DNA Oligonucleotide Purification and Labeling Protocol

- Modified from Daniel Zenklusen Labeling Protocol

1) Obtain probes and resuspend in 100 μl H_2O – check concentrations on Nanodrop
2) Depending on how many probes/gene, combine total of 10 μg DNA/gene (e.g. if have 5 probes/gene, then want 1 μg /probe)
3) Use QIAquick columns to purify probes according to **QIAquick Nucleotide Removal Kit Protocol**

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4) Add 10 volumes Buffer PN to total volume of combined probes and mix
5) Apply sample to QIAquick column – if total volume is greater than 750 μl you will have to spin down twice using half of volume in each spin
6) Let stand for 1 min
7) Centrifuge 1 min @ 6000 rpm
8) Wash with 750 μl Buffer PE
9) Centrifuge 1 min @ 6000 rpm
10) Dispose of flow through and re-spin column for 1 min @ 13000 rpm to dry
11) Place QIAquick column in new microcentrifuge tube and elute DNA with 50 μl H_2O – Make sure H_2O pH is within 7.0 - 8.5 and is placed directly on membrane
12) Let stand for 1 min
13) Centrifuge for 1 min @ 13000 rpm to elute DNA

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14) Lyophilize DNA at 45°C
15) Resuspend pellet in 10 μl labeling buffer and add to tube of dye **without touching the dye**
16) Repeat with another 10 μl labeling buffer
17) Vortex and spin down tube of dye and DNA
18) Cover tubes with aluminum foil and keep in dark at room temperature overnight to label

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19) Repeat QIAquick Nucleotide Removal Kit Protocol performing with 2 differences:
    - Add 200 μl PN buffer to probes and put labeled probes through columns **2x**
    - Perform **3 washes** with buffer PE in order to wash away any unattached dye before elution
20) After elution obtain concentration via nanodrop

Yeast in situ Hybridization

Modified from Daniel Zenklusen Protocol

Grow culture to OD600= 0.4-0.8 in YPD or SD Medium (Approx. 120 Klett)

1. Fix cells by adding 20% Paraformaldehyde directly to culture with final concentration of 4% (12ml chemostat culture + 3ml paraformaldehyde). Keep at room temp for 45 minutes.
2. Spin cells at 3000x g for 5 min. Transfer pellet to 2ml tube.
3. Wash cells 3x 1.8ml with Buffer B.
4. Resuspend pellet in 1ml spheroplast buffer plus lyticase (890ul Buffer B + 100ul VRC + 10ul Lyt + 2μl βME).
5. Incubate at 37C - Monitor digestion: ~90% phase dark (digested) cells.
6. Spin 5 min at 3500rpm (do not spin too hard as cells are fragile due to lyticase).
7. Wash with 1 ml Buffer B.
8. Optional: After digestion add 5 μl of 20 mg/ml Proteinase K to 1 ml Buffer B and add to tube. Incubate at 37°C for 30 min. Spin down and discard supernatant.
9. Resuspend pellet in 1.8 ml Buffer B and keep on ice.
10. Remove 150 μl and drop onto poly-L-lysine coverslips so that the whole coverslip is covered.
11. Repeat for all coverslips.
12. Incubate at 4C for at least 30 min.
13. Gently wash coverslips with 2-3ml Buffer B.
14. Gently add 2ml 70% ethanol.
15. Incubate at -20C for several hours or overnight. (If you are in a hurry you can incubate at 4°C for 1 hour instead.)

- Prepare solutions F and H, 2x SSC, 40% Formamide/2x SSC, and probes. Dry probes.

16. Remove ethanol and rehydrate coverslip by adding 2-3ml 2x SSC at room temp for 5 min. Repeat.
17. Wash once with 40% Formamide/2x SSC at room temp for 5 min.
18. Add 12ul solution F to probe tube (10ul if probes not dry, e.g only up to 2μl probe + tRNA/DNA). Heat at 95C for 3 min.
19. Allow resuspended probes to come back to room temp (5-10 min).
20. Add 12ul solution H to same tube (hybridization mix).
21. Drop 23ul hybridization mix onto parafilm. Place coverslip with cells side down on the drop.
22. Cover coverslips with parafilm and seal it around coverslip to avoid evaporation.
23. Incubate at 37°C overnight in the dark.

- Preheat 40% Formamide/2x SSC at 37°C.

24. Put 2 ml 40% Formamide/2x SSC in new 12 well tissue culture dish.
25. Place cover slips, cells side up, back into tissue culture dish with 40% Formamide/2x SSC. Incubate at 37C 15 min. Steps 24 and 25 with rocking.
26. Dump out and wash again with 40% Formamide/2x SSC at 37°C 15 min.
27. Wash with 2x SSC 0.1% Triton X-100 at room temp 15 min on shaker. Also, take mounting medium out of freezer to allow it to come up to room temp before mounting.
28. Wash with 1x SSC at room temp 15 min on shaker (wash twice if preferable).
29. Wash with 1xPBS plus DAPI (0.01μg/ml final) at room temp 2 min.
30. Wash with 1xPBS at room temp 2 min.

31. Remove coverslips and dip in 1x PBS to wash off remaining DAPI. Then dip in 95-100% ethanol to help dry for mounting.
32. Place coverslips, cells side up, on kimwipes to dry and place 3.5 ul of mounting medium (warm to 60^oC before pipetting) in center of slide (make sure not to have any bubbles in mounting medium).
33. After coverslip completely dries, place coverslip on mounting medium cell side down.
34. Allow mounting medium to cure overnight at room temp in the dark.
35. Next day, seal outside of coverslip with nail polish remover and take a look!

Solutions

<u>Labeling Buffer</u>

1.06g Sodium Carbonate
100ml DEPC H_2O
pH 8.6-9

<u>Buffer B</u>

- 1.2M Sorbitol
- 100mM KHPO4 pH 7.5
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e.g.

1MKH2PO4	8ml
1MK2HPO4	41.5ml
Sorbitol	109.3g

<u>Spheroplast Buffer</u>

- 1.2M Sorbitol
- 100mM KHPO4 pH 7.5
- 20mM Ribonucleoside-vanadyl complex (VRC; NEB #S1402S)
- 50U lyticase per OD of cells

<u>Lyticase</u>

- Sigma cat# L5263; resuspend in 1x PBS to 25000U per ml. Store at -20C in single use aliquots. Use 50U in 1 ml spheroplast buffer for slow growing cells.

<u>Solution F</u>

- 160ul Formamide
- 2ul 1M NaHPO4 pH 7.5
- <u>38ul</u> H2O

200ul

<u>Solution H</u>

Can vary concentration of Formamide from 40-50% depending on probe.

For final Formamide concentration of 50% in hybridization:

- 60ul H2O

- 40ul 20x SSC
- 40ul BSA (10mg/ml)
- 20ul VRC (100mM)
- 40ul Formamide

200ul

40% Foramide/ 2X SSC (50 ml 2X SSC= 5ml 20X SSC + 45 ml H_2O)

1 ml 20X SSC
4 ml Foramide
5 ml H_2O
10 ml

0.1% Triton X-100/ 2X SSC

5 ml 20X SSC
50 ul Triton X-100
45 ml H_2O
50 ml

1X PBS + DAPI

5 ul 1mg/ml DAPI
50 ml 1X PBS

Probe

Use 1.6 ng of each 50mer oligo probe per *in situ*. Use 20 μg of E. coli tRNA/ssDNA mix (1:1) per *in situ* (tRNA Roche 10 109 541 001, ssDNA Sigma D9156-1ml).

Preparation:

- Mix probe/s and 20ug tRNA/DNA mix
- Open lids and dry down in speed vac.

Mounting Medium

Prolong Gold antifade reagent – Invitrogen (P36934)

Preparation of Coverslips

Method 1:

1. Place coverslips on slides in vacuum chamber (the closer to the center the better)
2. Put vacuum chamber in microwave and make sure it is sealed
3. Turn on pump **then** turn on vacuum

4. Turn on microwave, and stop 5 seconds after you see plasma
5. Turn off vacuum then pump
6. Pull out vacuum chamber and remove coverslips with forceps (and that have fallen need to be cleaned again)
7. Place coverslips cleaned side up in 12 well plates
8. Go to step 6 wash with poly-L-lysine

Method 2:

1. Put one box of 18mm round coverslips into 500ml 0.1N HCL
2. Boil for 10 min
3. Rinse extensively with H_2O
4. Place in H_2O and autoclave
5. Store in 70% ethanol

Before Use:

6. Drop 150ul of 0.01% poly-L-lysine on coverslip (Sigma #P8920, dilute 1/10). Leave at room temp for 5 min
7. Remove poly-L-lysine carefully with suction and let air dry
8. Wash 3x with H_2O
9. Dump out H_2O and let air dry