Use of CDP-STAR in a fast and highly sensitive chemiluminescent detection procedure for VNTR loci with neutral and charged membranes.

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INTRODUCTION

The use of directly labeled alkaline phosphatase oligonucleotides (AP-probes) and chemiluminescent substrates for the analysis of VNTR loci has proven to be a simple, rapid and convenient format for DNA typing(Baum, 1990, Neuweiler 1992, Balazs 1994 and Baird 1994). The advent of new and more sensitive chemiluminescent substrates for alkaline phosphatase has provided the identity laboratory with even more powerful chemiluminescent probes for DNA typing. This paper discusses the development of a protocol for the use of a new, highly-sensitive alkaline phosphatase substrate, CDP-Star™ (Tropix Inc., Bedford, MA) with a number of AP-probes and with either charged or neutral membranes.

MATERIALS AND METHODS

VNTR probes were conjugated to alkaline phosphatase using the NICE™ method (Cellmark Diagnostics, Abingdon UK). They included; D1S7, D1S339, D2S44, D4S163, D5S110, D6S132, D7S467, D10S28, D17S26 and D17S79. Duplicate membranes were prepared using different amounts of Hae III digested DNA that was isolated, digested, electrophoresed and transferred to either Pall Biodyne B (charged) or MSI Magna (neutral) membranes as described (Balazs 1994). Charged membranes were baked after Southern Transfer and neutral membranes were baked and U.V cross-linked. NICE-labeled VNTR probes, QUICK-LIGHT™ hybridization solution and Buffers were supplied by LIFECODES CORPORATION.

1X QUICK-LIGHT Buffer, Wash I and Wash II were prepared according to manufacturer's instructions. CDP-Star was brought to room temperature and hybridization solution, Wash I and Wash II were equilibrated to 55°C for at least 90 minutes. Membranes to be hybridized were placed into an appropriate sized container containing pre-warmed Wash II (0.125 mL Wash II/cm^2 membrane) for 5 to 10 minutes. 0.5 - 1.0 µL NICE probe was added per mL of 55°C pre-warmed hybridization solution (hybridization solution volume = 0.0375 mL/cm^2 membrane). Wash II Solution was decanted and the NICE Probe/Hybridization solution was added. The membranes were incubated with the probe/hybridization solution for 20 - 30 minutes at 55°C with shaking. The membranes were then washed two times with pre-warmed Wash I solution (0.375 mL per cm^2 membrane) for 10 minutes at 55°C with shaking. The membranes were then washed two times with pre-warmed Wash II solution (0.375 mL per cm^2 membrane) for 10 minutes at 55°C with shaking. After the last Wash II solution was decanted, 0.125 mL/ cm^2 membrane of 1X QUICK-LIGHT Buffer was added to the container. The container is then covered and gently shaken. Using forceps, each membrane is carefully separated to allow complete wetting, the QUICK-LIGHT solution was decanted and this step was repeated three times. The membranes were left in the last QUICK-LIGHT rinse and one-by-one were transferred, first allowing excess buffer to drain off, to a clean, container with CDP-Star (0.025 mL CDP-Star per cm^2 membrane). The excess CDP-Star was drained off and the membranes were placed in an open

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Development Folder (LIFECODES). The folder was heat-sealed after the removal of air bubbles and excess CDP-Star. The surface of the folder was wiped dry and the folder was then placed into an X-ray cassette and exposed to Kodak XAR or Fuji RX film for up to 3 hours and overnight at room temperature. Prior to re-hybridization to other probes, membranes were incubated in Wash I for at least 30 minutes at 65°C with shaking to inactivate the AP-probe on the membrane. Membranes were air- dried for storage.

RESULTS AND DISCUSSION

CDP-Star, a 1,2-dioxetane, provides rapid, high intensity and prolonged light emission which permits the detection of 25-50 ng of genomic DNA bound to charged or neutral membranes in an exposure time of a few hours or less. The probes tested fell into three general groups based upon their sensitivity (Table 1). The first group consisted of D1S7, D1S339, D2S44 and D7S467, all of which gave strong signal with minimal noise. The second group consisted of D6S132, D17S79, D4S163 and D5S110 which gave good signal with minimal background and the third group consisting of D17S26 and D10S28 which gave adequate signal with minimal background. Additionally, D17S26 displayed a significant difference in allele signal intensity, with the lower allele of K562 DNA being much lighter than the upper allele which complicated the sensitivity determination.

Table 1. Sensitivity of NICE Probes using CDP-Star

Probe/Exposure	≤ 1 hour	≤ 3 hours	overnight
Group I D1S7, D1S339 D2S44, D7S467	<500ng	< 50ng	< 10ng
Group II D4S163, D5S110, D6S132, D17S79	500ng	50ng	< 50ng
Group III D10S28,D17S26	500ng	50-100ng	50ng

The results of Table 1 show that the VNTR probes in Group I provide sensitivity of less than 10ng of DNA after an overnight exposure using CDP-Star making them extremely valuable when the amount of DNA is limited.

An additional major advantage is CDP-Star's ability to be used with both neutral and charged membranes. This is due to CDP-Star's compatibility with SDS-detergent based hybridization/wash systems such as QUICK-LIGHT reagents. Another second-generation dioxetane/enhancer system, Lumi-Phos® Plus (Lumigen Inc. Detroit, MI) is incompatible with SDS-systems because the SDS quenches the enhanced chemiluminescence by interacting with enhancer (Price, in press). Such systems are precluded from using charged membranes because Tween-based systems cannot prevent non-specific binding of AP-probes to charged membranes. In this study, charged membranes gave slightly higher background but displayed better sensitivity than neutral membranes, especially with small allele sizes (Fig. 1).

Repeated stripping and rehybridization showed some loss of sensitivity which could be

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corrected by longer exposure times. For example, a Pall Biodyne B membrane stripped 4 times still yielded 100ng sensitivity after an overnight exposure when probed with D17S79 (data not shown).

The appearance of relatively weak artifact bands after overnight exposure, with more than 50 ng of genomic DNA, when using the more sensitive alkaline phosphatase substrates, has been previously reported (Price). Such bands were noted when membranes probed with D2S44, D6S132, D7S467 (Fig. 1) and D17S79 were over-exposed. These extra bands can be eliminated by reducing the amount of probe added to the hybridization solution. Such bands may be more evident in Tween-based systems due in part to the lower wash and hybridization stringencies. CDP-Star added sensitivity does require added care when preparing and performing hybridizations. Increased background due to contamination is more common, manifesting itself as heavy speckling all over the developed film. Using clean containers for the CDP-Star incubations as well as using freshly prepared reagents can eliminate background spots.

In summary, we have examined 10 NICE-labeled VNTR AP-probes using CDP-Star as the substrate and have found it provides sensitive detection, rapid turn-around times and allows the use of charged membranes in VNTR loci analysis.

D7S467 Hybridized to Uncharged and Charged Membranes

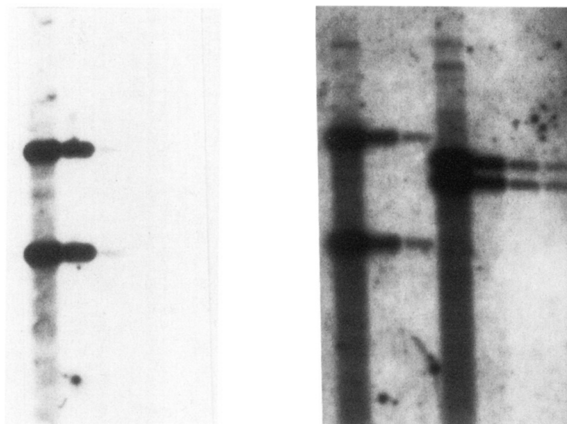

Figure 1. Left: Uncharged membrane containing 500ng, 50ng and 10ng of Hae III digested K562 genomic DNA. Right: Charged membrane containing 500ng, 50ng, 10ng Hae III digested K562 genomic DNA and 500ng, 50ng, 10ng, 5ng Hae III digested male genomic DNA. Both membranes were hybridized to NICE-labeled D7S467, incubated with CDP-Star and exposed overnight at room temperature.

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