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Detection of Nucleic Acids in the Attomole Range Using Polybiotinylated Oligonucleotide Probes

V. Zecchini, D. Caucheteux[1], J. Grétry, M. Lemaître[1] and J. Dommès[1]
CERISIL and [1]Eurogentec S.A., Liège, Belgium

ABSTRACT

This article describes the optimization of the hybridization signal obtained with biotinylated oligonucleotides. Optimal number and positions of biotin moieties on a 33-base oligonucleotide probe were determined. The quality of avidin-peroxidase conjugate and the choice of chromogenic substrate influenced detection sensitivity. A signal amplification method was also developed for avidin enzymatic conjugates. These improvements allowed the detection of less than 0.02 fmol of target DNA.

INTRODUCTION

In recent years, the results obtained from comparisons of bacterial rRNA sequences have revolutionized the methods of bacterial identification. Moreover, these results led to the development of rRNA-directed oligonucleotide probes that opened the way to the development of rapid bacterial detection methods. But, the widespread use of DNA probes in routine laboratory work is still dependent on the availability of a nonradioactive method, since nonradioactive probes are both safer and cheaper (8,10).

A study was begun in an attempt to optimize a well-known non-isotopic labeling system: the biotin/avidin system. This method involves the use of a biotin-labeled DNA probe, of avidin conjugated to an enzymatic activity and of a chromogenic substrate to detect this enzymatic activity. An optimization of the signal intensity related to the number of marker moieties attached to the probe has been performed. Different substrates have been studied with the intention of reducing the background and increasing the intensity of the signal and the sensitivity of the probe in dot-blot assays. In addition, a system of signal amplification was developed. The optimization of the avidin-biotin system led to a detection threshold of 0.1 fmol of the probe and 0.017 fmol of target DNA.

MATERIALS AND METHODS

Oligonucleotide Synthesis and Labeling

The oligonucleotide probe is a 33-mer with the following sequence: 5′-CCTTTCATCCAAATTCCATGTGAAACTT-GGCTT-3′. This oligonucleotide hybridizes specifically to the V2 variable region of 16S rRNA of *Clostridium tyrobutyricum* (9). Five differently labeled probes containing 1–5 marker moieties per oligonucleotide were synthesized on a Model 394-09 DNA Synthesizer (currently Perkin-Elmer/Applied Biosystems Division, Foster City, CA, USA) using a standard protocol (0.2 µM). The biotinylated oligonucleotides were synthesized using BioTEG™ phosphoramidite for 5′ labeling, biotin-dT for internal labeling and BioTEG-CPG for 3′ labeling. For the 5′ and internal labeling (BioTEG and biotin-dT), the coupling time was increased to 15 min. The 3′ "aminomodification" was done using 3′ "aminomodifier" C7 CPG phosphoramidite. Internal labeling was done using aminomodifier C6 dT. Finally, the 5′ aminomodification was performed using 5′ aminomodifier C6 phosphoramidite (all reagents are from Glen Research Corporation, Sterling, VA, USA). The aminomodifier dT was also introduced by increasing the coupling time to 15 min. Oligonucleotides were cleaved and deprotected in 30% ammonia for 2 h at room

temperature followed by 8 h at 55°C. All the oligonucleotides were purified by polyacrylamide gel electrophoresis (19% acrylamide, 1% bisacrylamide, 8 M urea, 0.9 M Tris-borate, 0.002 M EDTA). After elution, the purified oligonucleotides were desalted on a Sephadex® G-25 (Pharmacia Biotech, Brussels, Belgium) spin column. The percentage of biotinylation of the oligonucleotides was determined in a binding assay on magnetic beads coated with streptavidin (Promega, Madison, WI, USA). The results have shown that the internal and 3′ labelings reached high values: more than 90% biotinylation.

Probe Dot Blots

Successive 1:10 dilutions of the oligonucleotides were blotted on positively charged nylon membranes (Boehringer Mannheim, Brussels, Belgium). After UV fixation (3 min, 312 nm, 180 W), the membrane was blocked for 30 min in 30 mL/100 cm^2 blocking solution (5% [wt/vol] casein in 0.1 M maleic acid, 0.15 M NaCl, pH 7.5). It was then incubated for 30 min in 10 mL/100 cm^2 of blocking solution containing 8 µL/mL enzymatic conjugate. Four different enzymatic conjugates were tested: a commercially available NeutraLite™ avidin-peroxidase conjugate (Eurogentec, Seraing, Belgium) and three other NeutraLite avidin-peroxidase conjugates synthesized in our laboratory with NeutraLite avidin (Eurogentec) and three different grades of peroxidases (Sigma Chemical, St. Louis, MO and Genzyme, Cambridge, MA, USA). The coupling method for the synthesis of these conjugates involved oxidation of the peroxidase with periodate-generating aldehyde groups. The amino groups of the NeutraLite avidin were then able to react with these aldehyde groups (6). The membrane was then washed twice for 15 min in 50 mL/100 cm^2 washing buffer (0.1 M maleic acid pH 7.4, 0.03% Tween® 20). Peroxidase activity was developed in the presence of 4-chloro-1-naphthol (4CN; Bio-Rad, Brussels, Belgium), 3-3′ diaminobenzidin (DAB) or 3-amino-9-ethylcarbazol (AEC) (4). Reaction conditions for 4CN: 8.33 mM Tris-HCl pH 7.4, 10 mM NaCl, 5 mg/mL 4CN, 17% methanol, 0.03% H_2O_2. The deep blue coloration appeared after 15 min and was complete after 45 min. The reaction was stopped by washing with 10% sodium dodecyl sulfate (SDS). Reaction conditions for DAB: 50 mM Tris-HCl pH 7.6, 0.6 mg/mL DAB (Sigma Chemical), 0.03% H_2O_2, 0.03% $NiCl_2$. Reaction conditions for AEC: 0.1 M sodium acetate pH 5.2 with succinic acid (AEC revelation buffer), 268 µg/mL of AEC (Sigma Chemical), 0.03% H_2O_2. The coloration was complete after 30–40 min.

Signal Amplification

Signal amplification is described in Reference 3. Biotin was coupled to tyramine by incubating overnight under agitation in 100 ng of D-biotin-ε-aminocaproic acid *N*-hydroxysuccinimid ester (Boehringer Mannheim) and 40 mg tyramine (Sigma Chemical) in 2 mL dimethyl sulfoxide (DMSO). The solution of biotin-tyramine (BT) conjugate was used without any further purification, with a calculated concentration of 55 mg/mL (2). The signal amplification can be performed straight after hybridization or after a first coloration reaction. The membrane was layered on Whatman paper (Clifton, NJ, USA) soaked with the BT solution (100 µL BT in 2 mL 0.1 M phosphate buffer pH 8.5) for 30 min. The membrane was then washed twice for 15 min in 50 mL/100 cm^2 of washing buffer, incubated 30 min in 10 mL/100 cm^2 of blocking buffer containing 10 µL/mL NeutraLite avidin-peroxidase conjugate, washed twice for 15 min in 100 mL/100 cm^2 washing buffer and incubated in the AEC revelation buffer. The colorimetric reaction was stopped by placing the membrane in 1× phosphate-buffered saline (PBS) solution (0.14 M NaCl, 3 mM KCl, 10 mM Na_2HPO_4, 1.7 mM KH_2PO_4, pH 7.6 with HCl).

C. Tyrobutyricum DNA Extraction

The genomic DNA of *C. tyrobutyricum* was prepared by the following protocol and obtained from the compilation of three different methods (1,5,7): The bacterial cultures were centrifuged for 20 min at 3700 rpm, the pellet was resuspended in 10 mL THMS (30 mM Tris-HCl, pH 8.0, 3 mM

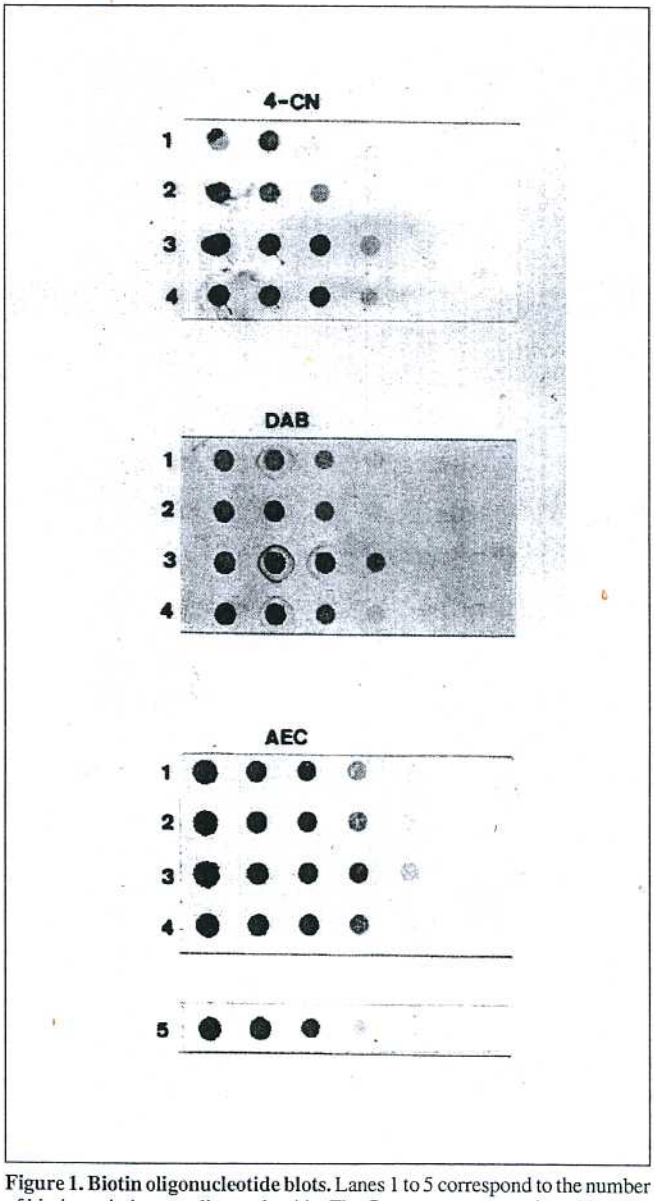

Figure 1. Biotin oligonucleotide blots. Lanes 1 to 5 correspond to the number of biotin moieties per oligonucleotide. The first spot corresponds to 10 pmol. The subsequent spots correspond to successive 1:10 dilutions of the oligonucleotides. Biotin detection was performed by using NeutraLite avidin-peroxidase. Chromogenic substrate was 4CN, DAB or AEC.

$MgCl_2$, 25% sucrose) and 10 mg lysozyme were added. After a 45-min incubation at 37°C, the suspension was centrifuged 10 min at 10000 rpm, and the pellet was resuspended in 10 mL STET (0.1 M NaCl, 10 mM Tris-HCl pH 8.0, 1 mM EDTA pH 8.0, 5% Triton® X-100) containing 0.1% (wt/vol) SDS, 0.1 mg/mL proteinase K. The mixture was incubated for 45 min at 37°C, followed by the addition of 1.6 mL of 5 M NaCl and 1.6 mL of 10% CTAB (cetyltrimethylammonium bromide)/0.7 M NaCl (1). The mixture was incubated for 10 min at 65°C. Following the addition of 14 mL of chloroform, the mixture was agitated and centrifuged for 10 min at 10000 rpm. Fourteen milliliters of phenol were added to the aqueous phase, and the mixture was agitated and centrifuged for 10 min at 10000 rpm. Then, 0.6 volumes of isopropanol were added, and the tubes were incubated overnight at room temperature. The tubes were then centrifuged for 30 min at 10000 rpm, washed in 70% ethanol, dried and resuspended in distilled water. The DNA concentration was estimated by fluorimetry. Successive 1:2 dilutions of the genomic DNA were blotted on positively charged nylon membranes.

Hybridization of the Probe to the Genomic DNA

The dotted nucleic acids were UV cross-linked to nylon membranes and prehybridized with 20 mL/100 cm^2 of membrane of hybridization solution (5× standard saline citrate [SSC], 2.5% blocking solution, 1% *N*-lauroylsarcosine, 1% [wt/vol] SDS, 10% dextran sulfate [Sigma Chemical]) in sealed plastic bags for 1 h at 65°C. The solution was discarded and replaced by 2.5 mL/100 cm^2 of fresh hybridization solution containing the biotinylated oligonucleotide probe at a concentration of 100–200 pM. Hybridization was performed for 6 h at 65°C under agitation. After hybridization, the membranes were washed twice for 5 min at room temperature in 50 mL/100 cm^2 of 2× SSC, 0.1% (wt/vol) SDS. This washing was followed by a stringent wash: twice for 15 min with 50 mL/100 cm^2 of 0.5× SSC, 0.1% (wt/vol) SDS at 65°C. The membranes were then directly used for the detection of hybridized probes as described above.

RESULTS AND DISCUSSION

In an attempt to improve the detection limits of our probes without having to use a radioactive label, we have initiated an optimization of the most widespread nonradioactive labeling system available on the market: the biotin system. Sensitivity was evaluated in relation to the colorimetric substrate and to the number of marker moieties per oligonucleotide. The enzymatic reaction substrate is another parameter that can influence the detection sensitivity. The three molecules most commonly used for this kind of peroxidase assay were considered: 4CN, DAB and AEC. Five labeled probes, differing in the number and position of the labels were synthesized (5′ labeling for the 1-marker-moiety probe; 5′ and 3′ labeling for the

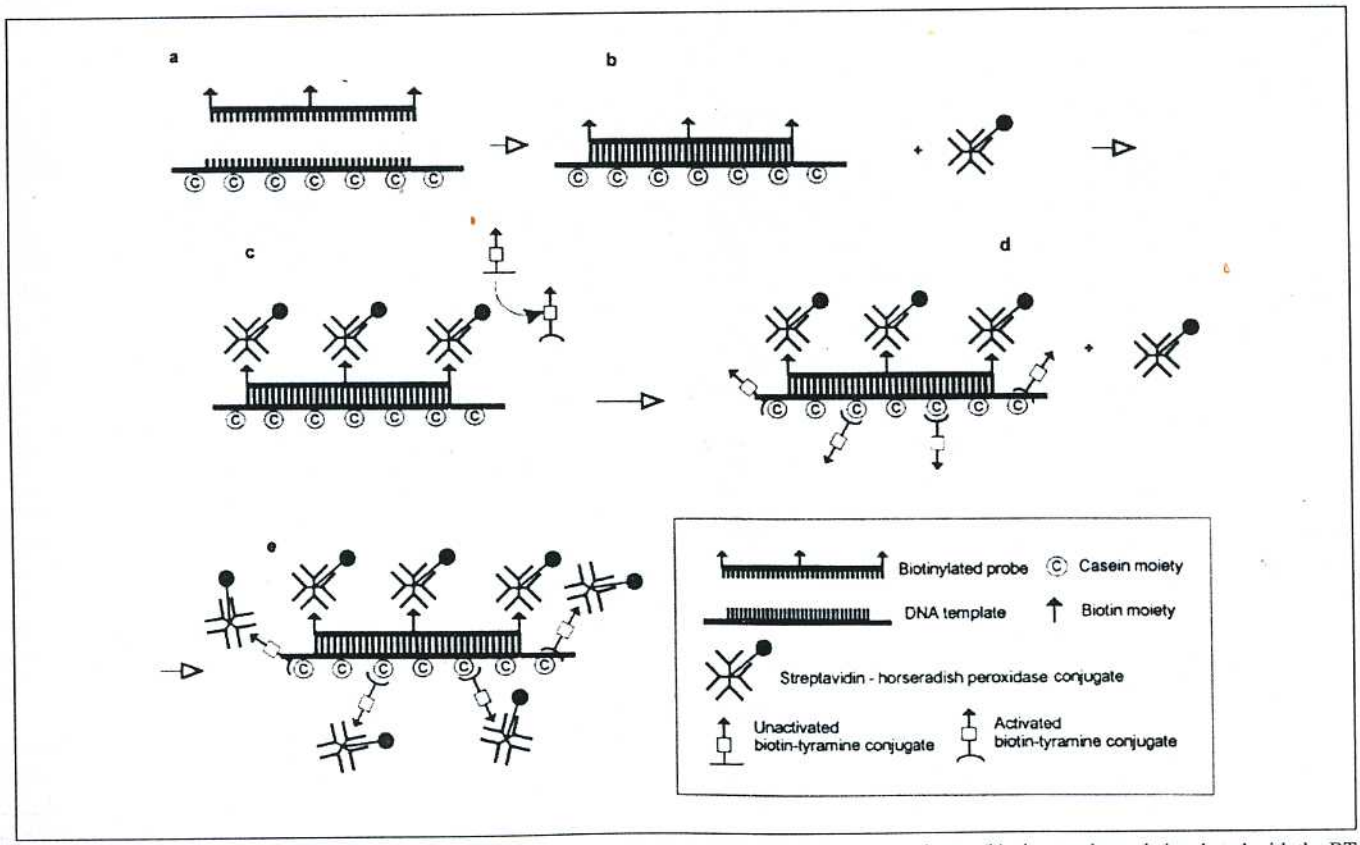

Figure 2. BT amplification method. After hybridization (a) and a first incubation with the peroxidase conjugate (b), the membrane is incubated with the BT solution for 30 min (c). The tyramine, activated by the peroxidase, is then able to bind to the casein moieties present around the probe (d). After washing, the membrane is incubated again with the NeutraLite-avidin peroxidase conjugate for 30 min (e). After a second washing step, the peroxidase activity is detected using the AEC colorimetric reaction.

2-marker-moieties probe; 5′, internal position 19 and 3′ labeling for the 3-marker-moieties probe; 5′, internal positions 8 and 21 and 3′ labeling for the 4-marker-moieties probe; 5′, internal positions 8, 15 and 21 and 3′ labeling for the 5-marker-moieties probe). To determine which type of labeling was the most efficient, successive 1:10 dilutions of these labeled oligonucleotides were blotted on positively charged nylon membranes. After incubation in NeutraLite avidin-peroxidase, the enzymatic activity was detected using either 4CN, DAB or AEC as chromogenic substrate. As shown in Figure 1, the best results were obtained with AEC as the peroxidase substrate. This component gave a red coloration with less background and led to a probe detection sensitivity of 0.1 fmol. The detection sensitivity increased with the number of marker moieties per oligonucleotide, reaching a maximum with 3 marker moieties per oligonucleotide for each of the 3 blots. The 4- and 5-marker-moieties probes showed a lower sensitivity. The lowest amount of labeled probe that could be detected was 10 fmol for the 1- and 5-biotin-moieties probes, 1 fmol for the 2- and 4-biotin-moieties probes and 0.1 fmol for the 3-biotin-moieties probe. The pattern of the signal obtained with the 1-, 2- and 3-marker-moieties probes showed

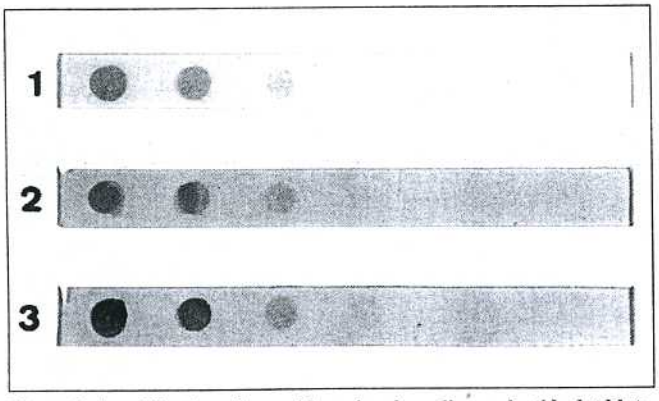

Figure 3. Amplification of peroxidase signal on oligonucleotide dot blots. The first spot corresponds to 10 fmol of the 3-biotin probe, the subsequent spots correspond to successive 1:10 dilutions. Lane 1: Control, no amplification, probe detection to 0.1 fmol. Lane 2: After 15-min BT amplification, probe detection to 0.01 fmol. Lane 3: After a 30-min amplification step probe detection to 0.01 fmol.

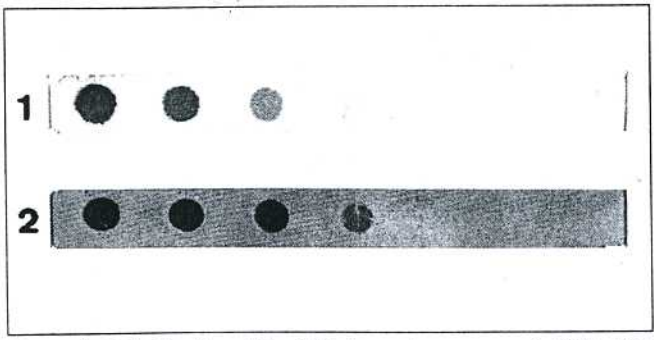

Figure 4. Hybridization of the 3-biotin probe on genomic DNA of *C. tyrobutyricum*. 1: The first spot corresponds to 40 ng of genomic DNA, the following spots correspond to successive 1:2 dilutions of this DNA. The signal is still visible for the fourth spot, i.e., 0.17 pg (0.017 fmol) of target DNA (33-nucleotide-long complementary sequence of our probe present at a level of four copies in the genomic DNA). 2: After BT amplification, the detection threshold is still 0.017 fmol of target DNA but the signal is more intense.

that the signal is proportional to the number of enzymatic conjugate moieties that the oligonucleotide can bind. The loss in sensitivity observed with 4 and 5 labels per oligonucleotide suggested that steric hindrance could make some of the avidin-binding sites inaccessible to the conjugates. Moreover, an internal labeling could also lead to a change in the configuration of the oligonucleotide, making the enzymatic conjugate binding sites less accessible. We also tested digoxigenin (DIG)-labeled probes, and the results showed that, as for the biotinylated oligonucleotides, the best sensitivity was obtained with 3 DIG moieties per probe (data not shown).

The quality of the enzymatic conjugate can also influence the background and the sensitivity of the detection. Preliminary experiments showed that the background signal almost completely disappeared when NeutraLite avidin was used instead of streptavidin. Four NeutraLite avidin conjugates were tested. The first to be studied was a commercially available conjugate (Eurogentec). In an attempt to improve the detection, three NeutraLite preparations were synthesized in our laboratory. They were produced by coupling NeutraLite avidin to horseradish peroxidase from different sources. These homemade conjugates proved to be less sensitive when detecting biotin-labeled probes. This low sensitivity was probably due to a lower peroxidase-specific activity as compared with the commercial molecule. The Eurogentec NeutraLite avidin-peroxidase conjugate was used in all further optimization experiments.

The presence of 3 marker moieties per oligonucleotide and the use of Eurogentec NeutraLite avidin-peroxidase conjugate with AEC as substrate allowed the detection of small quantities of oligonucleotide. Because this sensitivity might not be sufficient to detect very small amounts of DNA in a Southern blot assay for example, further amplification of the signal was investigated. A method (3) based on the use of BT complexes (Figure 2) was applied to the oligonucleotide blots. After the first color development reaction, the blots were incubated with the BT conjugate. In this reaction, the tyramine was activated by the peroxidase of the NeutraLite avidin conjugate bound to the biotinylated probe. The activated tyramine was then able to bind to the molecules of casein used to block the membrane. To avoid any diffusion of the activated BT conjugate, the membrane was placed on Whatman paper soaked with the BT conjugate solution. It was therefore possible to bind more molecules of peroxidase conjugate by incubating the membrane for a second time with NeutraLite avidin-peroxidase conjugate. A second colorimetric reaction was performed that resulted in a dramatic improvement in the detection limits (Figure 3). This method improved the signal intensity (deeper intensity of color), did not noticeably increase the background signal and permitted more sensitive detection (about 10-fold more) without significantly increasing the overall experimental time.

This optimized detection system was tested in a hybridization to *C. tyrobutyricum* genomic DNA. Successive 1:2 dilutions of 40 ng of the genomic DNA were blotted on a positively charged nylon membrane, and after a denaturation step in 0.15 *N* NaOH/1.5 M NaCl, the 3-biotin-moieties probe was hybridized as described in Materials and Methods. Figure 4 shows that the biotinylated oligonucleotides were able to detect less than 0.2 pg (0.02 fmol) of target DNA (sequence of 33 nucleotides of the genomic DNA that is able to hybridize to the oligonucleotide probe). To check its specificity, the probe was also hybridized to DNA extracted from other *Clostridia* strains (*C. sporogenes*, *C. butyricum*, *C. beijerinckii*, *C. lentoputrescens*, *C. bifermentens*). No signal was detected on these strains (data not shown).

In conclusion, by using 3-biotin-moieties-labeled oligonucleotides, NeutraLite avidin-peroxidase conjugate and AEC as the peroxidase substrate and by performing an amplification of the signal using the BT conjugate system, we were able to reach a detection threshold of 0.017 fmol of target DNA.

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Address correspondence to Jacques Dommès, Eurogentec S.A., Parc de Recherche de la Cense Rouge, 14 Rue Bois Saint Jean, B-4102 Seraing, Belgium. Internet: jdommes@rc1.vub.ac.be