Method: Purification for ssDNA Labeled FISH Probe (Part 3 of 3)

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Principle:

The purpose of making our dsDNA probe into a single-stranded probe is to prevent the situation of having the unlabeled complementary stand anneal back onto the labeled strand, rendering it useless for FISH by being unable to hybridize to the desired target sequence.

To make our probe single-stranded, we go through 3 main steps that are outlined here:

1. Digest dsDNA probe with nicking enzyme to create 3 different size fragments

2. Separate fragments out on a denaturing gel

3. Gel excise, extract, and purify our ssDNA labeled probe

Time Required:

The time required depends on the number and method of precipitation steps used. Our lab currently does overnight precipitations using the Ethanol method at -20ºC. Because our fragments are small (53bp in length), we've decided that this might prevent loss of product and increase our recovery (Ref 1,2)

Solutions / Reagents:

- Digestive Nicking Enzyme (Nb.BsrdI; NEB)*
- 0.4M NH_4OAc
- 100% EtOH
- Glycogen, Molecular Biology Grade (Fermentas #R0561)
- 2mL Safe-Lock Micro Tube (BioExpress; #C-3247-1)
- 0.7mL microcentrifuge tubes
- Pre-cast Criterion TBE-Urea Gel (Bio-Rad; #345-0091)
- Criterion™ Cell (Bio-Rad; #165-6001)
- UV Gel Box
- Typhoon™ 9400 (Optional – GE Healthcare; #63-0055-78)
- Repeater® stream/Xstream (Eppendorf; #022460811)
- Centrifuge (Beckman-Coulter; Allegra X22-R Centrifuge)
- 1X TBE Loading Buffer (1L; see notes at end of protocol)
- TBE-Urea Sample Buffer (Bio-Rad; #161-0768)
- GeneRuler™ Ultra Low Range DNA Ladder (Fermentas; #SM1213)
- Temperature Controlled Incubated Shaker
- Nano Drop 2000 (Thermo Scientific)

* Nicking enzyme used will be determined based on the primer sequence used when designing primers

Procedure:

This procedure should be followed once the "20mL Emulsion Breaking Protocol" is complete

1. Pull precipitations from -20ºC freezer, and spin at max speed (155,000 r.p.m) for 1 hour at 4ºC (Ref 1,2)

2. Remove ethanol, being careful not to disturb pellet at the bottom

3. Wash pellet with 500µL 70% EtOH to remove residual salt by carefully adding ethanol to side of tube, careful not to disturb pellet.

4. Carefully remove ethanol by inverting, and dry pellet either at RT or on 42ºC heat block to evaporate residual ethanol. *Be careful not to over dry, as it will make resuspending pellet difficult!*

5. Resuspend each pellet in 20µL dH_2O. Place samples at 37ºC for 1 hour or at 4ºC for an extended period to fully resuspend pellet.

6. Once fully resuspended, pool all samples together. The final volume can be calculated as the template volume for the digestion step.

   Important: Save 25µL of uncut dsDNA probe as a control for digestion!

Digestion:

7. Setup digesting with nicking enzyme using following mix in a 15mL falcon tube on ice:

   This is an example of the digestion mix that should be used if final template volume is 1.2mL (60 2mL tubes x 20µL/tube = 1,200µL)

1X	20µL Digest	1X 6mL Digest
2	µL 10X Buffer (#2)	600
2	µL Enzyme (Nb.BsrDI)	600
4	µL Template	1200
12	µL dH2O	3600
20	**µL Total**	**6000**

8. Invert digest 6-8 times to ensure solution is properly mixed. Aliquot 300µL of digest into 0.7mL eppendorfs, and digest at optimal temperature for nicking enzyme (65ºC) for 4 ½ hours to ensure dsDNA is completely digested.

Pause Point: Digest can be kept for extended period at 4ºC after completion

Note: This can be adjusted to save time depending on efficiency of nicking enzyme used. By periodically taking out aliquots at different time points and running on gel compared to uncut sample, you can optimize digestion time to ensure complete recovery.

9. After digestion, pool mix together, and re-aliquot 600μL of mix into 2mL eppendorfs to precipitate (~20 tubes). Add following volumes of 4M NH_4OAc, Glycogen, and 100% ice cold Ethanol to each tube ***in exact order*** shown below:

    - 600μL Digest mix
    - 40μL 4M NH_4OAc
    - 15μL Glycogen
    - 1400μL 100% ice cold EtOH

    2mL precipitation mix volume

    Invert tubes several times before placing in -20ºC freezer to precipitate O/N

Note: A repeat pippetor can be used in order to speed up the process of aliquoting and re-aliquoting. It can be also very useful when dealing with many samples.

10. Pull samples from -20ºC freezer, and repeat steps 1-6, **except this time, resuspend each sample in 40μL dH_2O**.

Example: If you had 20-2mL tubes, your final volume should be 800µL (20-2mL tubes x 40µL/tube = 800µL)

Seperation:

11. Heat up 1L of 1X TBE gel buffer in microwave for ~3 minutes; load Criterion Cell with 2 pre-cast 15% TBE-Urea denaturing gels and pre-run gels using a fix 200V for 20 minutes before loading gel.

12. Take digested, precipitated probe prep, and add a 1:1 volume of TBE-Urea sample buffer; at this time, you should also do the same with the 25μL of uncut sample from Step 6. Repeat with Ultra Low Range DNA Ladder

13. Denature samples by heating to 95ºC for 6 minutes before loading onto denaturing gel.

14. Before loading sample onto gel, using a P100 pipetteman, blast out each individual well of gel to prevent sample from floating away by removing heavy urea that is stuck in the wells; Load samples carefully into each lane, being careful not to loose sample

15. Run gel for ~30 minutes, until products are separated far enough to distinguish; afterwards, remove gel containers, pop off plastic seals, and image gels using a camera that can detect fluorescently labeled probe used for probe (i.e. GE Healthcare Typhoon)

16. After imaging fluorescent probe, stain gel with Ethidium Bromide for ~5 minutes in dH_2O, rinse 2-3 times, then image again using a gel-doc machine.

You should see 3 bands at this point. The 74bp complementary band, the 53bp ssDNA fluorescently labeled probe, and the 21bp digest product or leftover primer from PCR

Purification:

17. Gel excise 53bp band from gel, and place each individual gel fragment in a 2mL eppendorf. Place samples in freezer for ~30 minutes to dehydrate.

18. Pull gel fragments from freezer, and add 600μL of 0.4M NH_4OAc to each tube. Place samples in shaker at 55ºC and allow DNA to extract O/N. *Don't forget to cover samples!*

19. Pull gel extractions from shaker, and transfer aqueous phase to a fresh 2mL eppendorf

20. Precipitate samples using 100% ethanol; Refer to Step 9 for mix
    Invert samples, and place in -20º freezer O/N

21. Spin, wash, and dry samples; Refer to steps 1-6

22. After resuspending samples in 20μL dH_2O and pooling together, quantitate samples using Nano Drop 2000 and back calculate amount of total fluorophore recovered based on pmol/μL concentration

    Example: If final sample volume is 560µL, and the molar concentration of fluorophore based on Nano Drop is 10pmol/µL, your total recovery is 5600pmol of ssDNA probe

    560µL Sample x 10pmol/µL = 5600pmol ssDNA probe

Note: Currently, we are finding that the optimal amount of probe to use per hybridization is ~10pmol. For a sample that yields 5,600pmol, you can get ~560 good quality FISH hybridizations from this probe prep

Protocol Notes:

1X TBE Buffer (1L)
- 54g Tris Base
- 27.5g Boric Acid

Dissolve in 900mL dH2O
- add 20mL of 0.5M EDTA (pH 8.0)

Top off to 1L Volume

References:

1) Crouse J, Amorese D (1987). "Ethanol Precipitation: Ammonium Acetate as an Alternative to Sodium Acetate". *Focus* **9** (2): 3–5
2) Zeugin JA, Hartley JL (1985). "Ethanol Precipitation of DNA". *Focus* **7** (4): 1–2.