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Design and Evaluation of 16S rRNA-Targeted Oligonucleotide Probes for Fluorescence *In Situ* Hybridization

Philip Hugenholtz, Gene W. Tyson, and Linda L. Blackall

1. Introduction

Fluorescence *in situ* hybridization (FISH) of whole cells using 16S rRNA-targeted oligonucleotide probes is a powerful technique with which to evaluate the phylogenetic identity, morphology, number, and spatial arrangements of microorganisms in environmental settings ***(1)***. Probes can be designed to specifically target narrow to broad phylogenetic groups (from species to domain) by virtue of variable evolutionary conservation within the 16S rRNA molecule ***(2)***. The major steps in probe design are identifying short regions (usually 15–25 nucleotides in length) in a sequence alignment unique to the target group of interest, centralizing mismatches to nontarget organisms (where possible), and modifying the sequence to meet probe design criteria such as a minimum melting temperature.

The FISH method involves application of oligonucleotide probes to permeablized whole microbial cells. The probes enter the cells and specifically hybridize to their complementary target sequence in the ribosomes. If no target sequence is present in the cells ribosomes, probes are unable to hybridize and unbound probe is removed by a subsequent wash step. Hence only specifically targeted cells retain the probes under appropriate stringency conditions in the hybridization and wash steps. Probes are typically 5' end-labeled with fluorochrome reporters such as fluorescein or sulfoindocyanine (Cy3, Cy5) dyes and cells containing hybridized probes can be directly observed under epifluorescence microscopy owing to the natural amplification of the fluorescent signal by large numbers of ribosomes in any given target cell. An advantage is

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that multiple probes with varying target specificity can be used in the same preparation providing they are labeled with clearly distinguishable fluorochromes (i.e., well separated emission wavelengths). For instance, up to seven phylogenetically distinct groups of organisms can be visualized using a combination of three fluorochromes ***(3)***.

The method was first applied using radioactive reporters ***(4)***, which provided only limited microscopic resolution of cells and required an extra step (visualization by microautoradiography). The first demonstration of the FISH method in its modern form was by DeLong and co-workers ***(5)***, using a simple artificial microbial consortium. Subsequently, FISH has been applied in a variety of natural and artificial ecosystems confirming the great utility of the method ***(1)***. However, there are also a number of limitations associated with the method such as poor cell permeability, ribosome accessibility and content, and sample autofluorescence ***(1)***. FISH probes have been designed mainly to target 16S rRNAs but also 23S rRNAs ***(1)***. The aim of this chapter is to specifically address the design and evaluation of 16S rRNA targeted probes used in the FISH method.

2. Materials

2.1. Probe Design

1. Sequence database and phylogeny software: ARB, freeware available from the Department of Microbiology, Technical University, Munich: *http://www.arb-home.de/*. ARB runs on a UNIX platform, LINUX for PCs is recommended.

2. On-line public database search program: Basic local alignment search tool (BLAST): *http://www.ncbi.nlm.nih.gov/BLAST/*
3. On-line oligo parameter calculation program:
   Biopolymer calculator: *http://paris.chem.yale.edu/extinct.htm*

4. *E. coli* probe accessibility table: *http://aem.asm.org/cgi/content-nw/full/64/12/4973/T1*

2.2. Probe Evaluation

1. On-line probe synthesis companies: Interactiva (*http://www.interactiva.de/*), Genset oligos (*http://www.gensetoligos.com/*).
2. Teflon-coated glass slides with 8–12 individual wells (*http://www.superior.de/*).
3. Sterile milli-Q water.
4. 1× phosphate-buffered saline (PBS): 130 m*M* NaCl, 10 m*M* sodium phosphate buffer, pH 7.2. For pH 7.2, the ratios of disodium/sodium phosphates must be 2.57:1.

5. 0.2-μm membrane filters.
6. 2 *M* NaOH, 2 *M* HCl.
7. Fixative solutions: 4% Paraformaldehyde. Heat 65 mL of milli-Q water to 60°C. Add 4 g of paraformaldehyde. Add a few drops of 2 *M* NaOH solution and stir rapidly until the solution has nearly clarified (approx 1–2 min). Remove from the heat source and add 33 mL of 3× PBS. Adjust pH to 7.2 with 2 *M* HCl. Remove any remaining crystals by sterile filtration (0.2 μm). Quickly cool and store in 2-mL aliquots at –20°C.
8. 50%, 80%, 98%, and 100% ethanol.
9. Hybridization oven (e.g., Hybaid).
10. Two-milliter microcentrifuge tubes (sterile).
11. Hybridization and wash buffer ingredients: 5 *M* NaCl, 1 *M* Tris-HCl, 10% sodium dodecyl sulfate (SDS). All ingredients should be autoclaved except the SDS solution which should be prepared in sterile milli-Q water.
12. 100% Formamide in 2 mL aliquots.
13. 50-mL polypropylene screw-capped tube—one slide per tube for hybridization.
14. Paper towel.
15. FISH probes at working concentration (50 ng/μL).
16. 48°C water bath.
17. Antifading solution (e.g., Citifluor).
18. Large coverslips.
19. Epifluorscence or confocal laser scanning microscope.

3. Methods

Before embarking on the relatively lengthy process of FISH probe design and evaluation, it is worthwhile checking the literature to ensure a suitable probe does not already exist for your organism(s) of interest (target organism or group). This is a useful exercise because even well-designed oligonucleotides will not always be successful as FISH probes for reasons independent of probe design. Many on-line resources are available to search for existing FISH probes, including literature databases such as Web of Science (*http://wos.isiglobalnet.com/*, requires license) and PubMed (*http://www.ncbi.nlm.nih.gov/Entrez/*), which can be searched by keyword. A good combination of keywords to use is the name of your organism(s) of interest and the word "probe." Also, a number of rRNA probe databases are available on-line including Oligo Retrieval System (ORS; *http://soul.mikro.biologie.tu-muenchen.de/ORS/*) and Oligonucleotide Probe Database (OPD; *http://www.cme.msu.edu/OPD/*). These databases provide details of probes optimized for a number of applications, including FISH. The ORS database can be searched by keyword; however, the OPD database can be searched only by browsing through "Target Nucleic Acids and Data."

3.1 Probe Design

The objective of probe design is to select an oligonucleotide sequence completely specific (complementary) to a region of the target sequences which has at least one mismatch to the same region in all other (nontarget) sequences. A common rule of thumb is to centralize the mismatch or mismatches in the nontarget sequences to maximize the destabilizing effect of the mismatch ***(6)***. Initial probe design can be performed manually or by using computer programs, such as the freeware program ARB, which has a probe design function. A step-by-step description of probe design using ARB is given below; however, it is beyond the scope of this chapter to fully describe how to use the ARB program, and help is provided within the program. Pull down menus in ARB, used to perform the listed tasks, are indicated following a colon.

1. Unmark all sequences in the ARB database before proceeding: S*p*ecies/*U*nmark all Species
2. Mark sequences in the ARB database for which you wish to design a probe (target sequences): click on MARK button at top of left hand vertical command column and then click on individual sequences required in main window (*see* **Note 1**).
3. Open probe design window: *E*tc/Probe *F*unctions/Probe *D*esign…
4. Select a PT_SERVER (*see* **Note 2**), and define parameters. We usually only adjust the minimum percentage of group hits (by setting Min group hits (%) to 100), and maximum number of nongroup hits (*see* **Note 3**). The default target string length is 18 nt.
5. Click on GO. Results appear in PD RESULT window. Note you will not always get a result (*see* **Note 4**). Potential target sequences and associated parameters, such as length, location (*E. coli* number), G+C content, and melting temperature are displayed in the PD RESULT window. Often several close variations of a potential probe site (e.g., A) are displayed, shifted one or more nucleotides upstream (denoted as A+, A++, etc.) or downstream (A-, A––, etc.) relative to A. Highlight a target sequence of interest for further analysis by clicking on it.
6. Open probe match window: *E*tc/Probe *F*unctions/Probe *M*atch…A valuable feature of ARB is that fields are linked between windows. Therefore the highlighted target sequence in the PD RESULT window will appear in the Target String field of the PROBE MATCH window ready for further analysis.
7. Select a PT_SERVER (should be same PT-SERVER selected previously), and set Search depth to SEARCH UP TO NULL MISMATCHES. Click on MATCH. Sequences in the database with no mismatches to the target string will appear in the match window, and should more or less comprise the initial selection of target sequences used for the probe design confirming the putative probe specificity. A positional match in the target string is indicated by = in the match window.
8. Change the Search depth to one or more mismatches, and click on MATCH. This time nontarget sequences with one or more mismatches to the target string will

appear in the match window below the sequences with an exact match to the target string. Positional mismatches are indicated by the nucleotide that does not form a canonical pairing with its target string complement. Strong mismatches (A:A, C:C, G:G, U/T:U/T, A:C, C:U/T) are shown in uppercase and weak mismatches (a:g, g:u/t) are shown in lowercase. A useful probe will contain one or more mismatches to non-target sequences, ideally located in the middle of the target string. It may be possible to centralize the mismatch(es) by checking variations (A, A-, A+) proposed by the probe design program. If a nontarget sequence contains only a single weak mismatch to the target string, it may be necessary to design a competitor probe (*see* **Note 5**).

9. Once target strings are identified that have at least one mismatch to all nontarget sequences in the ARB database, further testing of the corresponding probe sequence can commence. It is important to note that the probe sequence is the **reverse complement** of the target string, as FISH probes target transcribed rRNA.
10. Confirm probe specificity against all publicly available DNA sequences using the BLASTN program at the National Center for Biotechnology Information website (*see* **Materials** and **Note 6**). An exact match will have a score (bits) in the BLAST Search Results descriptions output twice the value of the number of nucleotides of the submitted probe sequence, for example, an 18-mer probe will have an exact match score of 36. Sequences with mismatches to the probe sequence will have scores less than 36. Also, confirm the probe sequence is reverse complement by checking that "Strand = Plus / Minus" for a number of subject sequences in the BLAST Search Results alignments output. A target string will result in "Strand = Plus / Plus" (unless the subject sequence has been submitted to the databases as the reverse complement).
11. Confirm that the probe sequence has a melting temperature of ≥57°C using the nearest neighbor method (NN T_m, calculated using 50 m*M* NaCl and 50 μ*M* oligo). This can be performed on-line using websites such as the Biopolymer calculator or Primer calculator (*see* **Materials**). Empirical observations led us to believe that probes with NN T_m ≥57°C have a greater chance of success using the standard FISH protocol described below (originally described by Amann and coworkers ***[7]***) likely due to the hybridization and wash temperatures employed (46° and 48°C, respectively). If the NN T_m of the probe is <57°C, the T_m can be raised by increasing the probe length, often a one or two base extension is sufficient. This requires revisiting the ARB database and ensuring any additional nucleotides added to the probe do not compromise probe specificity.
12. Self-complementarity of oligonucleotides (hairpins or dimers) can result in disruption of duplex formation between probe and target sequences. However, we have noted no correlation between probe hairpin or dimer formation potential (up to consecutive 4 bp) and success of the probe in FISH analysis.
13. Check probe accessibility. Ribosome accessibility to probes is a well-recognized limitation with the FISH method ***(1)***. Fuchs et al. ***(8)*** systematically evaluated the accessibility of the *Escherichia coli* ribosome to more than 200 oligonucleotides complementary to the entire length of the *E. coli* 16S rRNA and found regions of

high and low relative probe accessibility (*see* **Materials**). These data can be used as a rough guide to regions of the 16S rRNA molecule which should be avoided as target sites (<10% relative accessibility) if possible. However, organisms phylogenetically remote from *E. coli* may be expected to have different ribosomal higher order structure and therefore different probe accessibility profiles. In these instances the *E. coli* accessibility profile may be of limited value. If the FISH probe does not work, and poor accessibility is suspected, accessibility may be improved by the use of helper probes that are unlabeled oligonucleotides targeting adjacent regions to the FISH probe and in theory help open the target site ***(9)***.

14. Name the probe. A number of naming systems exists for 16S rRNA-directed oligonucleotide probes. The most common shorthand nomenclature in use is a three-letter abbreviation of the target group followed by the nucleotide position that the 3' end of the probe hybridizes to, usually according to standard *E. coli* numbering ***(10)***. For example, the commonly used FISH probe EUB338 ***(11)*** targets most *Bacteria* (Eubacteria) and the 3' binding position of the probe is 338 (the probe hybridizes to positions 338–355). Recently a more comprehensive naming system has been proposed ***(12)*** whereby several features of the probe are indicated in the name, including the target gene, target group, target group level (e.g., domain, division, genus), 3' end of probe, and probe length. For example, by this system EUB338 is named S-D-Bact-0338-a-A-18. Accession numbers also have been used to identify probes (*http://soul.mikro.biologie.tu-muenchen.de/ORS/*), similar to the system of unique accession numbers used to identify DNA sequences submitted to the public databases.

3.2 Probe Evaluation

Once designed, a probe can be synthesized and evaluated. This process usually involves hybridizing the probe to pure cultures of target organisms and nontarget organisms (with the fewest mismatches to the probe sequence) at a range of stringencies. Stringency can be adjusted via a number of parameters, such as temperature and formamide (denaturant) concentration. We routinely use the method described by Manz et al. ***(7)*** in which stringency is varied using formamide concentration at set hybridization and wash temperatures. The objective is to determine the range of stringencies (formamide concentrations) at which the probe specifically hybridizes to the target organisms but not to the nontarget organisms. The optimal stringency usually is taken as the highest formamide concentration before specific hybridization signal is lost.

1. Synthesize FISH probes. Fluorescently labeled oligonucleotide probes can be synthesized commercially for approx US$60 (0.02 µmol synthesis scale) and provide enough probe for several thousand FISH reactions. Probes can be ordered on-line from companies such as Interactiva (Ulm, Germany) or Genset (worldwide) (*see* **Materials**). Fluorochromes are typically (and more cheaply) attached to the 5' end of the oligonucleotide and commonly available in fluorescein, Cy3

and Cy5. FISH probes can be synthesized and labeled in the laboratory, but this is no longer time or cost effective compared to commercially available probes.

2. Aliquot labeled probes into 2.5-µg aliquots in individual microcentrifuge tubes to avoid excessive freeze–thawing of probe stocks. Store stocks in the dark at –20°C. Resuspend each probe stock in a total volume of 50 µL of sterile milli-Q water to prepare a working concentration of 50 ng/µL (approx 9 pmol/µl for an 18-mer probe).
3. Sample fixation (*see* **Note 7**). For Gram-negative microorganisms add three volumes of 4% paraformaldehyde fixative to one volume of sample and hold at 4°C for 1–3 h. Pellet the cells by centrifugation (5000 *g*) and remove fixative. Wash the cells in 1× PBS and resuspend in 1× PBS to give 10^8 – 10^9 cells/mL. Add one volume of ice-cold 100% ethanol and mix. Fixed cells can be spotted onto glass slides or stored at –20°C for several months. For Gram-positive microorganisms add one volume of 100% ethanol fixative to one volume of sample and hold at 4°C for 4–16 h. Pellet fixed cells by centrifugation (5000 *g*) and remove fixative. Wash the cells in 1× PBS and resuspend in 1× PBS to give 10^8 – 10^9 cells/mL. Add one volume of ice-cold ethanol and mix. Ethanol-fixed cells should be prepared freshly for hybridization, as these samples do not store well.
4. Samples to include for probe evaluation are:
   - Fixed cells of log phase pure culture of a target organism (*see* **Note 8**)
   - Fixed cells of log phase pure culture of a nontarget organism (with fewest mismatches to the probe being evaluated).
5. Briefly vortex-mix fixed samples to resuspend settled material and apply 3–5 µL to wells on a Teflon-coated slide (*see* **Note 9**), air-dry thoroughly (to prevent cells detaching in subsequent steps), and dehydrate slides in an ethanol series (3 min each in 50%, 80%, and 98% ethanol). Slides can be stored at –20°C, but preferably should be hybridized soon after cell fixation and application.
6. For probe evaluation use a range of formamide concentrations in 10% increments, for example, 0–40% formamide. This will require five replicate slides, one for each formamide concentration. Determination of optimal formamide concentration can be refined by using smaller formamide concentration increments in the range of the broadly determined optimum.
7. Prewarm the hybridization oven to 46°C. Freshly prepare hybridization buffer in a 2-mL microcentrifuge tube (one tube of 2 mL of buffer per slide) in the following order:

   360 µL of 5 *M* NaCl (final concentration 0.9 *M*)
   40 µL of 1 *M* Tris-HCl (final concentration 20 m*M*, pH 7.2)
   x µL of 100% formamide (*see* **Table 1** and **Note 10**)
   y µL of autoclaved milli-Q water (according to volume of formamide; *see* **Table 1**)
   2 µL of 10% SDS (final concentration 0.01%; *see* **Note 10**)
8. Probe combinations for probe evaluation can include:
   - Test probe and domain-level probe (*see* **Note 11**)

Table 1
Formamide Volumes for Hybridization Buffer

% Formamide	Formamide volume *x* (µL)	Milli-Q water volume *y* (µL)
0	0	1598
5	100	1498
10	200	1398
15	300	1298
20	400	1198
25	500	1098
30	600	998
35	700	898
40	800	798
45	900	698
50	1000	598

- Nonsense probe (for nonspecific incorporation of probes into sample, *see* **Note 12**)
- No-probe control (for autofluorescence *see* **Note 13**)

9. Add 8 µL of hybridization buffer to each well containing sample on the slide. Fold a paper towel into a rectangle slightly larger than the slide, place the folded towel into a 50-mL polypropylene tube, and pour remaining hybridization buffer onto the paper towel. This prevents evaporation of buffer in the wells during hybridization. Add 0.5 µL of each probe (and competitor probe if required, *see* **Note 5**) at the working concentration of 50 ng/µL, and mix carefully with pipet tip (avoid touching surface of slide with pipet tip as this will disturb attached cells). Place the slide in the 50 mL tube containing the moistened towel. Screw on cap and place horizontally into hybridization oven at 46°C for 1–2 h.
10. During hybridization, prepare 50 mL of wash buffer in a fresh 50-mL polypropylene tube appropriate for hybridization buffer formamide concentration used (*see* **Table 2**) in the following order and prewarm to 48°C in a water bath:
    z µL of 5 *M* NaCl (*see* **Table 2**)
    1 mL of 1 *M* Tris-HCl (final concentration 20 m*M*, pH 7.2)
    Autoclaved milli-Q water up to 50 mL
    50 µL of 10% SDS (final concentration 0.01%)
11. Following hybridization, rinse wells immediately with 48°C wash buffer into the hybridization tube, using a pipet. Carefully remove slide from the hybridization tube, place into wash buffer tube, and hold at 48°C for 10–15 min. Remove slide from wash buffer, rinse briefly in a beaker of ice-cold distilled water, and thoroughly dry slide using compressed air (*see* **Note 14**). Rapid transfer of slides during these steps prevents cooling which can lead to nonspecific probe binding.
12. Mount slides in antifading solution such as Citifluor (**which is toxic** ; avoid inhalation and contact with skin). Apply a thin film of Citifluor to the slide and place

Table 2
NaCl Concentrations of Wash Buffers According to Formamide Concentration in Hybridization Buffer

Percent formamide in hybridization buffer	5 *M* NaCl volume *z* (µL)	Final NaCl concentration of wash buffer (*M*)
0	9000	0.900
5	6300	0.630
10	4500	0.450
15	3180	0.318
20	2150	0.215
25	1490	0.149
30	1020	0.102
35	700	0.070
40	460	0.046
45	300	0.030
50	180	0.018

a large coverslip over the slide to cover all wells. Press coverslip down gently to remove excess Citifluor.

13. Observe slides using an epifluorescence or confocal laser scanning microscope, starting with the lowest formamide concentration and working upwards:
    - No-probe wells—observe in all available channels for autofluorescence of sample.
    - Nonsense probe wells—observe in appropriate channel for probe fluorescence, confirm no fluorescence occurs.
    - Test probe and domain-level probe wells—observe in appropriate channels, confirm positive fluorescence and note formamide concentration (*see* **Note 15**).

    The optimal stringency for the test probe usually is taken as the highest formamide concentration before specific hybridization signal is lost. The window of specific hybridization stringencies is between the lowest formamide concentration at which the nontarget organism shows no fluorescence and the optimal probe stringency. Often nontarget organisms do not fluoresce, even at 0% formamide.

Notes

1. It is not recommended to design probes based on a single sequence, as sequencing errors could be present that may be inadvertantly incorporated into the probe. Multiple sequences reduce the chances of sequencing errors affecting probe design because the identified region must be identical in all target sequences. Where possible use full-length sequences for probe design, as this provides the maximum possible sequence data to locate potential probe sites. Do not include short

sequences (<500 nt) in the design if possible as they may have little or no overlapping (comparable) regions on which the probe design is based.

2. The PT_SERVER searches for patterns (such as regions specific to target sequences) in special searchable database files, which are essentially fragmented versions of standard ARB database files. Pattern searches cannot be performed directly on standard database files, hence the need for defining the PT_SERVER. Before the probe design tool can be used, searchable database files must be created from a standard database file as follows:
   a. Open PT-SERVER administration window: *E*tc/Probe *F*unctions/PT_SERVER *A*dmin…
   b. Select a PT_SERVER template into which your database file will be loaded, for example, SSU_rRNA.arb would be appropriate for a 16S rRNA gene database.
   c. Click on UPDATE SERVER under Functions. Updating takes several minutes, and will overwrite any preexisting files in the selected template. Therefore, caution should be exercised if several people are using the one ARB program, as the potential exists for multiple users to update the same template.
3. Probes are usually designed to target a monophyletic group of sequences, and such a group may comprise short sequences that should be excluded in the design process:
   a. Mark target group of sequences.
   b. Open Search and Query window: S*p*ecies/Search and *Q*uery.
   c. Search species that are marked: under DATABASE SEARCH click on "Search species that" in left-hand options and "are marked" in right hand options. Click on SEARCH. Marked sequences should appear in HITLIST window.
   d. Keep species that match the query: Under DATABASE SEARCH click on "Keep species that" in left-hand options and "match the query" in right-hand options.
   e. Under QUERY highlight the nucleotide (nuc) Search field and type >500 in Search string.
   f. Click on SEARCH. Sequences less than 500 nt long will be removed from the HITLIST window, but will remain marked. To unmark these short sequences click on MARK LISTED UNMARK REST. Only sequences greater than 500 nt long in the target group will be marked in the database.

   However, short sequences removed from the target group may contain probe sites inferred from analysis of full-length sequences, and this can be taken into account using the maximum number of nongroup hits. For example, if the monophyletic target group contains ten sequences, four of which are too short to include in the design process, then four should be entered into the Max. non group hits field, as these short sequences may contain target group probe sites inferred from the six full-length sequences. If Max non group hits is left at the default setting of zero, then the program may discount a potentially useful target group probe site

because a short target sequence containing that site is seen as a nontarget sequence.

4. If no potential probe sites are found for a group of target sequences the message "There are no results" will be displayed in the PD RESULT window. Less than optimal probe sites may be found for the target group by reducing the "stringency" of the design parameters. For instance, reducing the Min group hits (%) or increasing Max. non group hits may result in the location of potential probe sites. These type of suboptimal probes will usually either not target all sequences in the target group, or target sequences outside the target group. However, depending on the application, suboptimal probes may be adequate as FISH probes, usually in combination with other probes.
5. Nonspecific hybridization of a FISH probe to nontarget sequences with a single mismatch to that probe may not be discriminated from specific target sequence hybridization regardless of stringency. Inclusion of an unlabeled oligonucleotide complementary to these nontarget sequences (competitor probe) should prevent hybridization of the FISH probe (and therefore nonspecific signal) by competing for the target site. Competitor probes are often denoted by a lowercase c preceding the name of the FISH probe they are competing with, for example, cPLA886 is the competitor probe for planctomycete-specific FISH probe PLA886 ***(13)***.
6. A BLAST search also serves to confirm that the probe sequence has been transcribed correctly and is in the correct orientation. Where possible probe sequences should be cut and pasted between programs rather than typed manually.
7. Gram-positive cells over crosslink with paraformaldehyde (PFA) which can result in reduced permeability to oligonucleotide probes, hence ethanol has been suggested as an alternative fixative ***(14)***. In addition, enzyme pretreatments can aid in permeabilization of Gram-positive cell walls, such as lysozyme ***(15)***, mutanolysin ***(16)***, and numerous other chemical pretreatments ***(17)***. For fixation of microbial community samples containing a mixture of Gram-negative and-positive organisms, we recommend using the standard PFA fixation procedure as the samples can be stored successfully for longer periods and in most cases, the majority of cells will be sufficiently permeable for probe entry and hybridization (as determined by comparison of universal FISH probes to nucleic acid-binding dyes such as 4,6-diamidino-2-phenylindole [DAPI]).
8. It is a good idea to sequence the 16S rDNA of the target and nontarget pure cultures in the probe target zone to confirm the identity of the cultures and probe specificity. If no pure cultures of a target organism exist, a habitat sample known to contain the target organism should be used as the positive control. This is usually the case if a probe has been designed to target an environmental sequence (a sequence obtained using a culture-independent PCR-clone library approach).
9. Teflon-coated glass slides often are pretreated for use in FISH by cleaning in 10% KOH solution or warm detergent and coated with gelatin or silane. However, we have found commercially prepared slides can be used successfully for FISH without any pretreatment.
10. Store formamide in 2-mL aliquots at –20°C. After thawing an aliquot, restore at

4°C and use within a week; fresh formamide should be colorless. Formamide is toxic, so gloves should be worn when handling it and hybridization tubes should be tightly capped and incinerated after use. Add SDS last to avoid precipitation with the concentrated NaCl.

11. If the test probe targets a bacterium, the bacterial domain-level probe EUB338 (5'–GCTGCCTCCCGTAGGAGT; target site *E. coli* no. 338-355) should be used in concert with the test probe labeled with a complementary fluorochrome (e.g., test probe-Cy3 + EUB338-fluorescein). Similarly, for a test probe targeting *Archaea*, ARC915 (5'–GTGCTCCCCCGCCAATTCCT; target site *E. coli* no. 915-934) can be used. These domain-level probes can be used at all formamide concentrations ***(18)*** and act as positive controls for most microorganisms to confirm the FISH procedure was successful. Some groups of bacteria have mismatches to domain-level probes, such as the *Verrucomicrobia* and *Planctomycetes*, for which variants of EUB338 exist ***(18)***. Domain-level probes should not be used in combination with the test probe is if their target sites overlap.
12. A nonsense probe such as nonEUB338 (reverse complement of EUB338), which has no known rRNA target, can be included in the probe evaluation to ensure nonspecific incorporation of the probe into the sample does not occur. Nonspecific probe incorporation is usually only a significant problem with some habitat samples.
13. Many compounds present in the environment autofluoresce, including some cellular components (e.g., photosynthetic pigments, cofactor F420, some proteins), which can obscure specific FISH. This is particularly evident in habitats such as soils, sediments, and aquatic samples. The wavelengths at which autofluorescence occurs can be sample specific (e.g., rumen samples have high autofluorescence under blue excitation/green emission). Therefore, no-probe controls should be included to detect autofluorescent cells at different wavelengths.
14. Ensure that all droplets of water are removed from the wells as the probe can dissociate and leave the cells due to osmotic pressure.
15. Optimal probe stringencies also can be determined quantitatively using image analysis software ***(18)***.

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