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ADVANCES IN MOLECULAR HEMATOLOGY

Split-signal FISH for detection of chromosome aberrations

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Abstract
Chromosome aberrations are frequently observed in hematopoietic malignancies. These aberrations can deregulate expression of an oncogene, resulting in aberrant expression or overexpression, or they can form leukemia-specific chimeric fusion proteins. Detection of chromosome aberrations is an important tool for classification of the malignancy and for the definition of risk groups, which need different treatment protocols. We developed rapid and sensitive split-signal fluorescent in situ hybridization (FISH) assays for frequently occuring chromosome aberrations. The split-signal FISH approach uses two differentially labeled probes, located in one gene at opposite sites of the breakpoint region. In normal karyotypes, two co-localized green/red signals are visible, but a translocation results in a split of one of the co-localized signals. Split-signal FISH has three main advantages over the classical fusion-signal FISH approach, which uses of two labeled probes located in two genes. First, the detection of a chromosome aberration is independent of the involved partner gene. Second, split-signal FISH allows the identification of the partner gene or chromosome region if metaphase spreads are present, and finally it reduces false-positivity.

Keywords: *Fluorescence* in situ *hybridization (FISH), split-signal FISH, chromosome aberrations, fusion gene, peptide nucleic acid, leukemia, lymphoma*

Chromosome aberrations in hematopoietic malignancies

Chromosome aberrations play an important role in hematological malignancies [1]. Most of these aberrations concern balanced translocations involving genes that play key roles in the development and function of hematopoietic cells, such as transcription factors, cell cycle regulators, and signal transduction molecules. Chromosome translocations can result in deregulated expression of (onco)genes as a direct consequence of a translocation to a regulatory element, e.g., an immunoglobulin (Ig) or T-cell receptor (TCR) enhancer [2,3]. Ig and TCR-gene related chromosome aberrations are particularly found in mature B-cell malignancies, such as various types of B-cell non-Hodgkin lymphomas (B-NHL), and in immature T-cell malignancies, mainly T-cell acute lymphoblastic leukemias (T-ALL). Alternatively, translocations can result in fusion of two genes that encode leukemia-specific chimeric (fusion) proteins. Fusion genes with expres-

sion of aberrant fusion proteins are particularly found in precursor-B-ALL, acute myeloid leukemias (AML), and chronic myeloid leukemia (CML). The fusion proteins have functional features that differ from the corresponding wild type proteins and mostly play a role in oncogenesis. In addition to the new features of the fusion protein, loss of wild type activity due to the translocation (in some translocations enhanced by deletion of the second allele) might contribute to oncogenesis.

Several clinical studies have demonstrated that chromosomal translocations are useful markers contributing to classification of the malignancies and to the definition of risk groups, that need different treatment protocols. In precursor-B-ALL *MLL* gene translocations and t(9;22) with the *BCR-ABL* fusion gene are associated with a poor prognosis, while t(12;21) with the *TEL-AML1* fusion gene is associated with good outcome. Analogously, in AML inv(16) with *CBFB-MYH11*, t(8;21) with *AML1-ETO*, and t(15;17) with *PML-RARA* are associated

with good prognosis, while 11q32 (*MLL* gene) aberrations show poor outcome in AML as well [1].

Detection of chromosome aberrations

Several techniques can be used for the detection of chromosome aberrations, each having its inherent advantages and disadvantages (Table I). An advantage of *conventional karyotyping* is that it is highly informative as virtually all abnormalities can be detected. This includes not only structural abnormalities, but also numerical abnormalities such as hypo-, or hyperploidy. However, the interpretation may be difficult if the karyotype is complex. Another disadvantage is that for some samples no reliable results can be obtained because of a low mitotic index or poor chromosome morphology. In addition, some chromosome abnormalities are cryptic, i.e. they cannot be identified via conventional karyotyping, because changes in chromosome banding patterns are too marginal to be detected, such as t(12;21), t(5;14) and *SIL-TAL1* fusions [4].

Chromosome aberrations can also be identified via *Southern blot or PCR analysis on genomic DNA*. Southern blotting is considered to be technically demanding and laborious and the applicability to the detection of

chromosome translocations is limited, because the breakpoints in many translocations are scattered over large regions (>25kb). Nevertheless, Southern blot analysis has proven to be useful for detection of *MLL* translocations. As the *MLL* gene can have many translocation partner genes and the breakpoint region is relatively small (6.5 kb), Southern blot analysis is suitable for the detection of *MLL* rearrangements, independent of the partner gene [5,6]. Southern blotting has also been used for detection of *E2A (ETV6)* gene rearrangements, since the majority of *E2A (ETV6)* breakpoints are located in a breakpoint region of 15 kb [7].

PCR analysis on the DNA level is relatively easy for detection of *SIL-TAL1* fusion genes [8,9]. but much more complex for other translocations, mainly because PCR analysis needs multiple primers, if genomic breakpoint regions are larger than 2 to 4 kb [10–12].

An alternative approach, which is suitable for detection of chromosome translocations resulting in formation of fusion genes, is *detection of fusion genes or fusion gene transcripts* via (nested) RT-PCR analysis [13]. The advantage of this approach is that it reaches sensitivities of 1 cell in 10^4 to 1 cell in 10^6 cells, enabling detection of minimal residual disease

Table I. Techniques for detection of chromosome aberrations

Technique	Advantages	Disadvantages
Karyotyping	Detection of virtually all abnormalities (structural and numerical abnormalities)	Interpretation may be difficult if the karyotype is complex Sometimes low mitotic index or poor chromosome morphology Some chromosome abnormalities are cryptic
Southern blotting	Detection of chromosome aberration independent of partner gene/chromosome region	Technically demanding and laborious Detection of chromosome aberrations is limited, if breakpoints are scattered over large regions (>25 kb) Focused on a specific type of aberration, determined by the probe
PCR	Can be easy for some chromosome aberrations e.g. *SIL-TAL1* gene fusion Sensitive, allowing detection of minimal residual disease	Detection of chromosome translocations is limited, if breakpoints are scattered over large regions (2 to 4 kb) Variant translocations can easily be missed, if these variants are not covered by the used primers
RT-PCR	Suitable for detection of chromosome aberrations with formation of fusion genes Sensitive, allowing detection of for minimal residual disease	Variant translocations can easily be missed, if these variants are not covered by the used primers
FISH *general*	Dividing cells (metaphase nuclei) and non-dividing cells (interphase nuclei) can be analyzed Detection of cryptic aberrations	Focused on a specific type of aberration, determined by the applied probe set
fusion-signal	Detection of the fusion of two partner genes involved in the translocation	Translocations with other partner genes are missed 5–10% false-positivity
split-signal	Detection of chromosome aberration independent of partner gene/chromosome region Identification of partner gene/chromosome region if metaphase spreads are present Minimization of false-positivity	Partner gene/chromosome region can not be identified if metaphase spreads are absent

Table II. Examples of classical chromosome aberrations, which are easily detectable by split-signal FISH.

Disease category	Target gene for split-signal FISH	Detectable chromosomes aberration		Involved genes	Relative frequency per disease category
Precursor-B-ALL	E2A (TCF3)	(1;19)	(q23;p13)	E2A-PBX1	5–6%
		t(17;19)	(q22;p13)	E2A-HLF	~1%
	MLL	t(4;11)	(q21;q23)	MLL-AF4	4% (40% of infant ALL)
		t(9;11)	(p22;q23)	MLL-AF9	rare (10% of infant ALL)
		t(11;19)	(q23;p13)	MLL-ENL	1% (25% of infant ALL)
	TEL (ETV6)	t(12;21)	(p13;q22)	TEL-AML1	25% (childhood); <2% (adults)
	BCR	t(9;22)	(q34;q11)	BCR-ABL	4–7% (childhood); 25–45% (adults)
T-ALL	TAL1	del (1)	(p32;p32)	SIL-TAL1	20–25% (childhood); 10% (adults)
		t(1;14)	(p32;q11)	TCRD-TAL1	3%
	HOX11L2 (TLX3)	t(5;14)	(q34;q32)	TCRD-HOX11L2	15–20%
	LMO2 (RBTN2)	t(11;14)	(p13;q11)	TCRD-LMO2	7%
	CALM	t(10;11)	(p13;q14)	CALM-AF10	~10%
B-NHL	CCND1 (BCL1)	t(11;14)	(q13;q32)	IGH-CCND1	>90% of MCL
	BCL2	t(14;18)	(q32;q21)	IGH-BCL2	>80% of FCL; 25% of DLBCL
	MYC	t(8;14)	(q24;q32)	IGH-MYC	~90% of Burkitt lymphoma
		t(2;8)	(p11;q24)	IGK-MYC	~5% of Burkitt lymphoma
		t(8;22)	(q24;q11)	IGL-MYC	~5% of Burkitt lymphoma
	MALT1	t(11;18)	(q21;q21)	MALT1-API2	25–50% of MALT lymphoma
	PAX5	t(9;14)	(p13;q32)	IGK-PAX5	~50% of LPL
	BCL6	t(3;14)	(q27;q32)	IGH-BCL6	10–20% of DLBCL
	BCL10	t(1;14)	(p22;q32)	IGH-BCL10	5–10% of MALT lymphoma
	ALK	t(2;5)	(p13;q35)	NPM-ALK	~75% of ALCL
		t(1;2)	(q21;p13)	TPM3-ALK	~15% of ALCL

Abbreviations: MCL, mantle cell lymphoma; FCL, follicular lymphoma; DLBL, diffuse large B-cell lymphoma; ALCL, anaplastic large cell lymphoma; MALT, mucora associated lympoid tissue; LPL, lymphoplasmacytic lymphoma (immunocytoma).

(MRD) [13–15]. A disadvantage of PCR-based methods is that variant translocations can more easily be missed, if these variants are not covered by the used primers.

Detection of chromosome translocations with fusion genes can also be performed at the protein level via the specific detection of the fusion proteins. This technique, however, has not yet been implemented in routine diagnostics, due to lack of appropriate antibodies that specifically detect only the fusion protein and not the two wild type proteins of which the fusion protein is composed.

Fluorescence in situ hybridization (FISH) is a molecular cytogenetic technique, which uses fluorescently labeled probes for detection of specific chromosome aberrations. The advantage of this technique is that besides dividing cells (metaphase nuclei) also non-dividing cells (interphase nuclei) can be analyzed, which allows a rapid screening of a large number of cells even if the malignant clone did not divide under culture conditions. In addition, also cryptic aberrations can be detected [16,17]. A disadvantage of FISH analysis compared to cytogenetics is that this technique is focused on a specific type of aberration, determined by the applied probe set.

Fusion-signal FISH versus split-signal FISH

There are two main approaches of FISH probe design for use on (interphase) nuclei, i.e. fusion-signal FISH and split-signal FISH [18]. The classical fusion-signal FISH approach uses two differentially labeled probes, red and green, which flank the breakpoint regions of the two genes, which are involved in the translocation (Figure 1A). In normal karyotypes, i.e. without chromosome aberration, two red signals and two green signals are detectable. In case of a translocation, a red and a green signal will be juxtaposed giving rise to a co-localized green/red signal, which will generally appear as a yellow signal. In addition, separate green and red signals of the unaffected chromosomes will be visible.

The split-signal FISH approach also uses two differentially labeled probes, but these probes are located in only one of the two involved genes, hereafter called the target gene, and are positioned at opposite sides of the breakpoint region of the target gene (Figure 1B) [18–20]. In normal karyotypes, two co-localized green/red signals usually appearing yellow will be visible. A translocation will result in a split of one of the co-localized signals, resulting in a separate green and red signal together with a fused signal of the unaffected chromosome [19,20].

The split-signal FISH approach has several advantages over the more traditional fusion-signal FISH. First, the detection of a translocation is independent of the involved partner gene. This is particularly of great interest for target genes with multiple partner genes such as *MLL* and *E2A (ETV6)* (Figure 2) [19]. Although the detection is independent of the involved partner gene or partner chromosome, split-signal FISH in principle allows the identification of the partner chromosome, if metaphase spreads are present on the slide. As a result of the translocation, one
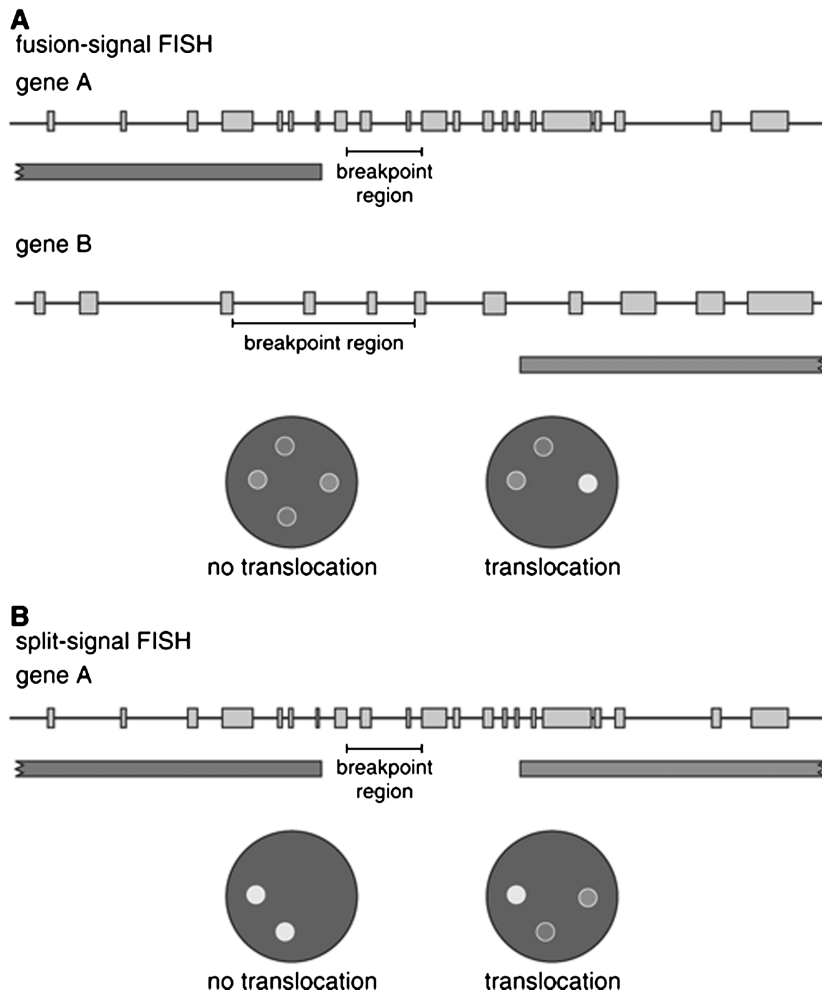
Figure 1. Differences between fusion-signal FISH and split-signal FISH. A. Fusion-signal FISH with two probes located in the two genes, which are involved in the chromosome translocation. In normal situations, two green and two red signals will be present. In case of a translocation, a green and a red signal co-localize generally appearing as rise to a yellow signal together with the separate green and red signals of the unaffected genes. B. Spit-signal FISH with two probes positioned at opposite sides of the breakpoint region in one of genes, which are involved in the chromosome translocation. In normal situations, two yellow signals will be present, while in case of a translocation separate green and red signals will be present together with the co-localized signal of the unaffected gene.

of the probes moves to the partner chromosome, i.e. der(partner), while the other probe remains on the der(target) chromosome. The split-signal approach therefore also allows the detection of new partner chromosomes or chromosome regions. Further molecular analysis can then be performed to identify the new partner gene, such as panhandle PCR or long distance inverse PCR [21,22].

Another advantage of split-signal FISH is absence of the traditionally high levels of false-positivity as observed via the fusion-signal FISH approach, which range between 5 and 10%. False-positivity occurs as a result of coincidental co-localization of two signals, which actually represent two separate signals in a three-dimensional nucleus, but due to the two-dimensional analysis of the nucleus, are visible as a single co-localized signal. On the other hand, one could argue that split-signal FISH can give rise to low frequencies of false-negativity due to the same type of coincidental co-localization of two separate signals making these

cells indistinguishable from normal nuclei. However, 5–10% false-negativity (percentage deduced from fusion-signal FISH) within the leukemic cell population will not alter the result in diagnostic material where the percentage of malignant cells is virtually always over 25%. Consequently, 10% reduction from 25% to 22.5% has no diagnostic meaning [18].

Reduction of background staining using PNA-based blocking

The successful use of large genomic probes for FISH is dependent on blocking of the undesired background staining derived from repetitive sequences present throughout the human genome. The finishing of the human genome project has shown that a large proportion of the human genome is comprised of tandem repeated sequences (i.e. arranged in blocks) and interspersed tandem repeated sequences (distributed all around the genome).
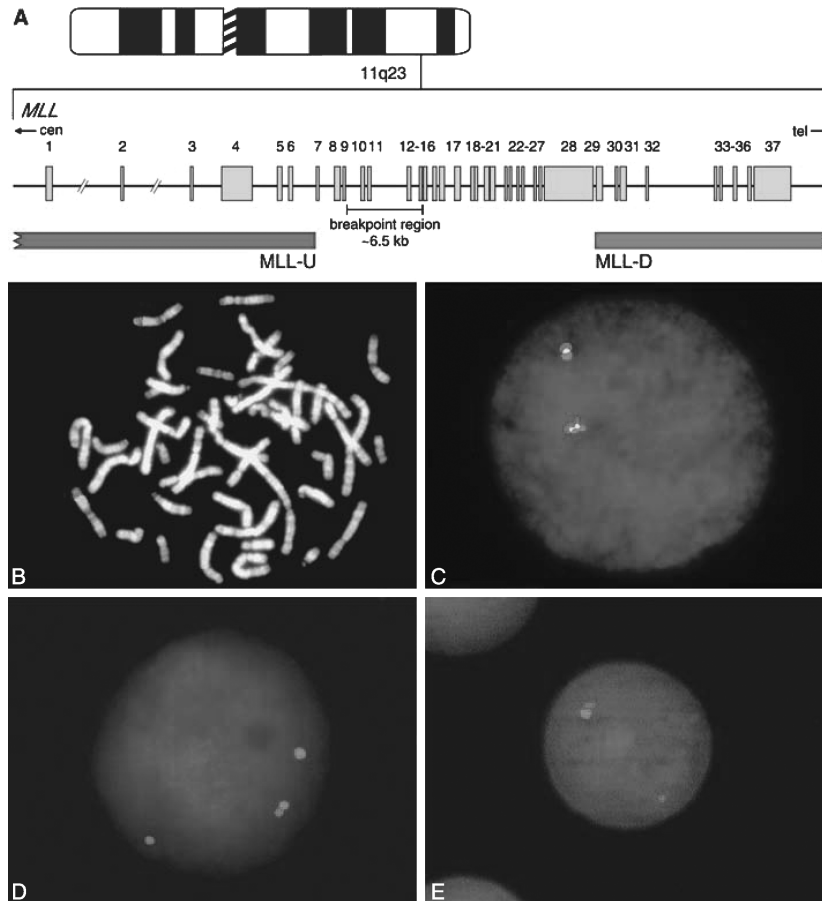
Figure 2. Split-signal FISH for detection of breaks in the *MLL* gene. A. *MLL* gene (11q23) with the position of the breakpoint region and the positions of the centromeric MLL-U probe (239 kb, red) and telomeric MLL-D probe (513 kb, green). B. Metaphase spread of a healthy donor. C. Precursor-B-ALL without *MLL* gene aberration. D. Precursor-B-ALL with *MLL* gene translocation. E. Precursor-B-ALL with *MLL* gene translocation with loss of 3' part of *MLL* gene.

Previously, heat denaturation and reannealing studies on DNA of higher organisms have distinguished three populations of genomic DNA: a slowly reannealing component (45% of the total DNA) containing unique sequences of protein-encoding genes, and intermediate and quickly reannealing components (30% and 25% of the total DNA, respectively) representing repetitive sequences [23]. The fast component contains small (a few nucleotides long), highly repetitive DNA sequences, while the intermediate component contains the interspersed repetitive DNA that can be classified as either SINEs (Short Interspersed Nuclear Elements), LINEs (Long Interspersed Nuclear Elements) or LTRs (Long Terminal Repeats) [24–27]. The repetitive units of the intermediate reannealing component the major reason that large genomic nucleic acid probes are not well suited for hybridization analysis without blocking the repetitive elements to prevent undesired staining.

Blocking of repetitive sequences can be achieved using a component of the total DNA, Cot-1 DNA, enriched with repetitive sequences [28]. Recently, a novel method has been developed based on selection of specific Peptide Nucleic Acid (PNA) oligos, directed against the Alu sequences, which is the most frequent repetitive element within and around genes. PNA is a DNA analogue in which the deoxyribose phosphodiester backbone is replaced by a pseudo-peptide backbone of N-(2-aminoethyl)-glycine units to which the nucleobases are attached through a methylene carbonyl linker (Figure 3) [29,30]. The charge of the pseudo-peptide backbone of PNA is neutral, whereas the charge of the deoxyribose phosphodiester backbone of DNA is negative. Because of lower electrostatic repulsion, a PNA–DNA interaction occurs faster and is stronger than a DNA–DNA interaction [31]. Different PNA oligos were selected in such a way that they cover both the upper and lower strand of the repetitive sequences and could therefore be used as a blocking reagent [32].

This novel PNA-based method for suppression of background staining is now included in our FISH procedure (DakoCytomation, Glostrup, DK, EU). A paraformaldehyde pre-treatment is used to improve the brightness of the fluorescence signals. The premixed ALL probe sets contain PNA oligos and the fluorescently labeled DNA probes, and are denatured together with the target DNA before hybridization in a humified environment overnight. Excess of
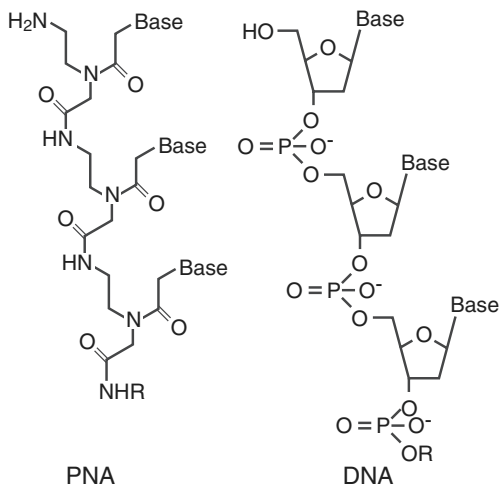
Figure 3. Chemical structure of a PNA and a DNA backbone molecule. "Base" indicates a purine (adenine, guanine) or a pyrimidine (cytosine, thymidine).

probe and PNA oligos is removed by washing under stringent condition, before embedding and examination of the hybridization area (Figure 4).

Concluding remarks

Split-signal FISH probe sets each consists of two differentially labeled probes (generally composed of several BAC/PAC clones), which are located in the target gene at opposite sides of the breakpoint region [17−20,33]. Directly labeled FISH probes work smoothly in combination with the newly developed PNA-blocking system, which allows combined blocking and hybridization in a single step. This single-step hybridization procedure makes split-signal FISH an easy, rapid, and sensitive tool for molecular cytogenetics (Figure 4).

The split-signal FISH approach has three major advantage over fusion-signal FISH. First, translocations involving the target gene can be detected independent of the involved partner gene. Second, split-signal FISH allows identification of the partner gene or partner chromosome region, if metaphases are present. The third advantage is the absence of high levels of false-positivity due to coincidental co-localization, as observed in the traditional fusion-signal FISH approach. One could argue that split-signal FISH can give rise to similar frequencies of false-negativity due to the same type of coincidental co-localization, but 5−10% false-negativity as deduced from fusion-signal FISH within the leukemic cell population will not alter the result in diagnostic material where the percentage of malignant cells is virtually always $>10\%$.

Pre-treatment
- Immersion of slide in 3.7% formaldyde for 2 min.
- Washing of slides in wash buffer for 2x5 min. at room temperature
- Dehydration in ethanol series (70%, 85%, 96%; 2 min. each)
- Air dry

- Application of *probe mixture* containing PNA oligos for blocking and fluorescent labelled DNA probes
- Adding coverslip and sealing with coverslip sealant

Co-denaturation & Hybridization
- Denaturation for 5 min. at 82°C
- Overnight hybridisation at 45°C

Stringent wash
- Washing of slide in stringent wash buffer for 10 min. at 65°C
- Washing of slides in wash buffer for 2 min. at room temperature
- Dehydration in ethanol series (70%, 85%, 96%; 2 min. each)
- Air dry

Mounting & Reading
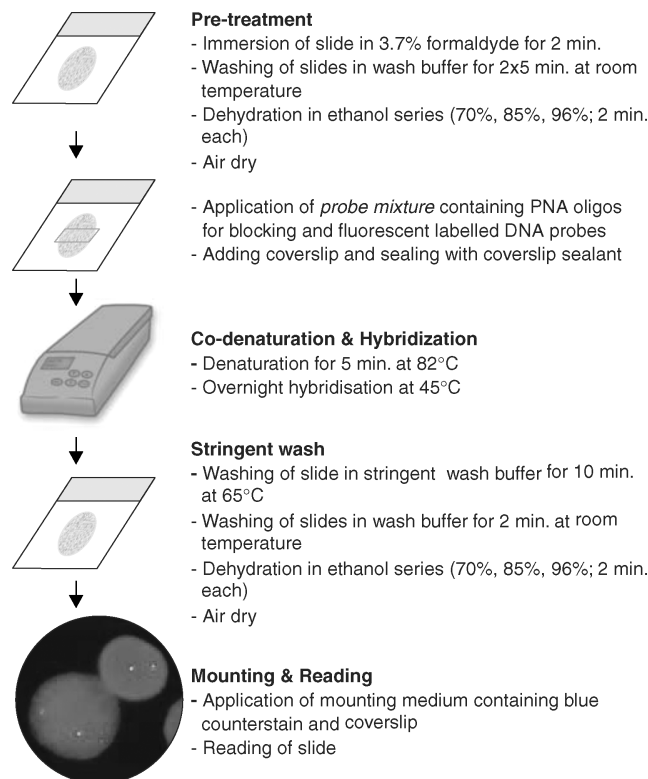- Application of mounting medium containing blue counterstain and coverslip
- Reading of slide

Figure 4. Protocol for FISH with PNA-based suppression of background staining. Slides with tissue or cytology preparation are pre-treated to increase the access of target DNA for the labeled probes. The probe mixture containing PNA oligos and fluorescent labeled probes is applied to the target DNA and co-denaturated, before hybridization. Unspecifically bound probe is removed by washing before the slide is scored with a fluorescent microscope. Normal cells present on the slides serve as control cells.

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