

## Technical report

# Improved in situ detection method for telomeric tandem repeats in metaphase spreads and interphase nuclei

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### Abstract

**Peptide nucleic acid technology (PNA) has become an extremely useful tool and promises to impact on molecular biology and diagnostics. These synthetic DNA analogues pair with DNA and RNA molecules according to Watson and Crick base pairing rules. This paper describes a sensitive and quick fluorescent in situ hybridisation (ISH) technique to determine DNA telomere repeat sequences (TTA GGG)<sub>n</sub> using epifluorescence microscopy. Telomeres are special, repeated structures at the end of each eukaryotic chromosome and serve as protective caps to prevent DNA rearrangements and fusion of chromosomes. A model system has been developed, using stimulated peripheral blood lymphocytes, which facilitates simultaneous detection of telomeres in metaphase as well as in interphase nuclei. A fluorescein isothiocyanate labelled PNA probe (18 mer) directed against complementary telomeric sequences at the end of each chromosome is used. In addition, a simple, easy to perform PNA-ISH protocol is described that overcomes common hybridisation problems encountered using DNA and RNA oligoprobes. Furthermore, the usefulness of a chromogenic immunocytochemical detection system is shown for PNA-ISH.**

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Recently, the synthetic construction of a DNA mimic molecule, peptide nucleic acid (PNA), has been reported.<sup>1–4</sup> This new class of uncharged molecules consists of a peptide (polyamide) backbone composed of N (2-aminoethyl) glycine units. The natural nucleobases are linked to the achiral backbone through methylene carbonyl bonds. Because PNA molecules have a higher affinity for RNA and DNA, the stability of PNA–RNA and PNA–DNA complexes is higher than the

corresponding DNA–DNA and RNA–RNA hybrids. This is because of the uncharged peptide like backbone. The uncharged PNA molecule reduces the time taken for in situ hybridisation experiments to be performed. Interestingly, only a low salt concentration is necessary to stabilise PNA–DNA hybrids. Accordingly, PNA probes should simplify non-isotopic hybridisation experiments for the detection of DNA and RNA in cytopins, paraffin wax embedded sections, and electron microscope preparations.

Eukaryotic chromosomes have physical tails called telomeres, which cap the ends of the chromosomes. Telomeres are conserved repeated sequences of several kilobases, which consist of (TTA GGG)<sub>n</sub>. They are believed to maintain chromosomal stability and protect from end to end fusion, DNA rearrangements, and chromosomal loss.<sup>5–6</sup> The numbers of telomeric repeats decrease with each round of mitotic replication in somatic cells<sup>7–9</sup> because of the inability of all known DNA dependent DNA polymerases to begin DNA synthesis de novo. DNA replication synthesis occurs only in the 5' to 3' direction. This “end replication problem” leads to the hypothesis of chromosome shortening and the “mitotic clock”. Thus, cell division is stopped as a matter of aging,<sup>10–12</sup> and this results in cell death. A ribonucleotide protein polymerase (telomerase) is able to compensate for the loss of telomeric ends during replication. The RNA component of the telomerase contains a short nucleotide template region that enables the synthesis of telomeric repeats on to the shortened chromosome.<sup>13</sup> In general, telomere length and telomerase activation are crucial counterparts in tumorigenesis and aging.

Here, we demonstrate the usefulness of a new simplified protocol to detect telomeres using fluorescein isothiocyanate (FITC) labelled PNA probes in metaphase and interphase nuclei. Using this in situ hybridisation protocol, we demonstrate that in situ hybridisation times can be reduced to less than one hour (30 minutes).<sup>14</sup>

### Materials and methods

Metaphase spreads of peripheral blood lymphocytes were prepared by the standard method of Seabright.<sup>15</sup> Heparinised blood (1 ml) was cultured in RPMI 1640 (10 ml; GIBCO BRL, UK) and phytohaemagglutinin (0.2 ml; GIBCO BRL) in a 5% CO<sub>2</sub> incubator at 37°C. After 72 hours, cell division was stopped with colcemid (GIBCO BRL) and cells were ruptured with hypotonic KCl (GIBCO BRL). The cells were washed several times with cold methanol/acetic acid (3/1) until a clean white cell pellet was obtained. Metaphase spreads were prepared with the resuspended cells in methanol/acetic acid. An area of satisfactory metaphase spreads was marked on the glass slide with a diamond pencil. A pretreatment solution (PNA-ISH detection kit; Dako, Glostrup, Denmark) that had been pre-heated at 37°C for 30 minutes was added to each slide. Slides were fixed in a series of graded concentrations of ethanol (70%, 85%, and 96%) for two minutes each and air dried. Denaturation and hybridisation were performed by adding 10 µl of a FITC conjugated telomeric probe (300 ng/ml) of the sequence (CCC TAA)<sub>3</sub> (Dako) in hybridisation solution. The marked area of the slide was covered with a coverslip (22 × 22 mm). The reaction took place in the dark to avoid fading of the signal. The probe was labelled at the N-terminus with one FITC molecule. The negative control was the hybridisation solution itself, containing 30% formamide, 0.1% Triton X100, and 0.3× SSC (saline sodium citrate). Slides were incubated on a hot plate at either 80°C or 65°C for three minutes, followed by a hybridisation step at room temperature for 30 minutes. The coverslips were removed and slides were immersed in a rinse solution (PNA-ISH detection kit; Dako) for five minutes at 65°C. Afterwards, the wet slides were counterstained and mounted in 25 µl of an antifade mounting medium (propidium iodide; Vector Laboratories) and the signals were visualised with a 100× immersion objective on a Nikon fluorescent microscope with blue filters (excitation, 535 nm; emission, 615 nm).

Using propidium iodide, positive signals appeared as yellow/green dots. Alternatively, the signals were detected by adding an alkaline phosphatase conjugated rabbit F(ab') anti-FITC antibody (1/100 in TBT: 50 mM Tris-HCl, 100 mM NaCl (pH 7.2), 3% bovine serum albumin (BSA), and 0.05% Triton X-100) after post hybridisation washes. Specimens were incubated at room temperature for 30 minutes followed by two washes in Tris buffered saline (50 mM Tris-HCl, 100 mM NaCl (pH 7.2)). NBT/BCIP (nitroblue tetrazolium/bromo-chloro-indolyl phosphate) was added as the substrate and the specimens were incubated for 30 minutes. The reaction was stopped with a final rinse in water. Slides were lightly counterstained in methyl green and evaluated by conventional light microscopy.

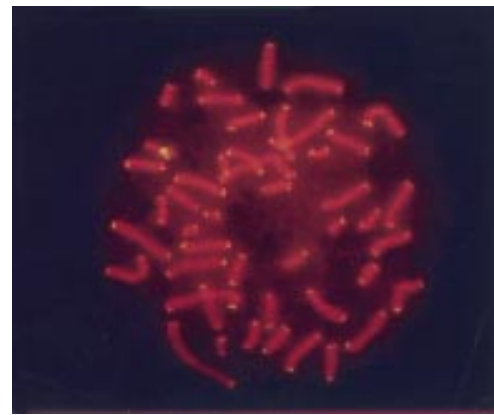


Figure 1 Peptide nucleic acid in situ hybridisation (PNA-ISH) telomere repeat assay in metaphase spreads showing signals at chromatid ends.

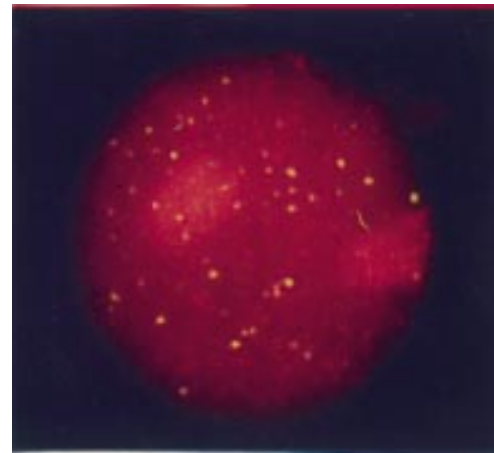


Figure 2 Peptide nucleic acid in situ hybridisation (PNA-ISH) telomere repeat assay of an interphase nucleus showing discrete intranuclear localisation of telomeric signals (for accurate counting an automated microscope should be used for planar focusing).

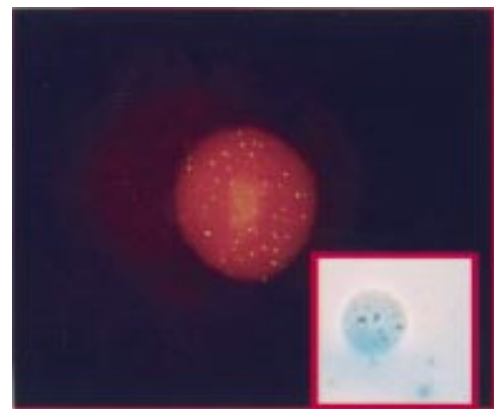


Figure 3 Peptide nucleic acid in situ hybridisation (PNA-ISH) telomere assay in interphase nuclei: fluorescent versus chromogenic detection (insert).

### Results

The FITC labelled peptide nucleic acid probe was used to detect several kilobases of tandem repeats at the end of each chromosome by in situ hybridisation. Using a PNA probe complementary to the telomeric repeat sequences, the telomeres appeared as green/yellow fluorescent spots at the end of both chromatids in the metaphase spreads (fig 1). In interphase nuclei, up to 96 dots can be visualised as scattered intra-

nuclear dots (fig 2). Because it is feasible to detect all chromatid telomere repeats in interphase nuclei, the fluorescent PNA-ISH is a very sensitive and efficient hybridisation method, superior to other oligonucleotide hybridisation protocols for telomeric repeats.<sup>16–18</sup>

The best results for hybridisation were achieved when the probe and cells were initially denatured at 80°C for three minutes. Results were also obtained for an initial denaturation step at 65°C. Hybridisation was performed for 30 minutes at 37°C, making this method faster than other PNA-ISH protocols.<sup>14</sup>

The decreased hybridisation time results from the enhanced hybridisation properties of the PNA molecule.

Chromogenic detection confirmed the FISH results (fig 3).

### Discussion

This paper shows that peptide nucleic acid in situ hybridisation (PNA-ISH) is an extremely sensitive method for the localisation of telomeric signals in both metaphase spreads and interphase nuclei. In contrast, similar hybridisation experiments (such as fluorescent ISH (FISH) or primer in situ synthesis (PRINS)) that use a fluorescent labelled RNA or DNA oligomer (20–35 mer) are not as sensitive or specific as protocols that use PNA oligonucleotides (unpublished data). A fluorescent PNA probe was used to hybridise to the DNA telomere repeat sequence (CCC TAA)<sub>n</sub>. In particular, the use of PNA technology reduced dramatically the time required from four to 12 hours to less than 30 minutes. Human telomeres can also be localised on fixed interphase nuclei by immunocytochemical chromogenic detection, using an anti-FITC alkaline phosphatase conjugate. This detection system had considerable disadvantages in terms of sensitivity and intensity of the signal.

Only a few protocols for PNA in situ hybridisation have been published,<sup>19–21</sup> although there is a huge potential for the use of PNA oligonucleotides as probes in molecular biology and clinical diagnostics.

The neutral, peptide like backbone of the molecule greatly facilitates the formation of PNA–DNA or PNA–RNA hybrids. No repulsion phenomena occur between any PNA and DNA or RNA target sequences. Importantly, these PNA hybrids are more stable and are only minimally affected by changes in salt concentration and hybridisation temperature. Therefore, the whole hybridisation process is easier and faster than existing protocols. We found that the best results were achieved for the detection of telomeric ends with an initial denaturation step of 80°C.

Because 30% formamide is added to the hybridisation solution, it decreased the melting temperature (T<sub>m</sub>) for the PNA probe as well as for the DNA target sequences. Excluding the probe from the hybridisation mixture gave no signal, thereby eliminating autofluorescence.

In conclusion, fluorochrome labelled, PNA based in situ hybridisation is a sensitive, quick, and reliable detection method for telomeric

ends of chromosomes. Results can be achieved in less than two hours. This protocol is a useful diagnostic tool for the assessment of interphase nuclear telomeric repeats in cells and tissue sections without having to resort to metaphase spreads, and could be valuable for evaluating malignant or premalignant cell growth. Because telomerase activity is important for continued growth during the progression of cancer,<sup>22–24</sup> the simultaneous detection of the two counterparts, telomeric length and telomerase, which both play an important role in tumorigenesis, will provide exciting insights into tumour cell biology.

- Eghohn M, Buchardt O, Nielsen PE, *et al*. Peptide nucleic acids (PNA): oligonucleotide analogues with an achiral peptide backbone. *J Am Chem Soc* 1992;114:1895–7.
- Nielsen PE, Eghohn M, Berg RH, *et al*. Sequence selective recognition of double-stranded DNA by strand displacement with a thymine substituted polyamide. *Science* 1991;254:1497–500.
- Eghohn M, Buchardt O, Nielsen PE, *et al*. Peptide nucleic acids (PNA): a novel approach to sequence selective recognition of double-stranded DNA. In: Epton R, ed. *Innovation and perspectives in solid phase synthesis peptides, polypeptides and oligonucleotides*. Andover, UK: Intercept Ltd, 1992:325–28.
- Eghohn M, Nielsen PE, Buchardt O, *et al*. Recognition of guanine and adenine in DNA by cytosine and thymine containing peptide nucleic acids. *J Am Chem Soc* 1992;114:9677–8.
- Blackburn EH. Telomeres: no end in sight. *Cell* 1994;77:621–3.
- Sandell LL, Zakian VA. Loss of a yeast telomere: arrest, recovery and chromosome loss. *Cell* 1993;75:729–36.
- Allsopp RC, Vaziri H, Patterson C, *et al*. Telomere length predicts replicative capacity of human fibroblasts. *Proc Natl Acad Sci USA* 1992;89:10114–18.
- Vaziri H, Schachter F, Uchida I, *et al*. Loss of telomeric DNA during aging of normal and trisomy 21 human lymphocytes. *Am J Hum Genet* 1993;52:661–7.
- Vaziri H, Dragowska W, Allsopp RC, *et al*. Evidence for a mitotic clock in human hematopoietic stem cells: loss of telomeric DNA with age. *Proc Natl Acad Sci USA* 1994;91:9857–60.
- Harley CB. Telomere loss: mitotic clock or genetic time bomb? *Mutat Res* 1991;256:271–82.
- Wright WE, Shay JW. Telomere positional effects and the regulation of cellular senescence. *Trends Genet* 1992;8:193–7.
- Shay JW, Wright WE, Werbin H. Defining the molecular mechanism of human cell immortalization. *Biochem Biophys Acta* 1991;1072:1–7.
- Feng J, Funk WD, Wang SS, *et al*. The human RNA component of human telomerase. *Science* 1995;269:1236–41.
- Landsdorp PM, Verwoerd NP, van de Rijke FM, *et al*. Heterogeneity in telomere length of human chromosomes. *Hum Mol Genet* 1996;5:685–691.
- Seabright M. A rapid banding technique for human chromosomes. *Lancet* 1971;2:971–2.
- Moyzis RK, Buckingham JM, Cram LS, *et al*. A highly conserved repetitive DNA sequence, (TTAGGG)<sub>n</sub>, present at the telomeres of human chromosomes. *Proc Natl Acad Sci USA* 1988;85:6622–6.
- Meyne J, Moyzis RK. In situ hybridisation using synthetic oligomers as probes for centromere and telomere repeats. *Methods Mol Biol* 1994;33:63–74.
- Meyne J, Baker RJ, Hobart HH, *et al*. Distribution of non-telomeric sites of the (TTAGGG)<sub>n</sub> telomeric sequence in vertebrate chromosomes. *Chromosoma* 1990;99:3–10.
- Taneja KL. Localization of trinucleotide repeat sequences in myotonic dystrophy cells using a single fluorochrome-labeled PNA probe. *Biotechniques* 1998;24:472–6.
- Thisted M, Just T, Pluzek K-J, *et al*. Detection of immunoglobulin kappa light chain mRNA in paraffin sections by in situ hybridisation using peptide nucleic acid probes. *Cell Vis* 1996;3:358–64.
- Hultdin M, Gronlund E, Norrback K-F, *et al*. Telomere analysis by fluorescence in situ hybridisation and flow cytometry. *Nucleic Acids Res* 1998;16:3651–6.
- Harley CB, Villeponteau B. Telomeres and telomerase in aging and cancer. *Curr Opin Genet Dev* 1995;5:249–55.
- Ishikawa F. Telomere crisis, the driving force in cancer cell evolution. *Biochem Biophys Res Commun* 1997;230:1–6.
- Nakayama J, Tahara H, Tahara E, *et al*. Telomerase activation by hTRT in human normal fibroblasts and hepatocellular carcinomas. *Nat Genet* 1998;18:65–8.



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