1. Principle:
The method described here enables the non-radioactive hybridisation of 43 samples with 43 different oligonucleotide probes in a single assay. In this method oligonucleotide probes are covalently attached to a membrane in parallel lines using a miniblotter. After binding of the oligos the membrane is rotated 90° in the miniblotter. The slots of the miniblotter which now are perpendicular to the oligo lines are filled with biotin labeled PCR products. Hybridization takes place in the miniblotter and is visualized using a peroxidase labeled streptavidine, which interacts with the biotin of the PCR product, followed by chemiluminescence detection. The blot with attached probes can be stripped and reused several times (>10x).

2. Procedure:

2.1 Covalent coupling of oligonucleotide probes to the membrane.
All oligonucleotide probes are synthesized with a 5' terminal aminogroup, which is used to covalently link the oligos to an activated negatively charged Biodyne C membrane.

1. Dilute the oligonucleotides to the optimized concentrations ranging from approximately 125 pmol to 5000 pmol in 150 µl 500 mM NaHCO₃ pH 8.4.
2. Activate the Biodyne C membrane by 10 min incubation in freshly prepared 16 % (w/v) 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDAC) in demineralized water, in a rolling bottle at room temperature.
3. Rinse the membrane with tapwater and place it on a support cushion in a clean miniblotter system. Turn the screws hand-tight. Remove residual water from the slots by aspiration.
4. Fill the slots of the miniblotter with 150 µl of the diluted oligonucleotide solutions but don't fill the first and last slots.
5. The first and the last slot are used to mark the edges of the membrane by filling these slots with drawing pen ink diluted 1:100 in water.
6. After all samples are added, incubate at least 1 min at room temperature.
7. Remove the oligonucleotide solutions by aspiration in the same order as in which they were applied.
8. Remove the membrane from the miniblotter using forceps and incubate the blot in 100 mM NaOH for 8 min in a rolling bottle to inactivate the membrane.
9. Wash the membrane in a plastic container under gentle shaking in 250 ml 2x SSPE/0.1% SDS for 5 min at 60°C.
10. The membrane is now ready for use.
11. If the membrane is to be stored at this point, wash the membrane in a plastic container under gentle shaking in 100 ml 20 mM EDTA pH 8 for 15 min at room temperature.
12. Seal the membrane in plastic to avoid dehydration of the membrane and store the membrane at 4°C until use.
2.2 In vitro amplification of DNA by PCR.

Reaction mixture:
- 5 µl template DNA
- 5 µl forward primer (B-F27, 5 pmol/µl)
- 5 µl reverse primer (R518, 5 pmol/µl)
- 2.5 µl dNTP’s (2.5 mM of each dNTP)
- 5 µl 10x PCR buffer
- 1.5 µl 50 mM MgCl$_2$
- 0.25 µl Taq polymerase
- 23.75 µl water.

Our forward primer is biotinylated.

**PCR program:**

<table>
<thead>
<tr>
<th>Temperature</th>
<th>Time</th>
<th>Cycle</th>
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<tbody>
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<td>1 cycle</td>
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<tr>
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2.3 Hybridization with PCR product and detection

All buffers should be prewarmed before use. Prepare the following buffers from concentrated stocks, using demineralized water for dilution. Diluted buffers should not be stored longer than 2 days.

Buffer 1: 250 ml 2xSSPE/0.1% SDS, room temperature.
Buffer 2: 500 ml 2xSSPE/0.5% SDS, 52°C (post hybridization temp.)
Buffer 3: 500 ml 2xSSPE/0.5% SDS, 42°C.
Buffer 4: 500 ml 2xSSPE, room temperature.

1. Add 175 ng PCR products to x µl 2x SSPE/0.1% SDS to obtain a total volume of 150 µl.
2. Heat-denyature the diluted PCR product for 10 min at 99°C (PCR machine) and cool on ice immediately.
3. Incubate the membrane for 5 min at room temperature in 250 ml 2xSSPE/0.1% SDS in a plastic container.
4. Place the membrane on a support cushion into the minblotter, such that the slots are perpendicular to the line pattern of the applied oligonucleotides and close the minblotter.
5. Remove residual fluid from the slots of the minblotter by aspiration.
6. Fill the slots with the diluted PCR product (avoid air bubbles). Empty slots adjacent to filled slots should be filled with 2xSSPE/0.1% SDS to prevent cross-flow. Hybridize for 60 min at 42°C on a horizontal surface. Avoid cross-flow to the neighbouring slots (no rocking or shaking).
7. Remove the samples from the minblotter by aspiration and take the membrane from the minblotter using forceps.
8. Wash the membrane twice in 250 ml 2xSSPE/0.5% SDS for 10 min at 52°C in a rolling bottle using a mesh.
9. Incubate the membrane with 10 ml of 1:4000 diluted SA-POD (peroxidase labeled streptavidin conjugate) in 2xSSPE/0.5% SDS, for 45 min at 42°C in a rolling bottle.
10. For chemiluminescent detection, the Solution A+B should be warmed to room temperature by mixing 15 ml of solution A and 150 µl solution B. Store at room temperature in the dark so it will be ready to use.

11. Wash the membrane twice in 250 ml 2xSSPE/0.5% SDS for 10 min at 42°C.
12. Wash the membrane twice in 250 ml 2xSSPE for 5 min at room temperature.
13. For chemiluminescent detection, incubate the membrane for 1 min. in 10 ml of BM Chemiluminescence Blotting Substrate. (Solution A+B).
14. Cover the membrane with an overhead sheet and expose an X-ray film to the membrane for 30 min or overnight (Depending on the signal).

2.4 Stripping of the membrane

1. Wash the membrane twice in 1 % SDS at 80°C for 30 min.
2. Wash the membrane in 20 mM EDTA pH 8, for 15 min at room temperature.
3. Seal the membrane in plastic to avoid dehydration of the membrane and store the membrane at 4°C until use.

3. Solutions and supplies

Miniblotter MN45
Immunetics order number MN45

Foam cushions
Immunetics order number PC200

Biodyne C Membrane 0.45 µm
Pall order number BNBCR3R

Streptavidine-POD-conjugate
Roche order number 1089153, or Uptima order number UP395888
The bottle contains 500 units of lyophilized conjugate, which should be diluted in 1 ml water. The solution can be stored at 4° C for half a year.

BM Chemiluminescence Blotting Substrate.
Roche order number 1500694, or Uptima Uptilight order number UP98049A
The substrate consists of two solutions (A and B) to be mixed prior to incubation. Mix prewarmed (25°C) solution A and starting solution B in a ratio of 100:1 and incubate for an additional 30 min at 15-25°C. The solution can be stored dark (!) at 4° C for 1 week, but should be prewarmed prior to use again.

20 x SSPE, 4 ltr.
Life technologies order number 15591-035, or Uptima order number 259773

SDS
BDH order number 44244H, or Uptima order number 089388

10 % (w/v) SDS
Dissolve 10 g SDS in a final volume of 100 ml water by stirring and heating. Store at room temperature for no longer than 1 month.

EDAC
Uptima order number UP52005A

16% (w/v) EDAC
Dissolve 1.6 g EDAC in 10 ml water.

100 mM NaOH
Dissolve 1 g NaOH in a final volume of 250 ml water. Store at room temperature for no longer than 1 month.
0.5 M NaHCO₃ pH 8.4
Dissolve 10.5 g NaHCO₃ in 240 ml H₂O. Adjust to pH 8.4 with 2 M NaOH. Add H₂O to a final volume of 250 ml.

20 mM EDTA
Dissolve 7.4 g EDTA in a final volume of 800 ml H₂O. Adjust the pH using a 100 mM NaOH solution. Add H₂O to a final volume of 1 L.

Remarks

1. Repeated freeze thawing of the biotin labeled oligo or PCR products may damage the biotin label.
2. The choice of SDS is of critical importance for the success of the reverse line blot. Some brands of SDS may kill the signal completely, whilst others result in high background.
3. Preferably the membrane should be stripped as soon as possible, but this can also be done a few days after the hybridization. However, it is important to prevent dehydration of the blot at this point. Dehydration of blots may result in intense background staining and irreversible binding of PCR products.
4. Use dedicated plastic containers for the washing. Some chemicals that are absorbed and released by the plastic may interfere with the assay.
5. For chemiluminescent detection, the Solution A+B should be warmed to room temperature for at least 30 min.
6. Temperature inside the hybridization incubator should be calibrated regularly.

References:


Rapid Hla-Dpb Typing Using Enzymatically Amplified Dna And Nonradioactive Sequence-Specific Oligonucleotide Probes
Bugawan Tl, Begovich Ab, Erlich Ha


(According to our literature search Dattagupta et al. and Saiki et al. were the first to apply reverse dot blot, Kaufhold et al. introduced reversed line blotting).