

DNA Analyst Training Laboratory Training Manual

Protocol 3.09 Slot Blot Colorimetric Procedure



This laboratory protocol (or part thereof) has been provided as an example of a laboratory SOP, courtesy of the National Forensic Science Technology Center. It has been included for training and example purposes only.

PRESIDENT'S
DNA
INITIATIVE



INTRODUCTION

The QuantiBlot® Human DNA Quantitation kit uses a probe complementary to a primate specific alpha satellite DNA sequence on chromosome 17 at locus D17Z1. Extracted DNA is first immobilized on a nylon membrane. The biotinylated probe, which is complementary to this sequence, is then hybridized to the DNA. This is followed by a colorimetric detection using streptavidin, horseradish peroxidase, and tetramethylbenzidine. The quantity of DNA is determined by comparing the intensities of the resultant bands to the intensities of the bands from a serial dilution of standard DNA ranging from 0.15 to 10.0 ng.

SAFETY CONSIDERATIONS

- Chromogen:TMB Solution is an irritant; avoid contact with skin and inhalation.
- SDS can cause lung irritation, wear a mask when working with powdered SDS.
- Refer to the Laboratory Safety Manual(s)

PREPARATIONS

Biodyne B Membranes

DNA Calibrator 1 (included in kit)

DNA Calibrator 2 (included in kit)

D17Z1 Probe (included in kit)

Enzyme Conjugate:HRP-SA (included in kit)

30% H₂O₂

Chromogen:TMB Solution

1. Warm Chromogen:TMB bottle to room temperature.
2. Tap bottle on counter to bring powder to the bottom.
3. Open carefully to prevent loss.
4. Add 30 ml room temperature absolute ethanol. Do not use ethanol that has been stored in a metal container. Seal.
5. Shake upright on an orbital shaker until dissolved, about 2 hours.
6. Store at 2° to 8°C.
Expiration date six months.

Citrate Buffer (0.1 M Sodium Citrate, pH 5.0) QuantiBlot®

1. 18.4 g trisodium citrate, dihydrate ($\text{Na}_3\text{C}_6\text{H}_5\text{O}_7 \cdot 2\text{H}_2\text{O}$)
2. 800 ml sterile deionized water
3. Dissolve. Adjust pH to 5.0 ± 0.2 with approximately 6 g citric acid monohydrate ($\text{C}_6\text{H}_8\text{O}_7 \cdot \text{H}_2\text{O}$).
4. Adjust final volume to 1 liter.
5. Store at room temperature.
Expiration date six months.

0.5 M EDTA, pH 8.0

1. 800 ml DI water
2. 186.1 g $\text{Na}_2\text{EDTA} \cdot 2\text{H}_2\text{O}$
3. 20 g NaOH pellets
4. Stir to dissolve.
5. Adjust pH to 8.0 ± 0.2 with 4N NaOH.
6. Bring volume to 1 liter with sterile deionized water.
7. Autoclave.
8. Store at room temperature.
Expiration date six months.

Human DNA Standards QuantiBlot®

1. Label seven 0.5 ml sterile tubes A through G.
2. Vortex DNA Standard A and quick spin. The quantity made may vary. The following is an example of the steps.
3. Pipette $14\mu\text{l}$ DNA Standard A to tube labeled A.
4. Pipette $7\mu\text{l}$ TE^{-4} , pH 8.0 into each of the remaining tubes.
5. Do a serial dilution by pipetting $7\mu\text{l}$ from tube A into tube B.
6. Mix.
7. Pipette $7\mu\text{l}$ from tube B into tube C.
8. Mix.
9. Continue dilutions through tube G.

DNA Standard	Concentration (ng/ μl)	Quantity of DNA in $5\mu\text{l}$
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

10. Store at 2° to 8°C. Make new each day of use.

Hybridization Solution (5X SSPE, 0.5% w/v SDS) QuantiBlot®

1. 250 ml 20X SSPE
2. 50 ml 10% w/v SDS
3. 700 ml DI water
4. Mix thoroughly.
5. Warm at 37° to 50°C to dissolve. All solids must be in solution before use.
6. Store at room temperature.
Expiration date six months.

Pre-Wetting Solution (0.4N NaOH, 25 mM EDTA)

1. 50 ml 4N NaOH
2. 25 ml 0.5M EDTA
3. 425 ml DI water
4. Mix and store at room temperature.
Expiration date six months.

4 N NaOH

1. 160 g NaOH pellets
2. 1 liter DI water
3. Dissolve.
4. Store at room temperature.
Expiration date six months.

20% (w/v) SDS

1. 200 g electrophoresis-grade (ultra pure) sodium dodecyl sulfate (SDS)
2. 800 ml DI water
3. Slowly dissolve with warming at 37° to 50°C. Adjust volume to 1 liter.
Dilute further with DI water as needed.
4. Store at room temperature.
Expiration date six months.
Use caution when weighing SDS. Wear protective mask.

Spotting Solution (0.4 N NaOH, 25 mM EDTA, 0.00008% Bromothymol Blue)

1. 75 ml Pre-wetting solution
2. 150 µl 0.04% Bromothymol Blue
3. Mix and store at room temperature.
Expiration date six months.

20X SSPE Buffer (3.6 M NaCl, 200mM NaH₂PO₄•H₂O, 20mM EDTA, pH7.4

1. 7.4 g disodium ethylenediaminetetraacetic acid dihydrate (Na₂EDTA•2H₂O)
2. 800 ml DI water
3. Dissolve.
4. Adjust pH to 8.0 ± 0.2 with 10 N NaOH.
5. 210 g sodium chloride (NaCl)
6. 27.6 g sodium phosphate, monobasic, monohydrate (NaH₂PO₄•H₂O)
7. Dissolve.
8. Adjust pH to 7.4 ± 0.2 with about 10 ml 10 N NaOH.
9. Adjust final volume to 1 liter. Dilute with DI water as needed for lower concentrations.
10. Autoclave.
11. Store at room temperature.
Expiration date six months.

TE-4 (10 mM Tris-HCl, 0.1 mM EDTA, pH 8.0)

1. 10 ml 1 M Tris-HCl, pH 8.0
2. 0.2 ml 0.5 M EDTA
3. 990 ml DI water
4. Dispense in 50 ml aliquots. Autoclave.
5. Store at room temperature or at 2° to 8°C.
Expiration date one year.

Wash Solution QuantiBlot® (1.5X SSPE, 0.5% w/v SDS)

1. 150 ml 20X SSPE
2. 100 ml 10% w/v SDS
3. 1750 ml DI water
4. Mix thoroughly. Warm at 37° to 50°C to dissolve. All solids must be in solution before use.
5. Store at room temperature.
Expiration date six months

INSTRUMENTATION

- Camera
- Centrifuge
- Pipettes
- Slot blot apparatus
- Vortex
- Water bath

MINIMUM STANDARDS & CONTROLS

- Positive extraction control
- Extraction blank – contains reagents only
- DNA calibrator 1 & 2-known standards included in kit

PROCEDURE OR ANALYSIS

Slot Blotting / DNA Immobilization

1. Determine the number of tubes needed for calibrators, controls, blanks and samples. Label 0.5 ml microcentrifuge tubes according to placement on the membrane (i.e. A1, A2, B1, B2, etc.).
2. Pipette 150 μ l of Spotting Solution into each tube.
3. Vortex Human DNA standards and calibrators. Quick spin. Pipette 5 μ l of DNA solution into the appropriate tube.
4. Vortex DNA samples. Quick spin. Pipette 1 to 20 μ l of sample DNA into the appropriate tube.
5. While wearing clean gloves, cut a piece of Biodyne B membrane 11.0 cm by 8 cm. Label in the upper right-hand corner to mark orientation. Place the membrane in the hybridization tray containing an adequate amount of Pre-Wetting Solution to wet the membrane. Leave for 1 to 30 minutes.
6. Using forceps, place the wetted membrane on the gasket of the slot blot apparatus. Place the top plate of the slot blot apparatus on top of the membrane. Turn on the vacuum source. Turn off the sample vacuum and turn on the clamp vacuum. Push down on the plate to ensure a tight seal.
7. Rinse the hybridization tray with DI water.
8. Slowly pipette each sample into the center of the appropriate well of the slot blot apparatus. Take care not to get bubbles on the membrane in the well(s).
9. Slowly turn on the sample vacuum. After all samples have been drawn through the membrane inspect each slot for a uniform blue band. Turn off the sample vacuum.
10. Turn off the clamp vacuum. Turn off the vacuum source. Disassemble the slot blot apparatus and remove the membrane. **Proceed immediately to hybridization or store the membrane in 5X SSPE at 4°C for up to 24 hours. Do not allow the membrane to dry out.**

11. Wash the slot blot apparatus with 0.1% SDS. Rub the gasket and the side of the top plate that touches the membrane. Rinse apparatus with an excess of DI water and allow to air dry. **Do not use bleach.**

Hybridization

1. Do not allow the membrane to dry out during any of the following steps. Warm the Hybridization Solution and the Wash Solution to 37° to 50°C before use. All solids must be in solution before use. Turn on the rotating water bath and allow to warm to 50°C. Check to see that the water level is 1/4 to 1/2 inch above the platform.
2. Place the membrane in the hybridization tray. Add 60 ml of pre-warmed Hybridization Solution. Add 3 ml of 30% H₂O₂. Place the lid on the tray and put in the 50°C water bath with a 1 kg lead weight on top of the tray. Rotate at 50-60 rpm for 15 minutes. Pour off solution.
3. Add 30 ml of pre-warmed Hybridization Solution to the tray with the membrane. Tilt the tray and add 20 µl of QuantiBlot® D17Z1 Probe into the Hybridization Solution. Place the lid on and rotate at 50-60 rpm and 50°C for 20 minutes. Pour off the solution.
4. Rinse the membrane in 60 ml of pre-warmed Wash Solution briefly. Pour off the solution.
5. Add 30 ml of pre-warmed Wash Solution to the tray. Tilt tray to one side and pipette 180 µl of Enzyme Conjugate:HRP-SA into the solution. Place the lid on the tray. Rotate at 50-60 rpm and 50°C for 10 minutes. Pour off solution.
6. Rinse with 60 ml of Wash Solution for 1 minute at room temperature. Pour off solution. Repeat.
7. Add 60 ml of Wash Solution. Place the lid on the tray. Rotate at 100-120 rpm at room temperature on an orbital shaker for 15 minutes. Pour off solution.
8. Rinse the membrane briefly in 60 ml of Citrate Buffer. Pour off the solution.

Detection

1. Add 30 ml of Citrate Buffer. Tilt the tray to one side and pipette 3 µl of 30% H₂O₂ and 1.5 ml of Chromogen:TMB Solution. Cover to protect from strong light.
2. Rotate at 50-60 rpm at room temperature on an orbital shaker for 20 to 30 minutes or until all the standard bands are seen. Pour off solution.

3. Stop the color development by washing with approximately 100 ml of DI water. Repeat for a total of 3 washes.
4. Photograph using a Polaroid MP4 camera with Type 553 film or equivalent. Normal camera settings are aperture f/16 and shutter speed 1/60 second. The color will fade on drying.
5. Clean the tray by washing with reagent alcohol followed by water rinse and DI water rinse.

Interpretation

1. DNA Standards must show an expected intensity decrease with decreasing concentration from 10 to 0.15 ng per 5 μ l. If the expected decrease is not seen, the test is inconclusive and must be rerun.
2. Negative controls must show no bands.
3. If a band is seen in the QuantiBlot® reagent blank slot, re-run the QuantiBlot®. It may be necessary to trouble-shoot the QuantiBlot® reagents and/or equipment for contamination if the problem persists.
4. If a band is seen in an extraction blank slot, the problem may be in the QuantiBlot® or the extraction. Re-run the extraction blank. If the problem persists, it may be necessary to re-extract the samples, which were extracted with that blank in order to eliminate any possible contamination.
5. The DNA Calibrator 1 must have an intensity between DNA Standards B and C.
6. The DNA Calibrator 2 must have an intensity between DNA Standards E and F.
7. Quantities of DNA in the samples are determined by comparing their band intensities with the band intensities of the DNA Standards. To get the DNA concentration in ng/ μ l, divide by the volume of sample added to the Spotting Solution.
8. If no DNA is seen in a sample slot, amplification may be attempted at the analyst's discretion.

[Return to Laboratory Training Manual User Guide](#)