PETITIONER'S EXHIBIT #22
Quanta Blot Kit

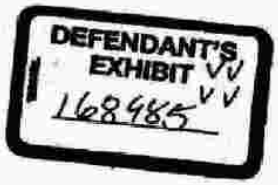
QuantiBlot®
Human DNA Quantitation Kit

PERKIN ELMER

Part No. N808-0114

FOR FORENSIC AND RESEARCH USE ONLY
NOT FOR USE IN DIAGNOSTIC PROCEDURES

READ ENTIRE PROCEDURE PRIOR TO USE OF KIT



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LIST OF KIT COMPONENTS

IMPORTANT: Do NOT freeze any of the kit components.

Store the QuantiBlot® Human DNA Quantitation Kit components at 2° to 8°C. Under these conditions, components of the kit are stable through the control date printed on the label.

Note: The QuantiBlot® Human DNA Quantitation Kit contains reagents for at least 10 hybridization reactions. Each hybridization reaction requires the DNA Standards and DNA Calibrators with space for testing up to 38 samples.

Reagent	Volume	Description
QuantiBlot® D17Z1 Probe	220 µL	1 vial containing 1 pmole/µL biotinylated DNA oligonucleotide probe of the following sequence: 5'-biotin-TAGAAGCATTCTCAGAA ACTACTTTGTGATGATTGCATTC-3'.
Enzyme Conjugate: HRP-SA	2.0 mL	1 bottle containing Horseradish Peroxidase-Streptavidin (HRP-SA) conjugate supplied in buffer with preservative.
Bromothymol Blue Solution	200 µL	1 vial containing 0.04% Bromothymol Blue in water.
QuantiBlot® DNA Standard A	250 µL	1 vial containing 2 ng/µL human genomic DNA in TE buffer.
QuantiBlot® DNA Calibrator 1	100 µL	1 vial containing 0.7 ng/µL human genomic DNA in TE buffer.
QuantiBlot® DNA Calibrator 2	100 µL	1 vial containing 0.1 ng/µL human genomic DNA in TE buffer.
Package Insert		QuantiBlot® Human DNA Quantitation Kit protocol.

INTRODUCTION

The QuantiBlot® Human DNA Quantitation Kit provides reagents and protocols necessary for the rapid quantitation of human DNA. The procedure is based on the hybridization of a biotinylated oligonucleotide probe to DNA samples immobilized on a nylon membrane.[1] The probe included in this kit is complementary to a primate-specific alpha satellite DNA sequence at the locus D17Z1.[2] Subsequent binding of Enzyme Conjugate:HRP-SA (horseradish peroxidase-streptavidin) to the biotin moiety of the probe allows for either colorimetric or chemiluminescent detection (protocols are provided for both detection schemes). In the case of colorimetric detection, the oxidation of 3,3',5,5'-tetramethylbenzidine (Chromogen:TMB) catalyzed by the horseradish peroxidase results in the formation of a blue-colored precipitate directly on the membrane. Alternatively, for chemiluminescent detection the oxidation of a luminol based reagent catalyzed by the horseradish peroxidase results in the emission of photons that are detected on standard autoradiography film.[3] This process is called Enhanced Chemiluminescence (ECL™). In both cases, the quantity of sample DNA is determined by comparison of the signal intensity to human DNA standards.

Using the protocols provided, 0.15 to 10.0 nanograms of human DNA can be quantitated. The entire protocol can be performed in less than 2 hours. DNA prepared by a variety of extraction methods, including phenol-chloroform, Chelex®, salting out[4], or binding to silica particles, can be quantitated.

The QuantiBlot Human DNA Quantitation Kit provides protocols for two alternative detection schemes: colorimetric and chemiluminescent. The advantages of the colorimetric detection scheme are as follows: 1) A darkroom equipped for X-ray film development is not necessary; and 2) The Chromogen:TMB reagent is available directly from Perkin-Elmer (**Part No. N808-0092**). The advantages of the chemiluminescent (ECL) detection scheme are as follows: 1) If desired, the sensitivity can be increased below 0.15 ng by performing long exposures to the X-ray film (see **Section 5.2.5**); and 2) The X-ray film result is a permanent record (no photography is required).

MATERIALS REQUIRED BUT NOT SUPPLIED

The items listed are those required for quantitation procedures. This list does not include reagents or equipment required for DNA extraction (for a list of DNA extraction materials see the AmpliType™ User Guide, Version 2, Section 3). Many of the items listed are available from major laboratory suppliers (MLS) such as Baxter Scientific Products, Fisher Scientific, or VWR unless otherwise noted. Equivalent sources may be acceptable where noted.

Materials	**Source**
AmpliType™ User Guide, Version 2	**Perkin-Elmer, Norwalk, CT (Part No. N808-0111).**
Reagents	
Use reagent grade unless otherwise noted.	
Citric acid, monohydrate, granular	JT Baker, Phillipsburg, NJ (Cat. No. 0110-05), or equivalent.
Deionized or distilled water	
Ethylenediaminetetraacetic acid (EDTA), disodium salt, dihydrate	GIBCO BRL, (Cat. No. 15575-012), Sigma (Cat. No. E4884), or equivalent.
Hydrochloric acid, concentrated	MLS
Hydrogen peroxide, 30%	Sigma Chemical Company, (Cat. No. H1009), or equivalent.
Sodium chloride	MLS
Sodium dodecyl sulfate (SDS), ultra pure electrophoresis grade	GIBCO BRL (Cat. No. 15525-025), or equivalent.
5N Sodium hydroxide solution	VWR, San Francisco, CA (Cat. No. JT5671-2).
Sodium hydroxide pellets	MLS
Sodium phosphate, monobasic, monohydrate	Sigma (Cat. No. S9638), or equivalent.
Tris base (Trizma base [FW 121.1])	GIBCO BRL (Cat. No. 15504-012), Sigma (Cat. No. T8404), or equivalent.
Trisodium citrate, dihydrate	Sigma (Cat. No. C8532), or equivalent.
Detection Reagents	
For colorimetric detection, the following reagents are needed:	
Chromogen:TMB	**Perkin-Elmer (Part No. N808-0092).**
Ethanol, 200 proof (100%) in glass container	Quantum Chemical Company, Cincinnati, OH (order by volume) or Gold Shield, Hayward, CA (Cat. No. EL200P24X1).
For chemiluminescent (ECL™) detection, the following reagents are needed:	
ECL™ Detection Reagents	Amersham, Arlington Heights, IL (Cat. No. RPN.2109).
Film processing GBX Fixer	Sigma (Cat. No. P-7167).
Film processing GBX Developer	Sigma (Cat. No. P-7042).

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General Equipment	Source
Autoclave	MLS
Hybridization Tray	**Perkin-Elmer (Part No. N808-0136).**
Hybridization Tray Retainer	**Perkin-Elmer (Part No. N808-0137).**
Balance, accurate to 1.0 mg	MLS
Waterbath with cover, rotating, Bellco Hot Shaker Plus™	Bellco, Vineland, NJ [Model No. 7746-22110 (110V), or Model No. 7746-22220 (220V)].
Thermometer, Total Immersion	Recommend: Cole Parmer, Niles, IL (Cat. No. H-08001-34, Graduated to 0.1°C range 49° to 57°C, specify NIST traceable when ordering). Alternative: Scientific Products (Cat. No. T2099-5, range 50°C to 80°C).
Weight, approximately 1 kg (e.g., lead ring)	VWR (Cat. No. 29700-048) Cole Parmer (Cat. No. G-06137-06), or equivalent.
Labware and glassware	MLS
Magnetic stirrer and stir bars	MLS
Polaroid® Camera and Type 55 or 667	MLS (black and white) film or Type 59 or 559 (color) film.
pH meter compatible with Tris solutions; Corning or equivalent, (need electrodes without silver chloride) and reference buffers	MLS
Pipettors; adjustable to deliver 1-20 µL, 20 - 200 µL and 200-1000 µL	Rainin, Woburn, MA (P20, P200, and P1000), or equivalent.
Refrigerator (2° to 8°C)	MLS
Shaker, variable speed, orbital platform (capable of 100-125 rpm)	Bellco, Lab-Line (Model No. 3520 for RPM X100).
Slot blot apparatus, The Convertible®	GIBCO BRL (Cat. No. 11055-019 and Cat. No. 11055-068).
Timer, 60 minute (±1 minute)	MLS
Vortex	MLS
Water bath, stationary, adjustable to 37°C or incubator	MLS
<u>Additional equipment needed for chemiluminescent detection</u>:	
Automatic film processor (optional)	Konica, Japan (Model QX-130 A plus Code No. 1521), or equivalent.
Film cassette	Amersham (Cat. No. RPN.1642).
Dark room	MLS

Supplies

Biodyne® B nylon membrane, 0.45 µm	GIBCO BRL (Cat. No 14811-012).
Gloves, disposable	MLS
Lab wipes or paper towels	MLS
Permanent ink marker (for marking tubes)	MLS
Pipet tips for adjustable pipettors with plugged tips (sterilized by irradiation or autoclaving)	GIBCO BRL or equivalent
GeneAmp® PCR Reaction Tubes (0.5 mL polypropylene tubes)	**Perkin-Elmer (Part No. N801-0180).**

Additional supplies needed for chemiluminescent detection:

Saran Wrap®	MLS
Whatman Benchkote® (polythene backed)	VWR (Cat. No. 52855-001).
X-ray film (Hyperfilm ECL or Kodak XAR5 film)	Amersham (Cat. No. RPN.2103), Kodak, or equivalent.

1.0 REAGENT PREPARATION

1.1 Preparation of reagents not supplied

Use reagent grade chemicals unless otherwise noted. Prepare all solutions using deionized or glass distilled water (identified below as DI H_2O). Wear gloves and follow safety recommendations provided by manufacturer for handling chemicals. Comply with any and all laws, regulations, or orders with respect to the disposal of any hazardous or toxic chemical, material, substance or waste. Store all reagents at room temperature (15° to 30°C) unless otherwise noted. Reagents are prepared as follows:

1.1.1 0.5 M EDTA (1 L).

Warning: EDTA is an irritant. Wear lab coat, gloves, mask, and protective eyewear when handling EDTA.

Warning: NaOH is corrosive and toxic. Wear lab coat, gloves, and protective eyewear. Use caution when handling. NaOH, when combined with water, results in an exothermic reaction. Dissove the NaOH pellets in the water gradually with 3 to 4 additions. Cover and allow the NaOH to dissolve completely and cool between each addition.

Add 186.1 g of disodium ethylenediaminetetraacetic acid dihydrate ($Na_2EDTA \cdot 2H_2O$) to 800 mL of DI H_2O. Stir vigorously on a magnetic stirrer. To dissolve the EDTA powder, adjust the pH to 8.0 (± 0.2) by adding approximately 20 g of NaOH pellets. Check the pH and add 5N NaOH solution if needed (for small pH adjustments, pellet addition cannot be accurately controlled). Adjust the final volume to 1 liter with DI H_2O and mix thoroughly. The solution should be autoclaved or filtered through a 0.2 µm Nalgene® filter.

1.1.2 20% (w/v) SDS (1 L).

Warning: SDS is an irritant. Avoid skin contact and inhalation. Wear lab coat, gloves, mask, and protective eyewear when handling SDS.

Slowly dissolve 200 g electrophoresis-grade (ultra pure) sodium dodecyl sulfate (SDS) in 800 mL DI H_2O. Warming (e.g., in a 37°C water bath) may be required to dissolve solids completely. Adjust the final volume to 1 liter with DI H_2O and mix thoroughly.

1.1.3 1 M Tris-HCl, pH 8.0 (1 L).

Warning: Hydrochloric acid (HCl) causes severe burns and is irritating to the eyes. When preparing this reagent, use a fume hood and avoid inhalation and contact with the skin. Wear a lab coat, gloves, and protective eyewear when handling.

Dissolve 121.1 g Tris base in 800 mL DI H_2O. Adjust to pH 8.0 (± 0.2) at room temperature by adding approximately 45 mL of concentrated HCl. Adjust the final volume to 1 liter with DI H_2O and mix thoroughly. Sterilize by autoclaving.

1.1.4 TE Buffer [10 mM Tris-HCl, 0.1 mM EDTA, pH 8.0 (1 L)]. Add 10 mL of 1 M Tris-HCL, pH 8.0 (**Section 1.1.3**) and 0.2 mL of 0.5 M EDTA (**Section 1.1.1**) to 990 mL DI H_2O and mix thoroughly. Dispense 100 mL aliquots and sterilize by autoclaving.

1.1.5 Citrate Buffer [0.1 M Sodium Citrate, pH 5.0 (1 L)]. Dissolve 18.4 g trisodium citrate, dihydrate ($Na_3C_6H_5O_7 \cdot 2H_2O$) in 800 mL DI H_2O. Adjust the pH to 5.0 (± 0.2) by addition of approximately 6 g of citric acid monohydrate ($C_6H_8O_7 \cdot H_2O$). Adjust the final volume to 1 liter with DI H_2O and mix thoroughly.

1.1.6 20X SSPE Buffer [3.6 M NaCl, 200 mM ($NaH_2PO_4 \cdot H_2O$), 20 mM EDTA, pH 7.4 (1 L)].

Warning: NaOH is corrosive and toxic. Wear lab coat, gloves, mask, and protective eyewear. Use caution when handling NaOH.

Dissolve 7.4 g of disodium ethylenediaminetetraacetic acid dihydrate ($Na_2EDTA \cdot 2H_2O$) in 800 mL DI H_2O. Adjust the pH to 8.0 (± 0.2) with 10 N NaOH solution. Add 210 g Sodium Chloride (NaCl) and 27.6 g Sodium Phosphate, monobasic, monohydrate ($NaH_2PO_4 \cdot H_2O$). Adjust the pH to 7.4 (±0.2) with 10 N NaOH (about 10 mL). Adjust the final volume to 1 liter with DI H_2O and mix thoroughly.

1.1.7 Hybridization Solution [5X SSPE, 0.5% w/v SDS (1 L)]. Add 250 mL 20X SSPE (**Section 1.1.6**) and 25 mL 20% w/v SDS (**Section 1.1.2**) to 725 mL DI H_2O and mix thoroughly. Hybridization Solution solids must be in solution before use; warming (e.g., in a 37°C water bath) may be required to dissolve solids completely. Preparation in a clear glass container is recommended to facilitate visual inspection for solids during warming.

1.1.8 Wash Solution [1.5X SSPE, 0.5% w/v SDS (2 L)]. Add 150 mL of 20 X SSPE (**Section 1.1.6**), and 50 mL of 20% w/v SDS (**Section 1.1.2**) to 1,800 mL of DI H_2O and mix thoroughly. Wash Solution solids must be in solution before use; warming (e.g., in a 37°C water bath) may be required to dissolve solids completely. Preparation in a glass container is recommended to facilitate visual inspection for solids during warming.

1.1.9 Spotting Solution [0.4N NaOH, 25 mM EDTA, 0.00008% Bromothymol Blue (75 mL)]. Add 6 mL of 5N NaOH, 3.75 mL of 0.5 M EDTA (**Section 1.1.1**) and 150 µL 0.04 % of Bromothymol Blue (provided in Kit) to 65 mL of DI H_2O and mix thoroughly. Spotting Solution is stable for at least three months at room temperature.

1.1.10 Pre-Wetting Solution [0.4N NaOH, 25 mM EDTA (500 mL)]. Add 40 mL of 5N NaOH, 25 mL of 0.5 M EDTA (**Section 1.1.1**) to 435 mL of DI H_2O and mix thoroughly.

1.1.11 3% Hydrogen Peroxide (1 mL) **[For Colorimetric Detection Only]**.
Add 100 µL of 30% H_2O_2 to 900 µL of DI H_2O and vortex to mix. Protect from light. Store at 2° to 8°C. 3% Hydrogen Peroxide has a shelf life of approximately 4 weeks when stored at 2° to 8°C.

1.1.12 Chromogen:TMB Solution. Bring the Chromogen:TMB (TMB) to room temperature (15° to 30°C). Before opening the bottle, tap it on the lab bench to shake the TMB to the bottom of the bottle. Remove the stopper carefully to prevent loss of the powder. Slowly add 30 mL of room temperature reagent grade 100% ethanol to the bottle. **Do NOT use ethanol that has been stored in a metal container. Do NOT use 95% ethanol or other alcohols.** Recap the bottle. Seal the stopper with Parafilm. Shake in an upright position on an orbital shaker for 30 minutes or until completely dissolved. Store in bottle at 2° to 8°C and protect from rust. Under these conditions the Chromogen Solution is stable for six months after preparation.

1.2 Preparation of reagents supplied

1.2.1 Human DNA Standards. Prepare a two-fold serial dilution of the DNA Standard A (provided in Kit) in TE Buffer (**Section 1.1.4**) as follows:

1. Label seven 0.5 mL autoclaved GeneAmp® PCR Reaction Tubes A through G.
2. Vortex the DNA Standard A to mix it thoroughly.
3. Transfer 120 µL of DNA Standard A into the tube labeled A.
4. Aliquot 60 µL of TE Buffer (**Section 1.1.4**) into each of the six remaining tubes labeled B through G.
5. Add 60 µL of DNA Standard A (tube A) to the 60 µL of TE Buffer in tube B. **Vortex to mix thoroughly.**
6. Add 60 µL of diluted DNA Standard B (tube B) to the 60 µL of TE Buffer in tube C. **Vortex to mix thoroughly.**
7. Add 60 µL of diluted DNA Standard C (tube C) to the 60 µL of TE Buffer in tube D. **Vortex to mix thoroughly.**
8. Continue the serial dilution through tube G.
9. If the dilution steps are performed as described in **Section 1.2.1** above, the seven DNA Standard tubes (tubes A through G) will have the concentrations of human DNA listed in **Table 1.**

Table 1: DNA Standards and Concentrations

DNA Standard	Concentration (ng/ µL)	Quantity DNA per 5 µL (ng)
A	2	10
B	1	5
C	0.5	2.5
D	0.25	1.25
E	0.125	0.625
F	0.0625	0.3125
G	0.03125	0.15625

Note: Store the diluted DNA Standards at 2° to 8°C. The DNA Standards A through G are stable for at least three months at 2° to 8°C.

2.0 INFORMATION REGARDING PROTOCOLS

This entire section should be read before slot blotting/immobilization of DNA.

The QuantiBlot® Human DNA Quantitation Kit contains reagents for at least 10 hybridization reactions. Each hybridization reaction must include the following ten control samples: seven DNA Standards, the two DNA Calibrators and one blank [Spotting Solution (**Section 1.1.9**) only]. An additional 38 samples can be spotted on the membrane, for a total of up to 48 samples per hybridization reaction. DNA Calibrators are provided as an internal control for DNA Standard performance.

3.0. SLOT BLOTTING/IMMOBILIZATION OF DNA

Note: Wear clean disposable laboratory gloves while preparing samples. Follow safety recommendations provided by manufacturer for handling chemicals. Comply with any and all laws, regulations or orders with respect to the disposal of any hazardous or toxic chemical, material, substance or waste.

Before beginning this section, assemble the required reagents, supplies and equipment as follows:

DNA Standards (A through G)
DNA Calibrator 1 (provided in Kit)
DNA Calibrator 2 (provided in Kit)
Slot Blot Apparatus
Spotting Solution (**Section 1.1.9**)
Pre-Wetting Solution (**Section 1.1.10**)
Biodyne® B nylon membrane

3.1 Determine the number of samples to be analyzed including the seven Human DNA Standards (A through G), the DNA Calibrators 1 and 2 (provided in Kit), and the one blank (Spotting Solution only). Aliquot 150 µL of Spotting Solution into a new 0.5 mL GeneAmp PCR Reaction Tube for each sample.

3.2 Label seven of the tubes containing 150 µL Spotting Solution as follows: A, B, C, D, E, F, and G and label two of the tubes containing 150 µL of Spotting Solution as follows: DNA Calibrator 1 and DNA Calibrator 2.

3.3 Vortex the seven DNA standards and the two DNA Calibrators. Add 5 µL of each solution to the corresponding labeled tube containing 150 µL of Spotting Solution.

Note: Sample DNA should be $MgCl_2$-free. See Troubleshooting Section.

3.4 Add 1 to 5 µL of each test sample DNA to the remaining tubes containing 150 µL of Spotting Solution.

3.5 While wearing clean gloves, cut a piece of Biodyne® B membrane to 11.0 cm x 7.9 cm. Cut a small notch in the upper right corner of the membrane to mark orientation. Place the membrane in the Hybridization Tray (**Part No. N808- 0136**) containing 50 mL of Pre-Wetting Solution. Incubate at room temperature for 1 to 30 minutes.

Note: The following protocol is for use with GIBCO BRL The Convertible® slot blot apparatus. Refer to GIBCO BRL instructions for additional details. The vacuum source must have a pressure of at least 8 to 10 inches Hg.

3.6 Using forceps, remove the membrane from the Pre-Wetting Solution. Place the membrane on the gasket of the slot blot apparatus, then place the top plate of the slot blot apparatus on top of the membrane. **Turn on the vacuum source** (i.e., turn on house vacuum line or vacuum pump). **Turn off the sample vacuum and turn on the clamp vacuum** on the slot blot apparatus. Push down on the top plate to ensure the formation of a tight seal. Pour off the Pre-Wetting solution and rinse the Hybridization Tray thorough with DI H_2O.

3.7 Use a new pipette tip for each sample. Pipet each sample (approximately 155 µL) into a different well of the slot blot apparatus. Slowly dispense each sample directly into the center of each well of the slot blot apparatus ensuring that the pipet tip is approximately 5 mm above the membrane.

3.8 After all samples have been pipetted into the wells of the slot blot apparatus, **slowly turn on the sample vacuum.** Leave the sample vacuum on until all of the samples have been drawn through the membrane (approximately 30 seconds). Inspect each slot that contains a sample for a uniform blue band. (If a uniform blue band is not visible, refer to the **Troubleshooting Section.**) **Turn off the sample vacuum.**

3.9 Turn off the clamp vacuum. Turn off the vacuum source. Disassemble the slot blot apparatus and remove the membrane. **Proceed to Section 4 immediately. Do NOT allow the membrane to dry-out.**

Note: After each use, soak the slot blot apparatus in a large volume of 0.1% SDS solution (approximately 5 to 15 minutes). Using a disposable lab towel, clean the gasket and the side of the top plate that contacts the membrane. Then rinse the slot blot apparatus with an excess of water and allow to dry at room temperature. Never use bleach.

4.0 DNA HYBRIDIZATION

The following section involves the hybridization of biotinylated QuantiBlot® D17Z1 Probe to DNA samples immobilized on the nylon membrane, the binding of Enzyme Conjugate:HRP-SA to the hybridized probe and a stringent wash to remove non-specifically bound probe.

Before starting the DNA Hybridization procedure, assemble the required reagents and equipment as follows:

QuantiBlot D17Z1 Probe (provided in Kit)
Enzyme Conjugate:HRP-SA (provided in Kit)
Hybridization Solution (**Section 1.1.7**)
Wash Solution (**Section 1.1.8**)
Citrate Buffer (**Section 1.1.5**)
30% Hydrogen Peroxide
Hybridization Tray and lid
Hybridization Tray Retainer

Do NOT allow the membrane to dry at any point in the protocol. Minimize the time the membrane is not submerged in solution. Use the Hybridization Tray with lid for all steps.

Warm the Hybridization Solution and the Wash Solution to between 37° and 50°C in either a water bath or an incubator. **All solids must be in solution before use.** Mix well.

Note: Clean, disposable gloves should be worn throughout the DNA Hybridi-zation (Section 4.0) and Detection steps (Section 5.0).

4.1 Pre-hybridization: Transfer the membrane to 100 mL of pre-warmed Hybridization Solution in the Hybridization Tray. Add 5 mL of 30% H_2O_2. Place the lid on the tray. Use the Hybridization Tray Retainer or a lead weight to keep tray from floating in the water bath. Rotate in a 50°C (±1°C) water bath (50 to 60 rpm) for 15 minutes (±2 minutes). Pour off the solution.

4.2 Hybridization: Add 30 mL of Hybridization Solution to the Hybridization Tray containing the membrane. Tilt the tray to one side and add 20 µL of QuantiBlot D17Z1 Probe to the Hybridization Solution. Place the lid on the tray. Rotate in a 50°C (±1°C) water bath (50 to 60 rpm) for 20 minutes (±2 minutes). Pour off the solution.

4.3 Rinse the membrane briefly in 100 mL of pre-warmed Wash Solution by rocking the tray for several seconds. Pour off the solution.

Note: Step 4.4 (below) describes the addition of two alternative volumes of Enzyme Conjugate:HRP-SA. The volume of Enzyme Conjugate:HRP-SA used depends on the detection scheme chosen: colorimetric (TMB) or chemiluminescent (ECL™) detection. (See Introduction on page 2 for more general information on these two alternative detection schemes.)

4.4 Stringent Wash/Conjugation: Add 30 mL of the pre-warmed Wash Solution to the Hybridization Tray. Tilt the tray to one side and add the Enzyme Conjugate:HRP-SA to the 30 mL of Wash Solution as follows: for colorimetric (TMB) detection add 180 µL of Enzyme Conjugate:HRP-SA **OR** for chemiluminescent (ECL) detection add 90 µL of Enzyme Conjugate:HRP-SA. Place the lid on the tray. Rotate in a 50°C (±1°C) water bath (50 to 60 rpm) for 10 minutes (±1 minute). Pour off the solution.

4.5 Rinse the membrane thoroughly for 1 minute in 100 mL of pre-warmed Wash Solution by rocking the tray or rotating it on an orbital shaker (100 to 125 rpm) at room temperature. Pour off the solution. Rinse again for 1 minute. Pour off the solution.

4.6 Wash the membrane by adding 100 mL of pre-warmed Wash Solution to the tray. Place the lid on the tray. Rotate at room temperature on an orbital shaker (100 to 125 rpm) at room temperature for 15 minutes. Pour off the solution.

4.7 Rinse the membrane briefly in 100 mL of Citrate Buffer by rocking the tray. Pour off the solution.

5.0 DETECTION STEPS

If 180 µL of Enzyme Conjugate:HRP-SA was used in Section 4.4, use the colorimetric detection steps in Section 5.1; if 90 µL of Enzyme Conjugate:HRP-SA was used in Section 4.4, proceed to Section 5.2 for chemiluminescent detection.

5.1 Colorimetric Detection

5.1.1 Prepare the Color Development Solution **not more than 10 minutes before use.** Add the following reagents in the order listed to a glass flask and mix thoroughly by swirling. **Do NOT vortex.** To 30 mL of Citrate Buffer (**Section 1.1.5**), add 1.5 mL of Chromogen:TMB Solution (**Section 1.1.12**) and 30 µL of 3% H_2O_2 (**Section 1.1.11**).

5.1.2 Add Color Development Solution to the membrane in the tray. **Cover the tray with the lid to protect the membrane from strong light.**

5.1.3 Shake at room temperature on an orbital shaker (50 to 60 rpm) for 20 to 30 minutes.

5.1.4 Remove tray from shaker, pour off liquid.

5.1.5 Stop the color development by washing in deionized H_2O (100 mL). Shake for 5 to 10 minutes (50 to 60 rpm) with the lid on the tray. Repeat for a total of three washes.

5.1.6 Photograph the membrane when it is wet. Saran Wrap® may be placed over the membrane during photography to prevent it from drying out.

1. Place the wet membrane on a flat non-absorbent surface. Keep the membrane wet throughout the photographic procedure. Minimize exposure to strong light.
2. Use a Polaroid camera with Type 55 or 667 (black and white) film or Type 59 or 559 (color) film.
3. Follow Polaroid film exposure and development instructions.

Note: For black and white photography, an orange filter (Wratten 22 or 23A) will enhance contrast.

4. Following photography, the membrane may be air-dried on any hard non-absorbent surface. Protect from light and oxidizing agents (e.g., acid treated paper, bleach and nitric acid). The blue color on the membrane will fade upon drying.

5.2 Chemiluminescent Detection

Before beginning this section, assemble the required reagents, supplies and equipment as follows:

ECL™ Detection Reagents
Whatman® Benchkote
Saran Wrap®
X-ray Film
Film Cassette

Note: ECL Reagents 1 and 2 should be stored separately (at 2° to 8°C) and not allowed to cross-contaminate each other.

5.2.1 To 5 mL of ECL Reagent 2, add 5 mL of ECL Reagent 1. **Do NOT prepare this mixture more than 5 minutes before use.** Add the 10 mL of ECL reagent mixture to the membrane in the Hybridization Tray and shake for exactly 1 minute at room temperature. Pour off the solution.

Note: For maximum sensitivity, expose the membrane to X-ray film within 10 minutes of incubation in ECL reagents.

5.2.2 Cut a piece of Benchkote to approximately 12 x 16 cm. Place the damp membrane DNA-side-up on the plastic-coated side of the Benchkote. Cover the membrane with a piece of Saran Wrap that is a few centimeters larger than the BenchKote. Use a paper towel to smooth out any wrinkles or air bubbles in the Saran Wrap. Fold the Saran Wrap behind the BenchKote on the top and bottom sides. Again, use a paper towel to flatten the Saran Wrap and remove any air bubbles. Fold the Saran Wrap behind the Benchkote on the two remaining sides.

5.2.3 In a darkroom, place a piece of Hyperfilm ECL or Kodak XAR5 film in the film cassette. Carefully place the covered membrane on top of the film such that the DNA side is in contact with the film. **Do NOT move the membrane once it is placed on top of the film; movement may cause blurring of the resulting image or a "double image".** Close the film cassette. **It is very important that the film is in tight, uniform contact with the covered membrane.**

5.2.4 Expose the film for 15 minutes at room temperature.

5.2.5 Process the film with an automatic film processor. If an automatic film processor is not available, use Kodak GBX Developer and GBX Fixer as follows:

1. Dilute the Developer and Fixer solutions as instructed on the reagent bottles.
2. In the darkroom, incubate the film in the Developer for 90 seconds before rinsing it in DI H_2O for a few seconds. Then incubate it in the Fixer for 90 seconds.
3. Rinse the film in a continuous flow of fresh DI H_2O for 30 minutes. Hang to dry.

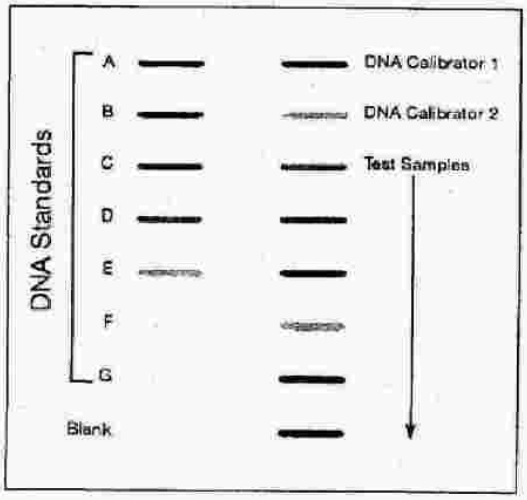
Note: If desired, additional X-ray film exposures can be obtained by repeating steps 5.2.3 through 5.2.5 above. Exposures longer than 15 minutes (up to several hours or overnight) will result in increased sensitivity. Preparation of additional further-diluted DNA Standards may be necessary to quantitate samples containing less than 0.15 nanograms of DNA.

6.0 RESULTS INTERPRETATION

Results are interpreted by comparing the signal intensity of the DNA test sample to the signal intensity obtained for the DNA Standards. The signal intensity for a sample reflects the total amount of DNA spotted on the membrane. The seven DNA Standards represent the following quantities of DNA spotted on the membrane: 10, 5, 2.5, 1.25, 0.625, 0.3125 and 0.15625 ng (**See Table 1**).

The DNA Calibrators are used to provide DNA of a known concentration to verify that the DNA Standards were correctly diluted and are providing correct results for the test samples. For example, the DNA Calibrator 1 has a stock concentration of 0.7 ng/µL. Five µL of this control was added to 150 µL of Spotting Solution and the entire 155 µL was spotted on the membrane. Thus, 3.5 ng of this sample was spotted on the membrane (0.7 ng/µL • 5 µL = 3.5 ng). The signal obtained for this control sample should have an intensity that is between the 2.5 and 5 ng DNA Standards. Likewise, the DNA Calibrator 2 should have an intensity that is between the 0.3125 and 0.625 ng DNA Standards. If not, see the **Troubleshooting Section.**

Figure 1: Example of QuantiBlot® results (using colorimetric (TMB) detection method). The seven DNA Standards represent the following quantities of DNA spotted on the membrane: A = 10 ng; B = 5 ng; C = 2.5 ng; D = 1.25 ng; E = 0.625 ng; F = 0.3125 ng and G = 0.15625 ng. DNA Calibrator 1 should have an intensity that is between DNA Standards B and C. DNA Calibrator 2 should have an intensity that is between DNA Standards E and F. Quantities for the test samples are determined by comparison of signal intensities to the DNA Standards.



The concentration of a DNA test sample is determined as follows:

1. Determine the quantity of DNA test sample spotted on the membrane by comparing its signal intensity to the intensity of the DNA Standards.
2. Divide this quantity by the volume of DNA test sample added to the Spotting Solution (typically 5 µL of DNA test sample is added to 150 µL of Spotting Solution).

This calculation gives DNA concentration in ng/µL .

PERFORMANCE CHARACTERISTICS

When the recommended protocols are followed, using the colorimetric detection method with Chromogen:TMB, the user of the QuantiBlot® Human DNA Quantitation Kit will be able to detect and quantitate 0.15 to 10 ng of human DNA per test sample.

TROUBLESHOOTING

Observation	Possible Cause	Recommended Action
1. No signal or low sensitivity (0.15 ng DNA Standard not visible).	Use of a membrane other than Biodyne® B.	Use Biodyne B nylon membrane. Do not use membranes that have a neutral charge.
	Incorrect NaOH or EDTA concentrations in Spotting Solution.	Prepare Spotting Solution correctly (**Section 1.1.9**).
	Water bath temperature too high.	Water bath temperature should be 50°C (±1°C).
	DNA Probe was not added at hybridization step.	Add QuantiBlot® D17Z1 Probe.
	Enzyme conjugate was not added.	Add Enzyme Conjugate: HRP-SA at indicated step in protocol. Use 180 µL of Enzyme Conjugate:HRP-SA for colorimetric (TMB) detection, and use 90 µL of HRP-SA for chemiluminescent (ECL™) detection.
	Hydrogen peroxide was inactive (colorimetric detection only).	Prepare a new Color Development Solution using a fresh bottle of hydrogen peroxide.
	Presence of $MgCl_2$ in the DNA sample	Concentrations of $MgCl_2$ >0.3 mM can result in reduced sensitivity. Prepare all DNA dilutions in TE Buffer. Any $MgCl_2$ can be removed from samples by microdialysis using Centricon® 100 spin units (follow manufacture's directions).
	Cross-contamination of ECL Reagents (chemiluminescent detection method only).	ECL Reagents 1 and 2 should be stored separately and should not be mixed until 5 minutes prior to use.
	ECL Reagents stored at room temperature (chemiluminescent detection method only).	Order fresh ECL Reag- ents. Store ECL Reag- ents at 2° to 8°C.
2. Areas of low sensitivity across the membrane.	Membrane slipped up onto the side of the Hybridization Tray during Hybridization or Stringent Wash steps.	Reduce the rotation rate of the water bath to 50-60 rpm. Check that the membrane is fully submerged in the bottom of the Hybridization Tray before shaking.
	Membrane dried-out significantly at some point in the protocol.	Do not allow the membrane to dry at any point in the protocol.

Observation	Possible Cause	Recommended Action
2. Areas of low sensitivity across the membrane.	Poor contact between the film and the covered membrane during film exposure for chemiluminescent detection.	Smooth-out any wrinkles or bubbles in the Saran Wrap® covering the membrane and make sure that the film cassette provides tight, uniform contact between the covered membrane and the film.
3. Non-uniform signal intensity within a slot.	Bubble(s) in slot blot wells when sample was pipetted into well, or when vacuum was applied.	Slowly pipet the Spotting Solution directly over the center of the wells of the slot blot apparatus, with the pipet tip raised approximately 5 mm above the membrane. Turn on the sample vacuum slowly, not all at once. After being drawn through the membrane, the sample should appear as a uniform blue band on the membrane. If the entire sample is not drawn through the membrane, turn off the sample vacuum. Pipet the sample back into the pipet tip; then pipet the sample back into the well of the slot blot apparatus. Turn on the sample vacuum to draw the sample through the membrane.
4. Filter background	No or low SDS in the Hybridization Solution or in the Wash Solution.	Prepare solutions with proper concentrations of SDS.
	Membrane was not pre-wetted prior to slot blotting.	Pre-wet the membrane in Pre-Wetting Solution prior to slot blotting.
	Too much Enzyme Conjugate:HRP-SA was add-ed.	Use 180 µL of Enzyme Conjugate:HRP-SA for colorimetric detection or 90 µL of Enzyme Conjugate:HRP-SA for chemiluminescent detection.
	Lack of thorough rinsing at **Section 4.5** of the DNA Hybridization protocol.	Thoroughly rinse twice, for 1 minute each, using 100 mL of pre-warmed Wash Solution at this step. These two rinse times can be extended beyond 1 minute if necessary.
	Slot blot apparatus not cleaned thoroughly after last use.	Immediately after each use, soak the slot blot apparatus in a large volume of 0.1% SDS solution. Never use bleach.

Observation	Possible Cause	Recommended Action
5. Blurry or mis-shapen slots (chemiluminescent detection only).	Bubbles or wrinkles in Saran Wrap covering membrane during film exposure.	Carefully smooth-out wrinkles or air pockets in the Saran Wrap that covers the membrane during film exposure.
6. Blurry slots and/or double image (chemiluminescent detection only).	Membrane was moved after it was placed in contact with the film.	Carefully place the membrane directly on top of the film. Do not move the membrane once it has been placed on top of the film.
7. The DNA Calibrators do not quantitate correctly with respect to the DNA Stan-dards.	DNA Standard serial dilutions prepared incorrectly.	Prepare two-fold serial dilutions of DNA Standard A in TE Buffer as described. Add 5 µL of each dilution to 150 µL of Spotting Solution for slot blotting.
8. Signals obtained for non-human DNA samples.*	Water bath temperature too low.	Water bath temperature should be 50°C (±1°C).
	SSPE concentration too high in Wash Solution.	Check that the 20X SSPE solution and the Wash Solution were prepared correctly.

* DNA from primate species may give signals similar to those obtained from equivalent amounts of human DNA. In Roche Molecular Systems (RMS) laboratories, 30 ng to 300 ng quantities of non-primate DNA samples result in either no signals or signals that are less than or equal to the signal obtained for 0.15 ng of human DNA. The following non-primate DNA samples have been tested in RMS laboratories: *E. coli*, yeast, dog, cat, mouse, rat, pig, cow, chicken, fish and turkey.

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