**TB-Hybridization chain reaction (TB-HCR)**

**Method developed and optimised by Paul Campbell, Fiona Filardo and Peter Vukovic.**

**See attached recipes for listed buffers.**

**Tissue printing**

* Blot plant stems onto nitrocellulose membrane (Amersham Protran 0.45 uM supplied by Merk, Cat # GE10600018).
* UV-crosslink membrane in HL-2000 hybrilinker at 1500kj/cm2 for 1 min – aids in DNA/RNA hybridizing to membrane

Note:- hairpins can be prepared at any stage (see process below) and buffers should be prewarmed

**Probe hybridization stage**

* Rinse membrane 3 times in 2 x SSC + 1.0 % SDS at room temperature (RT) for ≥5 mins each.
* Prepare Hybridisation (Hyb) Buffers (2 separate lots) - enough to cover membrane by at least 2mm (e.g. ~ 3mL for membrane 5 x 2 cm in small container) and pre heat to 55oC - 60 oC (temperature depends on the probe).
* Wash membrane in pre-heated Hyb Buffer and incubate at 55 oC - 60 oC for ≥5 mins.
* Prepare Probe: Add 5-8 uL of each 10uM probe (diluted in TE-low EDTA) per 1 mL **fresh** warmed Hyb Buffer (eg 3 mL pre-heated hyb buffer + 15 uL of each probe).
* Transfer membrane to Fresh Hyb buffer containing probe.
* Incubate membrane at 55oC - 60 oC for 10-30 min with gentle shaking.
* Remove excess probes by washing 1x with 0.5 x SSC + 0.1% SDS at 55oC, then 2x with 0.2 x SSC + 0.1 % SDS at 37oC for ≥5 mins each.

**Amplification stage**

* Prepare each labelled hairpin separately: Add 3 μL HP (100UM) to 10 uL of 5 x SSC. Heat HP at 95°C for 90 seconds then allow to come to room temp for ~30 min.
* Transfer membrane from wash buffer to pre-warmed (37oC) amplification buffer, enough to cover blots (~3 mL).
* When HPs are ready, transfer membrane to **fresh** pre-warmed amplification buffer (3 mL) and add each of the hairpin solutions (total 13 uL per HP).
* Incubate at 37°C with gentle shaking for 10 min - 1 hr.

**Detection stage – for biotin labelled HP’s**

* Transfer membrane and wash twice with detection buffer + 3% BSA at RT.
* Transfer membrane to fresh detection buffer + 3% BSA + streptavidin-AP (~1:5,000 – 10,000 dilution for new Invitrogen bottle). Ie. 5 mL buffer + 120 mg BSA + ~0.4ul strep-AP.
* Incubate with shaking for 10 min at RT.
* Transfer membrane and wash 2x with RT substrate buffer for ≥5 mins each.
* Transfer membrane to fresh substrate buffer + substrate solutions A & B (5 uL / mL Substrate Buffer e.g. 25 uL each in 5 mL buffer) and leave for ~ 10 - 30 min at RT watching colour development.

**Detection stage – for fluorophore HP’s**

* Add HP and watch for fluorescence after 10 min – 1 hr.

**SOLUTIONS**

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| **20X SSC, 1L**  175.3 g NaCl (3.0 M NaCl)  88.2 g Na citrate (0.3 M Na citrate; C6H5O7Na3)  + HCL to pH 7 | **Amplification buffer**  100 mL of 5 X SSC  100 ul Tween 20 (final is 0.1 % Tween)  10 mg Dextran Sulphate (final is 0.01 %) |
| **2 x SSC + 1 % SDS, 1L**  100 mL of 20 x SSC  100 ml 10 % SDS  Make up to 1L with ddH20 | **Detection Buffer – pH7.5**  100 mM Tris-HCL  100 mM NaCL  2mM MgCl2  0.05 % Triton X-100  3% BSA at time of use. |
| **5 x SSC, 200 ml**  50 mL of 20 x SSC  150 mL with ddH20 | **1L Detection Buffer**  15.76 g Tris-HCL  5.884 g NaCL  0.4066 g MgCl2.6H2O  pH to 7.5 with NaOH  500 ul Triton X-100  3% BSA at time of use. |
| **0.5 x SSC + 0.1% SDS, 400 ml**  10 ml of 20 x SSC  4ml 10 % SDS  Make up to 400 ml with ddH2O | **TE buffer with low EDTA (for diluting probes)**  **10 mM Tris-HCL pH 8.0 & 0.1 mM EDTA – 50ml**  500 ul 1M Tris pH 8.0  20ul EDTA pH 8.4. 250 mM  49.48 ml H2O |
| **Substrate buffer 1L**  NaCl – 5.84 g  100 mM Tris – 12.12 g  5mM MgCL2.6H2O - 1.02g. pH to 9.5 | **0.2 x SSC + 0.1% SDS, 400 mL**  4 ml of 20 x SSC  400 ul of 10 % SDS |

**Hybridization Buffers:**  
ULTRAhyb™ Ultrasensitive Hybridization Buffer (Invitrogen, Cat#: AM8670)

Rapid-hyb buffer Amersham, (Cat#: RPN1635/6)

**PerfectHyb – Sigma, (Cat#: H7033) – can get in 1L cheaper**

Streptaviden AP – Invitrogen (Cat#: 434322)

AP conjugate substrate kit – Bio-Rad (Cat#: 1706432)