Telomere PNA Kit/FITC for Flow Cytometry

Code K5327

6th edition

For detection of telomeres by flow cytometry using fluorescence in situ hybridization and a fluorescein-conjugated PNA probe.

The kit contains reagents for 20 tests with duplicate determinations.

For research use only. Not for use in diagnostic procedures.

Contents

Recommended Use

Telomere PNA Kit/FITC for Flow Cytometry is intended for research use only. The kit is recommended for the detection of telomeres in nucleated haematopoietic cells using a fluorescein-conjugated peptide nucleic acid (PNA) probe. The kit may be used for the detection of telomeres from all vertebrate nucleated haematopoietic cells. Results are evaluated by flow cytometry using a light source with excitation at 488 nm.

Introduction

Prior to mitotic cell division, all cellular DNA is duplicated by the action of a DNA polymerase. However, this enzyme does not replicate the very ends of the chromosomes, the so-called telomere region. As a consequence, the telomeres get shorter after each cell division. In normal somatic cells, this shortening contributes to cellular senescence and the telomeres act as a “mitotic clock”. Telomeres in all vertebrates are composed of a sequence of six nucleotides (TTAGGG) repeated from a few hundred to several thousand times. The length of telomeres is species-specific and ranges from 5-20 kilobases in man to 20-150 kilobases in mice. Telomeres are considered to have a protective function, keeping the chromosome ends intact, and thereby protecting the underlying genes and avoiding fusion of chromosome ends.

Some specialized cells maintain their capacity to divide. Examples are germ cells, fetal cells, haematopoietic stem cells, and basal cells of the epidermis. These cells overcome the ageing problem by the action of the enzyme, telomerase (1). Telomerase maintains telomere length by adding hexameric repeats to the telomeric ends of the chromosomes, thus compensating for the continued shortening of telomeres that would otherwise occur.

The length of the telomeres depends both on the age of the cell donor and on the number of times the cell has divided. In general, fibroblasts cultured from a foetus are able to divide more times in culture than those from a child, which in turn may divide more times than those from an adult. However, all normal diploid cells are “mortal” and have a limited capacity to proliferate in culture. In contrast, established tumour cell lines can divide forever and are “immortal” mainly due to reactivation of telomerase. Almost 90% of human primary tumours express telomerase, while the cells of most normal tissues lack this enzyme activity. Recently, the hypothesis that the activation of telomerase is necessary for extension of the life-span of human cells by avoiding telomere shortening has been proven (1). Telomerase activity and the preservation of telomere length are, therefore, important for the cancerous process and for the sustained growth of most solid tumours both in vivo and in vitro.

The telomere length can be measured by quantitative FISH in both human (2) and other vertebrate cells (3). Chromosome-specific factors regulate the length of the individual telomere. Short telomeres on the human chromosome 17p might be responsible for the frequent loss of 17p alleles in human cancer, e.g. the tumour suppressor gene for the p53 protein (4). The genetically determined variation in telomerase activity between individuals (5) makes telomere length measurements relevant for the study of age-related diseases. The average telomere length of chromosomes in a cell population can be estimated by quantitative FISH in flow cytometry (6, 7).

Summary and Explanation

The Telomere PNA Kit/FITC for Flow Cytometry provides a convenient method for detection of the telomeric sequences in nucleated haematopoietic cells from vertebrates. The probe of this kit does not recognize subtelomeric sequences, and in contrast to traditional telomere restriction fragment (TRF) measurements, the Dako kit, therefore, allows an estimation of the telomere length without inclusion of subtelomeres. The kit has been designed so that post-hybridization treatments are kept to a minimum and formamide washes are avoided. The probe of the kit recognizes telomeric repeats, but also potential intrachromosomal TTAGGG repeats will be included in the fluorescence signal. In the literature the prevalence of non-telomeric TTAGGG sequences vary (10, 11), but were found to be few and short using the sensitive Q-FISH method with a PNA probe (4). These data and the good correlation with Southern blotting support the idea that intrachromosomal telomere sequences are of less significance for the total hybridization signal detected by flow cytometry (6).

It is recommended that all samples are run in duplicate and that the mean values of the duplicate determinations are used for data analysis.

PNA is a synthetic DNA/RNA analog capable of binding to DNA/RNA in a sequence-specific manner obeying the Watson-Crick base pairing rules. In PNA the sugar phosphate backbone has been replaced by a neutral peptide/polyamide backbone keeping the distances between the bases exactly the same as in DNA (8). The neutral peptide backbone gives PNA excellent properties for hybridizing to DNA/RNA. In addition, PNAs are highly resistant to degradation by DNases, RNases, proteinases, and peptidases.

This kit uses PNA probes, which are superior to DNA probes in terms of sensitivity and specificity (2). The method is optimal for estimation of telomere length, as the fluorescence intensity of the cells is directly correlated to the length of the telomeres (6, 7).

Principle of the Procedure

This kit provides all key reagents needed for performing fluorescence in situ hybridization in solution for detection of telomere sequences by flow cytometry. On a single cell suspension (consisting of a mixture of sample cells (nucleated haematopoietic vertebrate cells) and control cells, both provided by the user), the sample DNA is denatured for 10 minutes at 82 °C in a microcentrifuge tube either in the presence of hybridization solution without probe or in hybridization solution containing fluorescein-conjugated PNA telomere probe. Then hybridization takes place in the dark at room temperature (RT) overnight. The hybridization is followed by two 10-minute post-hybridization washes with a Wash Solution at 40 °C. The sample is then resuspended in an appropriate buffer for further flow cytometric analysis. It is recommended to use the DNA Staining Solution included in the kit for identification of $G_{0/1}$ cells. DNA Staining Solution may also be provided by the user, or the cells may be resuspended in Wash Solution if no DNA staining is wanted. After flow cytometric analysis, the data obtained can be used for determination of a relative telomere length (RTL).

The RTL value is calculated as the ratio between the telomere signal of each sample and the control cell (1301 cell line) with correction for the DNA index of $G_{0/1}$ cells. Sample cells and control cells should be analyzed separately for DNA ploidy in order to be able to make an accurate compensation for the cellular DNA content, e.g. using Dako Staining Kit for DNA Analysis (CN300/CN301) or propidium iodide staining according to Vindeløv et al. (12). This correction is performed in order to standardize the number of telomere ends per cell and thereby telomere length per chromosome. The propidium iodide staining of DNA included in the Telomere PNA Kit/FITC procedure is undersaturated and should not be used for DNA content determination.

A positive reaction may after cytocentrifugation also be recognized in a fluorescence microscope as a green fluorescence signal at the sites of hybridization.

Reagents and Materials

A. Reagents provided

HYBRIDIZATION SOLUTION

Hybridization Solution (ready-to-use)
Vial 1 (12 mL)
Hybridization solution without probe.
Contains 70% formamide.

TELOMERE PNA PROBE/FITC

Telomere PNA Probe/FITC in Hybridization Solution (ready-to-use)
Vial 2 (12 mL)
Fluorescein-conjugated telomere PNA probe in hybridization solution.
Contains 70% formamide.

WASH SOLUTION (X 10)

Wash Solution (x 10)
Vial 3 (20 mL)
10 x concentrated buffer for post-hybridization washing of samples. Dilute in pure water.

DNA-STAINING SOLUTION (X 10)

DNA-Staining Solution (x 10)
Vial 4 (4 mL)
10 x buffer with propidium iodide and RNase A for staining of DNA before flow cytometric analysis. Dilute in pure water.

B. Reagents required but not provided

1. Phosphate-buffered saline (PBS) for washing of cells prior to in situ hybridization procedure.
2. Pure water.

C. Materials required but not provided

1. General laboratory equipment for flow cytometric procedures.
2. Sample cells.
3. Control cells.
4. Bürker chamber.
5. Heating block, adjustable to 40 °C and 82 °C.
6. 1.7 mL microcentrifuge tubes, micropipettes, and tips.
7. Microcentrifuge, adjustable to 500 x g.
8. Flow cytometer capable of excitation at 488 nm and emission analysis at 530 nm and 617 nm.

D. Preparation of reagents

Wash Solution (working solution)

At 2-8 °C crystals may form in Vial 3. If crystals are present, leave the vial at RT until the crystals dissolve. Do not dilute the Wash Solution (x 10) until all crystals have dissolved.

Dilute required volume of Washing Solution (x 10) Vial 3, 1:10 in pure water. Stability after dilution: 6 months at 2-8 °C.

DNA Staining Solution (working solution)

At 2-8 °C crystals may form in Vial 4. If crystals are present, leave the vial at RT until the crystals dissolve. Do not dilute the DNA Staining Solution (x 10) until all crystals have dissolved.

Dilute required volume of DNA Staining Solution (x 10) Vial 4, 1:10 in pure water. Prepare fresh for each experiment and protect from light after dilution.

E. Storage of reagents

The reagents of the kit should be stored at 2-8 °C. Vials 1 and 2 contain formamide, which is sensitive to light. This is also the case for the fluorescein-conjugated probe in Vial 2 and for the propidium iodide in Vial 4. Therefore Vials 1, 2 and 4 should be protected from the light. Keep all vials tightly closed.

F. Precautions

1. The device is not intended for clinical use including diagnosis, prognosis, and monitoring of a disease state, and it must not be used in conjunction with patient records or treatment.
1.2. The Hybridization Solution and the Telomere PNA Probe/FITC in Hybridization Solution (Vials 1 and 2) contain 70% formamide and are labelled:
Toxic.
R61 May cause harm to the unborn child.
S45 In case of accident or if you feel unwell, seek medical advice immediately (show label where possible).
S53 Avoid exposure - obtain special instructions before use.
S60 This material and/or its container must be disposed of as hazardous waste.
Exclusively for professional use.

As a main rule, persons under 18 years of age are not allowed to work with this product. Users must be carefully instructed in the proper working procedure, the dangerous properties of the product and the necessary safety instructions. Please refer to the Material Safety Data Sheet (MSDS) for additional information.

Assay Procedure

The procedure has been developed for use with single cell suspensions of haematopoietic cells, and has been adjusted to give optimal results when using the Telomere PNA Kit/FITC for Flow Cytometry, code No. K5327.

NOTE: All incubations are at RT unless otherwise stated.

Day 1: Start by pre-warming the heating block to 82 °C. Place Vials 3 and 4 at RT and leave until Day 2. Follow the procedure described below for Day 1. On Day 1 also reserve some sample cells for DNA index measurement, e.g using propidium iodide staining according to Vindeløv et al. (12). DNA index measurement can be omitted if the sample cells are known to be diploid and the DNA index of the control cells is known.

Day 2: Start by pre-warming the heating block to 40 °C. Make sure that crystals in Vials 3 and 4 have dissolved before diluting the solutions to working strengths. Follow the procedure described below for Day 2.

Day 1

Pre-treatment	Wash sample cells and control cells in PBS.	
	Count cells in Bürker chamber[1].	
	For each sample, mix 2 x 10^6 test cells and 2 x 10^6 control cells. Add PBS to a total of 6 mL. Divide the mixture into four 1.5 mL aliquots (1 x 10^6 cells) and place in 1.7 mL microcentrifuge tubes, labeled A, B, C, and D. Close the lids.	
	Centrifuge cells at 500 x g.	5 min
	Remove as much as possible of supernatant by suction/aspiration.	
Denaturation	Add 300 µL of Hybridization Solution, Vial 1, to each of the 2 tubes A and B (controls) and 300 µL of Telomere PNA Probe/FITC in Hybridization Solution, Vial 2, to each of the other 2 tubes C and D. Close lids and mix by using a vortex mixer. Make sure the cell pellets are properly resuspended.	
	Place the 4 tubes in a pre-warmed heating block adjusted to 82 °C[2].	10 min
Hybridization	Mix by using a vortex mixer and place the tubes in the dark at RT.	Overnight

Day 2

Washing	Add 1 mL of Wash Solution (Vial 3 diluted 1:10) to each of the 4 tubes. Close lids and mix by using a vortex mixer.	
	Place the tubes in a pre-warmed heating block adjusted to 40 °C.	10 min
	Mix by using a vortex mixer and centrifuge tubes at 500 x g.	5 min
	Gently decant the supernatant into appropriate waste container.	
	Add 1 mL of Wash Solution (Vial 3 diluted 1:10) to each microcentrifuge tube. Close lid and mix by using a vortex mixer.	
	Place the tubes in a pre-warmed heating block adjusted to 40 °C.	10 min
	Mix by using a vortex mixer and centrifuge tubes at 500 x g.	5 min
	Gently decant the supernatant into appropriate waste container.	

DNA staining	Add 0.5 mL of DNA Staining Solution (Vial 4 diluted 1:10) to each of the 4 tubes. Close lids and mix by using a vortex mixer.	
	Transfer the 4 cell suspensions, A, B, C, and D to 4 flow cytometry tubes labeled A, B, C, and D. Mix the samples by using a vortex mixer just before the transfer to minimize cell loss caused by adherence of cells to tube walls.	
	Leave tubes in the dark at 2-8 °C[3].	2-3 h
Analysis	Analyze samples on a flow cytometer using logarithmic scale FL1-H for probe fluorescence and linear scale FL3-H for DNA staining. Save forward scatter, side scatter, FL1-H and FL3-H (optionally FL3-A and FL3-W) values.	
	Analyze data as described under Interpretation of Results.	

Procedural notes

1. To achieve a good reproducibility it is important that the initial cell count for the control cells is accurate. If cells are counted in a Bürker chamber, we recommend that 64 fields should be counted. The 1301 cell line should be preferred as control cells as they have very long telomeres. 1301 is a sub-line of the EBV-genome negative T-cell leukaemia line, CCRF-CEM (9).
2. Denaturation should be performed at minimum 80 °C and maximum 84 °C. Check the temperature of the heating block carefully. Denaturing temperatures below 75 °C impair results seriously.
3. The 2-3 hours DNA staining time in the protocol should be regarded as the minimum time required. If it is more convenient to stain the samples for longer (up to 24 hours), then this can be done without affecting the results if samples are kept in the dark at 2-8 °C.

Interpretation of Results

Samples hybridized with the Telomere PNA Probe/FITC should exhibit a fluorescence signal in FL1, which is higher than the background/autofluorescence signal obtained from the sample of the same cells hybridized with the hybridization solution without probe. When analyzing flow cytometry data for cells hybridized with or without the Telomere PNA Probe/FITC it is important only to look at cells in the $G_{0/1}$-phase of the cell cycle where the cell has one copy of its genome. This is achieved by setting the correct gates, see Figures 1 and 2. If cells in S or G_2/M-phase are not removed by gating, the estimated RTL will not be per genome equivalent.

Example

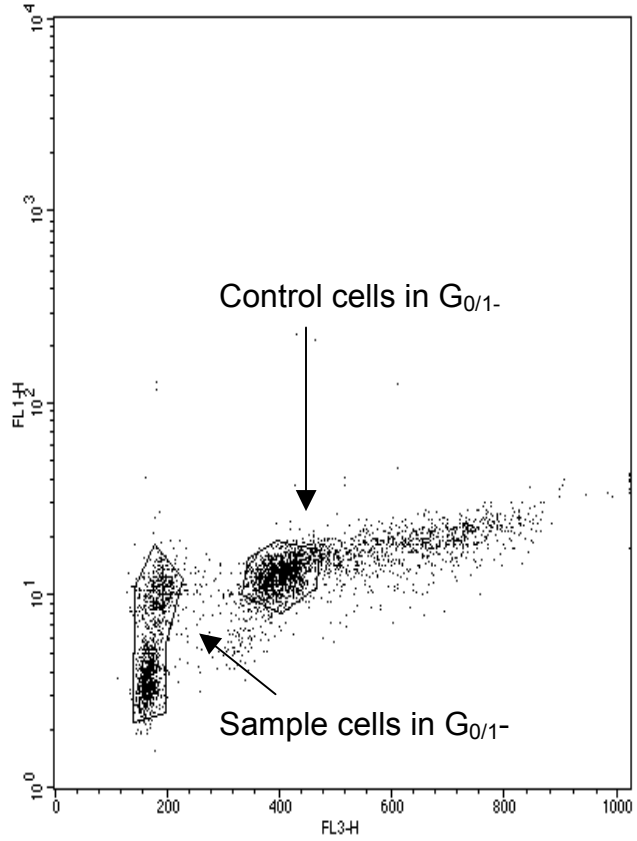

Figure 1. Dot plot of FL1-height versus FL3-height of cells hybridized with Hybridization Solution (Vial 1) *without* Telomere PNA Probe. Gates are set around cells in the $G_{0/1}$-phase for both sample cells (human mononuclear cells isolated from blood) and control cells (1301 cell line).

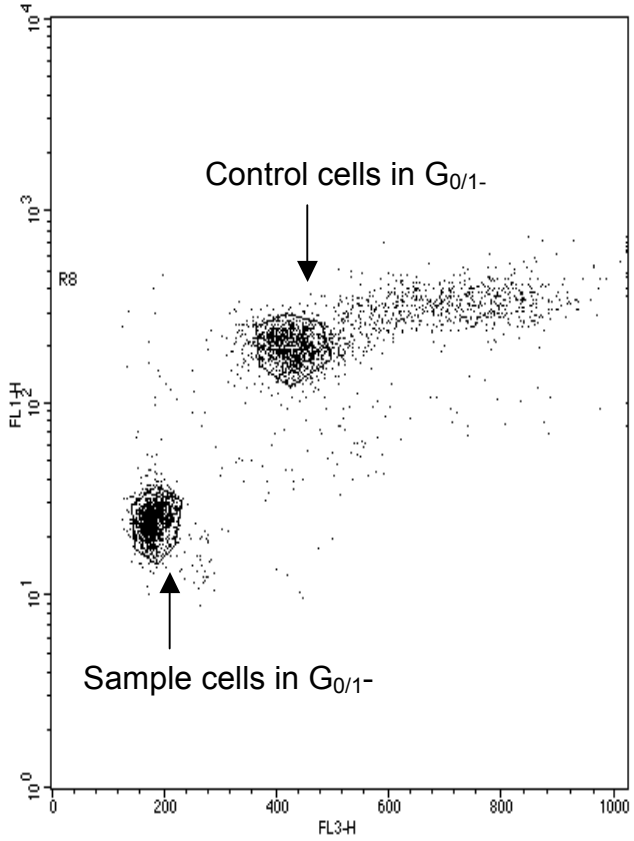

Figure 2. Dot plot of FL1-height versus FL3-height of cells hybridized with Telomere PNA Probe/FITC in Hybridization Solution (Vial 2). Gates are set around cells in the $G_{0/1}$-phase for both sample cells (human mononuclear cells isolated from blood) and control cells (1301 cell line).

In the example, human mononuclear cells isolated from blood by gradient centrifugation were analyzed. The cells were mixed 1:1 with control cells (1301 cell line). If preferred, the single cell populations can also be selected using a gate set in the dot plot of FL3-width versus FL3-area. The gated cells can then be displayed in the FL3-height versus FL1-height dot plot. As shown in the FL3-height versus FL1-height dot plots (Figures 1 and 2), it is possible to identify cells in the $G_{0/1}$-phase and set gates around these populations. Statistical data on these cells, together with the DNA index of the cells determined by traditional DNA measurement, are then used for calculation of RTL of the sample cells compared to the control cells in the following way:

$$\text{RTL} = \frac{\text{(mean FL1 sample cells with probe - mean FL1 sample cells without probe) x DNA index of } \mathbf{control} \text{ cells x 100}}{\text{(mean FL1 control cells with probe - mean FL1 control cells without probe) x DNA index of } \mathbf{sample} \text{ cells}}$$

In the example the figures were:

$$\text{RTL} = \frac{(24.6 - 6.3) \times 2 \times 100}{(197.8 - 12.9) \times 1} = 19.8\%$$

The above RTL-value indicates that the average telomere fluorescence per chromosome/genome in the sample cells (normal human mononuclear cells isolated from blood) is about 19.8% of the telomere fluorescence per chromosome/genome in the control cells (1301 cell line).

As can be seen from Figure 1, the population of sample cells consists of two subpopulations, monocytes and lymphocytes. The monocyte population has a slightly higher autofluorescence/background than the lymphocyte population.

Reproducibility

A reproducibility study of the Telomere PNA Kit/FITC for Flow Cytometry was performed at Dako's laboratories.

The complete study showed RTL-values with a standard deviation of 8-13% for single determinations and 6-9% for duplicate determinations.

Some variation in RTL was observed when different persons performed the assay.

Only minimal variability was observed when two different persons performed the analysis of flow data (gating and calculation of RTL).

Variation between flow cytometers

If results obtained on different flow cytometers are to be compared, it is essential that proper quality control of the flow cytometer has been performed. We have observed a difference in the results obtained when analyzing a single sample on different flow cytometers. For example, one sample gave an RTL value of 9.1% on one flow cytometer and 5.6% on another. When the two flow cytometers were checked using Dako FluoroSpheres calibration beads, code No. K0110, it was revealed that they had different linearity. In a plot of observed FL-values against known bead fluorescence, one flow cytometer had a slope of 1.0 and the other had a slope of 0.8. It is mandatory that this type of variation between individual flow cytometers is taken into account when results from samples analyzed on different flow cytometers are compared.

Sample Preparation and Storage

The Telomere PNA Kit/FITC for Flow Cytometry is recommended for use on nucleated haematopoietic cells from all vertebrates. The cells can be used fresh or after storage in 10% DMSO in liquid nitrogen or at –80 °C. Cells must not have been submitted to fixation before use in the assay.

As control cells we recommend the cell line 1301, which is tetraploid and has very long telomeres (> 30 kilobases). The 1301 cell line is very easy to distinguish from most other cell types because of its characteristic features: Tetraploidy (seen in FL3) and long telomeres (seen in FL1). In principle, other cell types that are easily distinguished from the sample cells can be applied.

The 1301 cell line can be ordered through the European Collection of Cell Cultures in the U.K. (www.ecacc.org.uk) and has accession No. 01051619.

Appendix A: Data acquisition, including set-up of flow cytometer

1. Set up the flow cytometer using standard operating procedures.
2. Select logarithmic amplification for FL1-height and linear amplification for FL3-height, optionally FL3-width and FL3-area. Forward scatter and side scatter can be set at the user's preference.
3. Establishment of the Window of Analysis: Make pre-adjustments of the PMTs of the relevant fluorescence detectors to assure that cells hybridized with and without Telomere PNA Probe/FITC are displayed on scale as shown in Figures 1 and 2, respectively.

When using flow cytometry for quantitative purposes, it is important to perform quality control of the flow cytometer. Linearity of the instrument should be checked by using e.g. Dako FluoroSpheres calibration beads, code K0110. This will give a good estimation of the linearity of the instrument.

References

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Related Products

Dako Telomere PNA FISH Kit/FITC, code K5325

Dako Telomere PNA FISH Kit/Cy3, code K5326

These kits are for the detection of telomeres by fluorescense microscopy using fluorescein or Cy3 as fluorochrome, respectively.

Explanation of symbols

REF	Catalogue number		Keep away from sunlight (consult storage section)		Use by
	Consult instructions for use		Contains sufficient for <n> tests		Manufacturer
	Temperature limitation	LOT	Batch code		Toxic

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