCR. Subsequently, the in cell amplified product is detected with an internal labelled oligoprobe or PCR generated single or double stranded probe.

#### LABELLED PRIMER DRIVEN RNA IN CELL AMPLIFICATION (DIRECT METHOD)

This is a special modification of the basic in situ RT PCR technique using labelled primers (such as biotin, digoxigenin, or DNP) for in situ RNA amplification within cells and tissues.

#### IN SITU 3SR (IS 3SR)

This refers to self-sustained sequence replication and it is a two enzyme isothermal in vitro amplification technique based on multiplying mRNA into cDNA intermediates that contain promoter sequences for RNA transcription. RNA polymerase (T7 or SP6) then produces several copies of RNA, which increases the sensitivity of the reaction.<sup>28</sup> The obvious advantage of this technique for in cell amplification is that it is isothermal (carried out at one temperature), thereby preserving tissue morphology. In addition, dedicated IS PCR equipment is not needed.

#### How do I perform in cell amplification of DNA?

The technique follows three basic steps<sup>29</sup>:

- cell/tissue pretreatment
- in cell DNA amplification
- detection of amplified product (direct or indirect systems)

Table 3 Different fixatives used for in cell amplification

Crosslinking	Non-crosslinking
10% Neutral buffered formaldehyde (NBF)	Acetone
2% Paraformaldehyde	Ethanol
Glutaraldehyde	Methanol/acetic acid (3:1)
Bouin's solution (formalin with picric acid)	Streck's fixative
Zenker's solution (formalin with heavy metals)	Heat fixation
Permeafix (Ortho, Raritan, USA)	

#### PRETREATMENT STEPS

To perform slide based DNA in cell amplification, cells and tissues have to be immobilised on to glass slides coated with binding reagents such as APES (aminopropyl triethoxysilane, Sigma, St Louis, USA), Denhardt's solution, poly-L-lysine, or Elmer's glue. Binding depends upon electrostatic or hydrophobic interactions between the glass surface and chemical groups of the coating agent. The first crucial step is to preserve the cell/tissue morphology and to fix nucleic acids rigidly to the cytoskeleton by means of a suitable fixative. Table 3 lists commonly used fixatives for in cell DNA amplification. Formaldehyde based fixatives cause extensive crosslinking of DNA to DNA, and of DNA to histone proteins and other charged molecules within the nucleus. This has implications for DNA amplification, where DNA-histone protein crosslinks might interfere with Taq DNA polymerase progression during the elongation phase of PCR.<sup>30</sup> After fixation, cells or tissue sections must be permeabilised. The fixed and permeabilised cells function as "amplification sacks" with semi-permeable membranes so that: (1) the PCR reagents can penetrate into the cell to reach the nucleic acid targets, and (2) the PCR amplicon does not leak out of the cell. The amount and time of proteolytic digestion varies from assay to assay, depending on the type of specimen examined. An adequate balance between digestion and permeabilisation procedure has to be determined, with the aims of both maintaining cellular morphology and avoiding diffusion of the PCR product out of the cell. Therefore, in all cases, the optimum concentration of protease for particular tissues or cells has to be titrated empirically. The commonly used proteolytic agents include: proteinase K (Boehringer Mannheim), pepsin/HCl, proteinase K, and SDS (sodium dodecyl sulphate; Sigma). In addition, acid hydrolysis (0.02–0.2 N HCl) can be used and microwave irradiation can be used to unmask nucleic acid.

Depending on the enzyme system used for immunocytochemical detection (alkaline phosphatase or horseradish peroxidase), it is recommended that endogenous enzyme activity is blocked before amplification and detection to avoid false positive results. Incubation with a solution of 0.1% sodium azide and 0.3% hydrogen peroxide for 15 minutes is sufficient to quench endogenous peroxidase activity in most cells and tissues; alternatively, 20% ice cold acetic acid for 10 seconds quenches endogenous alkaline phosphatase activity. Occasionally, levamisole is needed to quench endogenous alkaline phosphatase activity.<sup>31</sup>



Figure 7 The IS-PCR system 1000 (Perkin Elmer), which is designed specifically for in cell amplification.

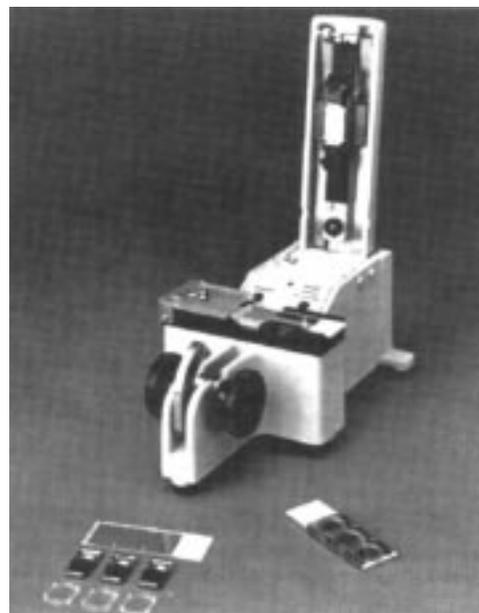


Figure 8 The sealing chamber, consisting of the assembly clip and disc for the IS-PCR 1000 system (Perkin Elmer). This allows compartmentalisation of the PCR solution during slide based PCR assays.

#### IN CELL AMPLIFICATION

Since the original description of in cell amplification by Haase *et al*,<sup>32</sup> dedicated equipment has become available to perform in cell DNA amplification. Attaining the correct temperatures for denaturation, annealing, and extension at the individual cell level on the glass slide is crucial for the success of in cell PCR. Previously, we have reported the phenomenon of "thermal lag", encountered when using a standard PCR thermal cycler.<sup>33</sup> The difference between the block temperature and that of the cell can be as great as 5°C. The newly designed in cell amplification thermocyclers (IS-PCR 1000; Perkin Elmer Applied Biosystems, Foster City, USA. Hybaid Omnigene/Omnislide; Teddington, UK. MJB in situ thermocycler; Watertown, USA) and microprocessor controlled ovens (fig 7) specifically correct for this, allowing successful in cell amplification.

Haase *et al* amplified lentiviral DNA segments in single, fixed cells suspended in a PCR buffer (very much like immersion histo PCR) and subsequently cytospun on to microscopic slides.<sup>32</sup> The amplicon was detected by in situ hybridisation. Initially, microscope slides were

Table 4 Reagents for different DNA PCR assays

DNA solution phase PCR	DNA in situ PCR
200–400 $\mu$ M dNTPs	200–400 $\mu$ M dNTPs
0.2–0.4 $\mu$ M Primer	0.2–1.0 $\mu$ M Primer
1–2.5 mM MgCl <sub>2</sub>	1–5 mM MgCl <sub>2</sub>
1–2 U Taq DNA polymerase	5–10 U Taq DNA polymerase
10 mM Tris-HCl, 50 mM KCl, pH 8.3	10 mM Tris-HCl, 50 mM KCl, pH 8.3

All reagents are supplied by Perkin Elmer Applied Biosystems, Foster City, USA and Warrington, UK.

placed in an “aluminium foil boat” on top of the heating block of a standard solution phase PCR machine. To minimise evaporation during the thermocycling process, the PCR mixture was overlaid with mineral oil and glass coverslips, and sealed with nail polish.<sup>34</sup> Despite these modifications, the PCR reaction was often suboptimal, with reaction failure reported commonly. Newly designed tight sealed, non-evaporating amplification chambers (Perkin Elmer Applied Biosystems) increase amplification efficiency, obviating the need for nail polish or rubber cement (fig 8).

The PCR reagents required for in cell DNA amplification differ somewhat from those used in standard DNA solution phase PCR.<sup>35–41</sup> Table 4 compares the typical reagent set up for a standard solution phase DNA PCR and an in cell DNA PCR. Success of the in cell reaction is dependent upon several variables: the efficiency of pretreatment, the maintenance of tissue/cellular morphology, primer design, and reaction kinetics. To simplify the in cell amplification procedure, a two step PCR protocol

Table 5 Controls needed for in cell amplification

Control	Purpose
Reference control gene (for example, pyruvate dehydrogenase)	Control of specificity and sensitivity of the amplification method
DNase digestion	Abolishment of genomic signals (negative control) For RT: reduction of false positives
RNase digestion	Reduction of false positives For RT: abolishment of RNA signal (negative control)
Omission of primers	Detection of artifacts related to DNA repair mechanism
Omission of RT step (only relevant for RNA assay!)	Detection of mispriming
Omission of labelled nucleotides	Control of the detection system
Omission of Taq polymerase	Detection of primer oligomerisation
Omission of ligase	Detection of primer oligomerisation in IS LCR reaction
Mixtures of known positive and negative cells; identification of different cell types by immunohistochemistry	For RT: control of specificity/sensitivity of the method

Table 6 Reagents used for different RT PCR assays

Solution phase RT PCR	In situ RT PCR
Using rTth polymerase	
200–400 $\mu$ M dNTPs	200–400 $\mu$ M dNTPs
0.4–0.5 $\mu$ M Primer	0.2–1.0 $\mu$ M Primer
1–3.0 mM Mn(OAc) <sub>2</sub>	1–5 mM Mn(OAc) <sub>2</sub>
1–3 U rTth DNA polymerase	5–10 rTth DNA polymerase
50 mM Bicine, 115 mM potassium acetate, 40% (wt/vol) glycerol, pH 8.2	50 mM Bicine, 115 mM potassium acetate, 40% (wt/vol) glycerol, pH 8.2
For cDNA synthesis followed by PCR	
0.4–1 $\mu$ M dNTPs	0.4–1 $\mu$ M dNTPs
0.75 mM Downstream primer	0.75–1 mM Downstream primer
1–5 mM MgCl <sub>2</sub>	2–5 mM MgCl <sub>2</sub>
50 U MMLV	50–100 U MMLV
30 U RNase inhibitor	30 U RNase inhibitor
10 mM Tris-HCl, 50 mM KCl, pH 8.3	10 mM Tris-HCl, 50 mM KCl, pH 8.3

cDNA is followed by PCR (table 4[t4]).

All reagents are supplied by Perkin Elmer Applied Biosystems, Foster City, USA and Warrington, UK.

MMLV, moloney murine leukaemia virus.

has been adopted by many investigators, resulting in a combined annealing/extension step. Denaturation times and annealing/extension times are also increased to facilitate Taq DNA polymerase accessibility to its target sequence in the setting of a volume limited nucleus. The “hot start” PCR modification (preheating the PCR solution and the slide to 70°C) is also used, and minimises primer dimerisation and mispriming events.

If one uses in situ ligase chain reaction (IS LCR), the concentration of thermostable ligase used is two to three times that of a standard solution phase LCR. Primers (four in number) are also increased in concentration, with similar increases in other reagent concentrations as is needed for in cell PCR (table 4).

#### DETECTION OF THE AMPLIFIED PRODUCT

Depending on whether direct or indirect techniques have been used, the amplified product is either labelled or unlabelled. Standard immunocytochemical detection techniques are used as for in situ hybridisation and have been described previously.<sup>19–31</sup> The numbers of immunocytochemical steps largely depend on the sensitivity required, keeping in mind what can be achieved using standard NISH and the specific detection protocol (table 1). For indirect techniques, an in situ hybridisation step is performed using an oligoprobe or genomic probe. Maximum specificity is achieved using an internal oligoprobe, whereas genomic DNA probes will hybridise with the amplicon, the primers, and other sequences outside the amplified region. Other strategies can be adopted to increase the specificity of the hybridisation reaction, including concatameric probes (probes with overlapping ends) or tandemly repeated internal probes (three to six for each amplicon). In addition, the numbers of reporter molecules attached to a probe can vary enormously, depending on the type of labelling reaction used.

Conventionally, when using 5' and 3' end labelling, only one reporter molecule is attached to each probe. Thus, for each hybridisation event, only one reporter can be seen. 3' tailing (using the Genius 3' tailing kit; Boehringer Mannheim, Indianapolis, USA) adds between five and 10 reporter molecules for each probe. This increases the signal, but at the expense of increased background. One must choose the system that gives the optimal signal to noise ratio for the particular application. The stringency of the hybridisation must also be established correctly. Typically, medium stringency conditions should be used first.<sup>21</sup> A typical hybridisation solution for oligoprobes would be: 2 $\times$  saline sodium citrate (SSC), 10% formamide, and 5–10% dextran sulphate. For genomic probes, the concentration of formamide could be increased to 50%. Typically, hybridisation temperatures are 37°C, but higher temperatures can be used, which of course increases the stringency. Typically, hybridisation can be carried out at T<sub>m</sub> –10°C to –20°C, where T<sub>m</sub> is defined as the theoretical solution temperature at which half of the probe is annealed to its complementary strand

Table 7 Troubleshooting for in cell amplification assays

Problem	Explanation	What to do
Loss of cells	Insufficient coating	Coat slides with APES or use precoated microscope slides
Evaporation of PCR mix	No tight seal	Choose semi-automated closed chamber system
Weak or no signals	Reagent loss resulting from non-specific adsorption to the slide	Increase the amount of manganese/magnesium
Signal outside the cell (high background)	Ineffective amplification	Increase numbers of cycles
	Leakage of product/diffusion of product	Incorporate biotin substituted dNTPs (bulkier amplicon)
		Increase size of product or use multiple primer pairs
		Fix after PCR with 100% ethanol or paraformaldehyde
False positive results	Non-specific signals "diffusion artifacts"	"Hot start technique"
		Generate more complex PCR products
		Reduce PCR cycles
		Optimise fixation/permeabilisation
False positive results within the nuclei	Endogenous priming/DNA repair mechanism (a special problem of direct in situ PCR)	Dideoxy blocking
		Super denaturation
		DNase digestion or UV crosslinking of DNA
		Use of labelled primers
		Use of Stoffel DNA polymerase

APES, aminopropyl triethoxysilane.

and half is not annealed. This value is solution dependent, being subject to the ionic strength of the solution, and it differs in tissue sections. Here it is called the  $T_m$ , a value calculated as a midpoint temperature between the temperature at which one can see a hybridisation signal retained on the tissue section and the temperature at which it disappears when altering post-hybridisation washing conditions.<sup>42</sup> The stringency of the post-hybridisation wash can also be adjusted by decreasing the salt concentra-

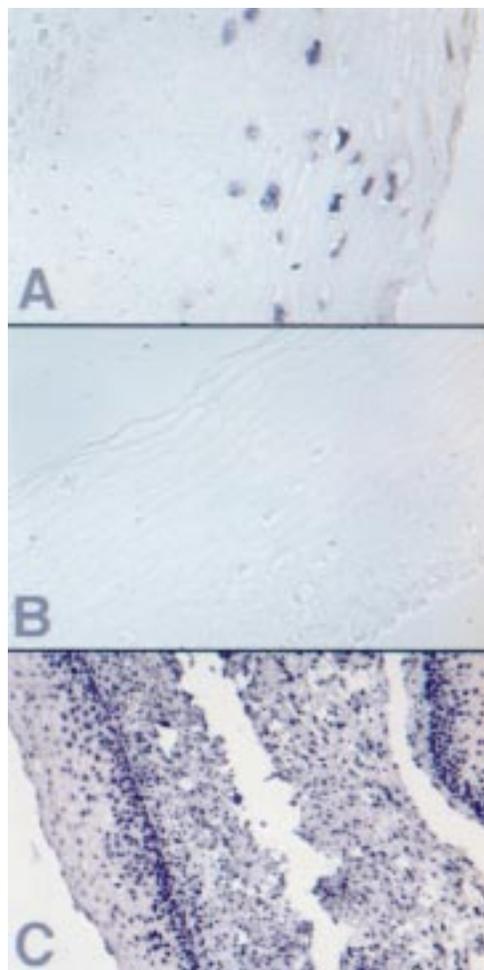


Figure 9 Non-specific incorporation phenomena seen with DNA IS PCR when a labelled nucleotide is included in the reaction mix. This is because of end labelling of nicked (damaged) DNA in fixed cells and tissue specimens.

tion of the washing solution, from  $2\times$  SSC to  $0.1\times$  SSC, and increasing the temperature. In addition, formamide can be added, but we feel that this complicates many assays and adds another variable to the establishment of the correct washing stringency.

The other difficulty to be overcome is retention of the amplicon. This is a delicate balancing procedure. It is achieved largely by correct pretreatment, amplifying to a level that is just detectable, and creating a PCR product that is big enough to remain within the cell. There have been many attempts to prevent diffusion or back diffusion from target positive cells to negative bystander cells. One strategy uses concatameric primers (that is, overlapping primers), which creates a large PCR product.<sup>19</sup> Another approach is to use a bulking agent, such as biotin 11dUTP (Sigma), incorporated into the PCR product during the PCR and then detected with a digoxigenin labelled oligoprobe. Another strategy is to allow a second in cell PCR to proceed in parallel with the first (using a reference gene such as actin) to increase the density of the intranuclear space, making it less likely for product diffusion to occur, because of volume limitation. We suggest that running a parallel asymmetric PCR (with the accumulation of a single stranded product) can prevent back diffusion, because much of the phenomenon is caused by single stranded forms of the target sequence adhering to cytoplasmic membrane sticky sites in neighbouring cells (J J O'Leary *et al*, unpublished data).

To prevent loss of the product, we advise a post-amplification fixation step with 100% ethanol or 2% paraformaldehyde. If product diffusion is a major problem, we suggest that investigators decrease the numbers of rounds of amplification (perform only 10–15 rounds, instead of the typical 30–40 rounds). The numbers of rounds of amplification needed is determined largely by the numbers of copies of the target sequence for each cell and how well these copy numbers are preserved during the fixation process.

The importance of adequate and appropriate controls for each PCR experiment cannot be over emphasised because there are many, undesired pitfalls. Table 5 lists the minimum controls

Table 8 Summary of in cell applications

	Method	Reference
<b>DNA</b>		
Lentivirus DNA	Indirect in situ PCR, cells in suspension	Haase <i>et al</i> 1990 <sup>32</sup>
Human papillomavirus DNA	Indirect in situ PCR, tissue sections	Staskus <i>et al</i> 1991 <sup>47</sup>
	Indirect in situ PCR, tissue sections	Nuovo <i>et al</i> 1991, 1992 <sup>34,19</sup>
HIV DNA	Direct in situ PCR, cyocentrifuge preparation	Zehbe <i>et al</i> 1992 <sup>38</sup>
	Direct in situ PCR, cyocentrifuge preparation	Spann <i>et al</i> 1991 <sup>49</sup>
Mouse mammary tumour virus DNA	Direct in situ PCR, cyocentrifuge preparation	Nuovo <i>et al</i> 1992 <sup>19</sup>
	Indirect in situ PCR, cyocentrifuge preparation	Bagasra <i>et al</i> 1992, 1993 <sup>35,36</sup>
Cytomegalovirus DNA	Indirect insitu PCR, tissue sections	Embretson <i>et al</i> 1993 <sup>41</sup>
	Indirect in situ PCR, tissue sections	Chiu <i>et al</i> 1992 <sup>50</sup>
Hepatitis B virus DNA	Indirect in situ PCR, cells in suspension	Komminoth <i>et al</i> 1994 <sup>51</sup>
Herpes simplex DNA virus 2 DNA	Indirect in situ PCR, tissue sections	Long <i>et al</i> 1993 <sup>22</sup>
Human herpes virus 8 DNA	Indirect in situ PCR, tissue sections	Long <i>et al</i> 1993 <sup>22</sup>
Gene rearrangemets, DNA	direct in situ PCR, tissue sections	Gressens and Martin 1994 <sup>52</sup>
Chromosomal translocations, DNA	Indirect in situ PCR, cells in suspension	Boshoff <i>et al</i> 1995 <sup>38</sup>
Chromosome mapping, DNA	Indirect in situ PCR, cells in suspension	Komminoth <i>et al</i> 1992 <sup>53</sup>
	Direct in situ PCR, metaphase preparation	Long <i>et al</i> 1993 <sup>22</sup>
		Gosden and Hanratty 1993 <sup>54</sup>
<b>RNA</b>		
HIV mRNA	Indirect in situ RT PCR, cells in suspension	Patterson <i>et al</i> 1993 <sup>55</sup>
JC virus RNA	Direct in situ RT PCR, tissue sections	Kelleher <i>et al</i> 1994 <sup>56</sup>
Gene rearrangements, mRNA	Indirect in situ RT PCR, cells in suspension	Embleton <i>et al</i> 1992 <sup>57</sup>
Nerve growth factor mRNA	Direct in situ RT PCR, tissue sections	Staecker <i>et al</i> 1994 <sup>58</sup>
Granzyme A, perforin mRNA	Direct in situ RT PCR, tissue sections	Chen and Fuggle 1993 <sup>59</sup>
Epidermal growth receptor mRNA	Direct in situ RT PCR, tissue sections	Patel <i>et al</i> 1994 <sup>60</sup>

required for DNA and RNA in cell amplification and the reasons for these controls.

### How do I perform in cell amplification of RNA?

To amplify RNA in cells and tissue sections we must create a cDNA template. First, antisense oligonucleotide primers anneal complementary to their specific site in the mRNA. Then a reverse transcriptase enzyme such as MMLV, AMV, or Superscript I and II (all from GIBCO BRL, Gaithersburg, USA) catalyse the synthesis of cDNA. This newly created cDNA is then the target for Taq DNA polymerase catalysed PCR amplification.

Alternatively, a one step reaction using rTth DNA polymerase and a bicine buffer system (Perkin Elmer Applied Biosystems) can be used to carry out in cell RNA amplification more efficiently. This enzyme possesses reverse transcriptase and DNA polymerase activities. Amplification of RNA with this system has some advantages, primarily that reverse transcription with rTth polymerase can be carried out at higher temperatures (up to 60°C) compared with AMV or MMLV (42°C), thereby reducing RNA secondary structure and facilitating cDNA synthesis. In addition, the use of a single bicine buffer system greatly facilitates

the performance of in cell RNA amplification, obviating the need to change buffer systems during amplification or to use chelating agents. Table 6 illustrates the difference between a typical solution phase RNA PCR and an in cell RNA assay.

To prevent amplification of genomic DNA, a DNase digestion step should be carried out before in situ amplification takes place. However, it is often difficult to achieve full DNase digestion when using paraffin wax embedded tissue and thus newer strategies can be applied. Moreover, incomplete DNase digestion leads to undesired "DNA repair mechanism" artifacts generated by the exonuclease activity of the DNA Taq polymerase, particularly if a two enzyme RNA amplification system is used. By using specially designed primers for cDNA amplification ("junctional primers") (fig 6), which overspan splice junction sites, only RNA molecules will be amplified. Because RNA is extremely labile in tissues and cells, and because of the effect of the pretreatment process, endogenous RNases can easily degrade RNA. Therefore, it is recommended that only solutions and tools that have been treated with diethylpyrocarbonate (DEPC) at a final concentration of 0.1% should be used.

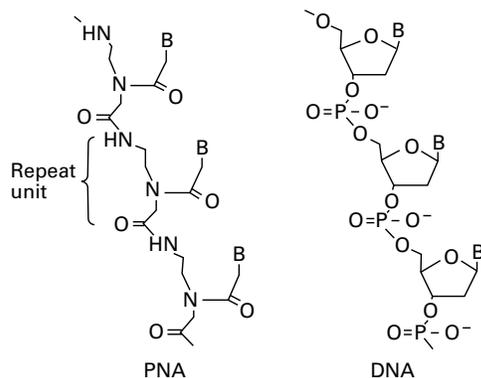
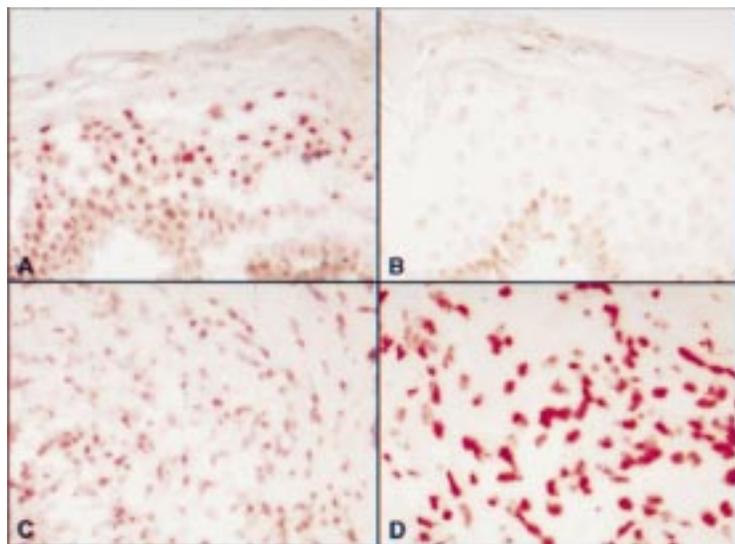


Figure 10 Schematic structure of PNA as compared with DNA. Note that PNA is an uncharged molecule, making it ideally suited for NISH and PCR ISH assays.

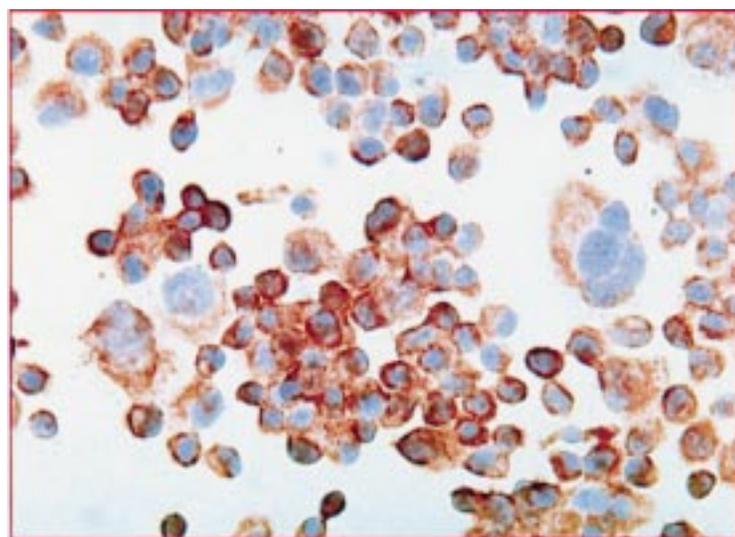
### What problems are associated with performing in cell amplification?

In the final analysis, critical review of the results is needed, with careful assessment of the control slides and familiarity with the problems and their solution. Table 7 illustrates some of the common problems and the possible ways of troubleshooting them.

False positive results for direct in cell amplification can be caused by non-specific incorporation of labelled nucleotides (such as digoxigenin-11-dUTP) into damaged DNA. Fragmented or nicked DNA strands undergo a "DNA repair mechanism" catalysed by the exonuclease activity of Taq DNA polymerase. The newly repaired DNA strand will have incorporated the labelled nucleotide, giving a



**Figure 11** DNA PCR ISH. (A) Beta globin gene PCR ISH revealing amplicons in many nuclei. (B) Illustrates the result with an oligoprobe: hybridisation signals are very faint. (C) and (D) illustrate the magnesium chloride drive effect on the PCR in cell DNA amplification: (C) and (D) show beta globin gene amplification at 3.5 mM magnesium chloride and 4.0 mM, respectively. Note the increase in the intensity of signals when the higher magnesium chloride concentration is used.



**Figure 12** RNA in cell amplification using the labelled primer driven RT PCR technique. The model used here comprises DRD2 (dopamine receptor 2) transfected chinese hamster ovarian epithelial (CHO) cells. Note the discrete cytoplasmic staining in all cells in this field.

labelled PCR product, which is non-target dependent and occurs independently of the primer pairs used. This reaction even occurs at room temperature. Thus, IS PCR with labelled nucleotides should not be used when performing DNA in cell amplification (fig 9).

#### Alternative techniques of signal amplification

##### TYRAMIDE SIGNAL AMPLIFICATION (TSA)

Because most non-isotopic methods of ISH cannot detect low copy RNA or DNA, an alternative detection system has been developed for use following ISH; this system amplifies the hybrid signal, thereby increasing the sensitivity of detection. This technique is based on the peroxidase catalysed deposition of biotinyl tyramide and it is now available commer-

cially as TSA detection (NEN Life Sciences, Boston, USA and Gen Point, Dako, Glostrup, Denmark) (fig 2). After oxidation with horseradish peroxidase, tyramides are converted into an intermediate, reactive compound, generating an extremely quick reaction with electron rich aromatic amino acids in proteinaceous specimens. Afterwards, the signal is detected by either streptavidin/alkaline phosphatase or streptavidin/peroxidase and a suitable substrate. The procedure produces high amplification of the signal at its hybridisation site. For the system to work, a biotinylated probe or antibody must be used. Biotinylated detection systems often cause high background problems because of endogenous biotin and avidin binding sites. More recently, another tyramide (DNP-tyramide) has become available, minimising high background and non-specific binding phenomena.

##### PNA ISH COMBINED WITH TSA DETECTION

The introduction of PNA, consisting of a peptide backbone composed of N-(2 aminoethyl) glycine units to which nucleobases are attached by carbonyl methylene linkers, brings new possibilities for in situ hybridisation technology (fig 10).<sup>43-46</sup> PNA is an uncharged molecule. While in situ hybridisation procedures with DNA or RNA probes are very time consuming, non-charged PNA probes reduce the hybridisation process dramatically. Typically, hybridisation times can be reduced to 5–10 minutes as compared with three to four hours for DNA oligoprobes and 24 hours for genomic probes. Hybridisation conditions are also easier to establish because it appears that a wide range of hybridisation buffers work for most PNA probes (J J O'Leary *et al* 1998, unpublished data). Furthermore, PNA in situ hybridisation affords greater flexibility when establishing hybridisation stringency conditions. A mismatch in a DNA/PNA duplex lowers the  $T_m$  by on average 15°C, in contrast to 5–10°C for a DNA/DNA duplex, making it possible to introduce this methodology for the screening of single base pair mutations and for allele specific detection. Because of the high specificity and higher  $T_m$  of PNA/PNA duplexes compared with DNA/DNA complexes, it is possible to use PNA probe technology for the detection of short DNA or RNA sequences in cells and chromosome spreads (such as, telomeric repeats). In combination with PCR ISH, this new technique greatly simplifies the in situ hybridisation component of the reaction. However, the cost of PNA oligomers still limits their use in standard ISH applications. The combination of PNA and TSA technology is obviously attractive and will offer detection as sensitive as most in cell PCR/LCR reactions.

##### Conclusion

As we have seen, there is now a wide repertoire of in cell amplification techniques for the detection of low copy viral, protozoal, and mammalian DNA and RNA sequences in tissue sections and cells (table 8; figs 11 and 12). The choice of reaction is totally dependent on the particular target being amplified and the

skill and experience of the operator. These techniques are now robust and with newer chemistries and novel methodologies (such as IS-“Taq Man” PCR) becoming available many more targets can be examined.

In many ways, the world of in cell amplification mirrors the early days of immunocytochemistry and in situ hybridisation: “greeted by enthusiasm, scorned by failure and established by the dedicated”!!!

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