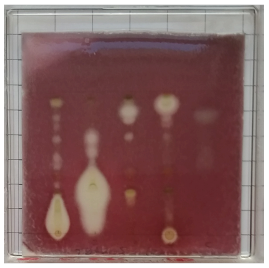
Oct 01, 2020 Version 2

Thin Layer Chromatography (TLC) Bioautography V.2



DOI
https://dx.doi.org/10.17504/protocols.io.bmxik7ke

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DOI: https://dx.doi.org/10.17504/protocols.io.bmxik7ke

Protocol Citation: Dane Lyddiard 2020. Thin Layer Chromatography (TLC) Bioautography. **protocols.io** https://dx.doi.org/10.17504/protocols.io.bmxik7keVersion created by Dane Lyddiard

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Protocol status: Working
We use this protocol and it's working

Created: October 01, 2020

Last Modified: October 01, 2020

Protocol Integer ID: 42698

Keywords: identification of antibacterial constituent, plants for antibacterial constituent, essential oils for antibacterial activity, antibacterial constituent, constituents of plant extract, plant extract, antibacterial activity, possessing antibacterial activity, essential oil, tlc bioautography, tlc bioautography protocol, direct bioautographic tlc, journal of natural product, natural product, thin layer chromatography, screening plant, constituent, myoporaceae, compound, bioautography, tlc

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Abstract

A TLC bioautography protocol to test the constituents of plant extracts and essential oils for antibacterial activity was adapted and optimised from the methods described in Smith et al. (2007) and Hamburger & Cordell (1987). The protocol described here has been used successfully with a number of non-fastidious organisms, plant extracts and essential oils. The procedure is economical, easy to undertake and the results easily interpreted. In addition to initially screening plants for antibacterial constituents, the TLC bioautography can inform compound isolation work.

References:

Hamburger, M. O., Cordell, G. A., 1987. A direct Bioautographic TLC assay for compounds possessing antibacterial activity. Journal of Natural Products 50, 19-22.

Smith, J. E., Tucker, D., Watson, K., Jones, G. L., 2007. Identification of antibacterial constituents from the indigenous Australian medicinal plant Eremophila duttonii F. Muell. (Myoporaceae). J Ethnopharmacol 112, 386-393.

Materials

MATERIALS

Nutrient Broth (Dehydrated) **Thermo Fisher Catalog #**CM0001B

Mueller Hinton Agar (Dehydrated) **Thermo Fisher Catalog #**CM0337B

Oxoid™ Agar Bacteriological, (Agar No. 1) **Thermo Fisher Catalog #**LP0011B

Iodonitrotetrazolium Chloride **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**I8377

Square Petri Dish 90 mm (Sterile) **Sarstedt Catalog #**82.9923.422

Aluminium TLC plates (TLC Silica gel 60 F254) **Merck Millipore (EMD Millipore) Catalog #**1.05554.0001

Tea Tree Oil **Thursday Plantation**

Generic Materials

- Plant extract(s) (in solvent) or plant oil(s)
- Solvents for TLC (e.g., methanol, dichloromethane, ethyl acetate, hexanes)
- Disposable transfer pipettes (sterile)
- Sterile tubes (e.g., 10 ml disposable centrifuge tubes)
- TLC spotter or syringe needle
- Swabs and/or loop (sterile)
- Glass test tubes (10-50 ml)
- Sterile 0.9% NaCl solution
- Round Petri dishes (sterile)
- 0.5 McFarland Standard
- TLC stains
- Bacterial organisms [non-fastidious organisms which grow satisfactorily under aerobic conditions within 20 h at 35°C; *Pseudomonas aeruginosa* (ATCC27853), *Escherichia coli* (ATCC25922), *Staphylococcus aureus* (ATCC29213) *and Staphylococcus epidermidis* (ACM3978) have been used successfully].

Equipment

- Chromatography tank
- Lamina flow cabinet
- Incubator (35ºC)
- Small spray bottle (sterile)
- UV lamp (optional)

Safety warnings

- Some organisms noted in this protocol are rated at BSL-2 and should be handled following BSL-2 precautions.
- Molten agar can cause burns and should be handled with caution.
- Solvents and stains can be hazardous and should be handled as per their MSDS.
- UV lights can cause eye and skin damage; follow manufacturer instructions.

1 **Grow Bacteria (Overnight)** 1d

Prepare streak plates of bacteria on appropriate solid agar media (e.g., nutrient or Mueller-Hinton agars). Incubate overnight (aerobically) at 35°C ±1°C

2 **Select TLC Solvent System** 1h

Cut TLC plates (handle with gloves) to 9 × 9 cm (or smaller for testing solvent systems). Using a TLC spotter or syringe needle, spot a few TLC plates with plant extract (in solvent) or oil approx. 1 cm from the base of the plate. In the case of plant extracts in solvent, allow the solvent to evaporate. Test the plates using different solvent systems (e.g., 5% methanol in dichloromethane) with the aim of getting a solvent system producing good separation of the extract/oil constituents (you may need UV light and/or TLC stains to visualise the plates).

3 **Prepare TLC Plates** 30m

Prepare a TLC tank with approx. 0.5 cm of solvent in the base. Pencil a line 1 cm from the base of a cut TLC plate and spot approx. 1 µl of oil or 0.5 mg of extract (in solvent, allowing the solvent to evaporate) onto the line, leaving a 1.5 cm gap between the sides of the card and between each spot (total of 5 lanes). Include a control extract or oil (i.e., with known antimicrobial activity such as tea tree [*Melaleuca alternifolia*] oil).

4 **Develop TLC Plates** 15m

Place the TLC plate into the developing tank (submerge the base of the plate but avoid submerging the spots into the solvent). Allow the solvent front to rise sufficiently to obtain adequate separation (leave at least 1-2 cm from the top of the plate). Remove the plate, indicate the solvent front in pencil and keep the plate under sterile conditions (e.g., under lamina flow) until the solvent has evaporated. Store in a closed sterile square (10 × 10 cm) Petri dish.

5 **Repeat Steps 3-4**

Repeat steps 3-4 for as many organisms as will be tested (and for extra plates for TLC staining, if required).

6 **Prepare Media** 2h

Prepare nutrient broth as per manufacturer's instructions, but with 10% less water than recommended. Add 0.4 g bacteriological agar per 90 ml volume. Sterilise by autoclaving (as per manufacturer's recommendations) and maintain molten (e.g., in a near-boiling heat bath) until use.

7 **Prepare Bacterial Suspensions**

30m

From bacterial streak plates (step 1), select isolated colonies and suspend in sterile saline solution to a 0.5 McFarland turbidity (approximately 1-2 × 10^8 CFUs/ml for *E. coli*). Turbidity can be assessed visually by comparison to an 0.5 McFarland standard held in front of a card with black and white stripes or text (alternatively, a device such as a McFarland nephelometer can be used). If the inoculum is too dense, add more saline solution. If the inoculum is too light, suspend more bacteria from the plates. This solution should be used soon after preparation and cannot be stored.

8 **Prepare TLC Bacterial Overlay** 10m

Add 9 ml of molten agar into sterile 10 ml tubes. Once the temperature has dropped to approx. 40°C (under 45°C), add 1 ml of bacterial suspension to the 9 ml tube and mix quickly but gently to minimise bubbles. This will lead to a final inoculum density of approximately 1-2 × 10^7 CFUs/ml and agar concentration of 0.4% w/v.

9 **Overlay TLC Plates** 5m

Pour the 10 ml molten solution gently on to the TLC plate (pour above the solvent front) ensuring the entire card is covered (gently tilting the Petri dish may assist the even spread of the solution across the plate). Allow to cool to a gel and place the lid on the Petri dish.

10 **Repeat Steps 7-9**

Repeat steps 7-9 for each organism to be tested.

11 **Overnight Incubation** 1d

Transfer the plates into an incubator (aerobic) for 20-22 h at 35°C ±1°C. Keep an open beaker of water in the incubator to ensure the plate does not dehydrate.

12 **Tetrazolium Development** 2h

After 20-22 h, spray each plate (using a small sterile spray bottle) with approx. 2-3 ml iodonitrotetrazolium chloride (2 mg/ml) solution evenly across the plate. If excessive pooling occurs, gently tilting the Petri dish will allow the liquid to drain off the TLC plate. Reincubate under the same conditions as step 11 for 2 h.

13 **Read Plate** 15m

Review plates: clearings indicate bacterial inhibition and pinkish colour indicates bacterial growth. R_f values of clearings (and comparison to TLC plates visualised with UV or stains) can assist in compound characterisation and inform isolation of active compounds.