Standard fixation of samples for FISH:
I) For gram-negative cells (page 1)
II) For gram-positive cells (page 3)

For modifications or other fixation procedures, consult special literature or check FISH course www.microbial-systems-ecology.de/fish_course_new.html

Fixation protocol I (generally recommended for gram-negative prokaryotes but may also work occasionally on certain gram-positive prokaryotes, Archaea and Eukarya). Modified from Amann, 1995:

Chemicals (Advice: Prepare 1 Liter stock solution of the following three solutions. If autoclaved, they can be stored for longer periods. Use aliquots in e.g. falcon tubes for daily work).

1) Sterile Na_xPO_4 stock solution (phosphate buffer):

A) Na_2HPO_4 200 mM = 35.6 g/l (prepare 1 litre)

B) NaH_2PO_4 200 mM = 27,6 g/l (prepare 250 ml)

Adjust pH to 7.2-7.4 of solution A by using solution B

2) Sterile 1 x phosphate buffered saline solution (PBS); pH 7,2):

130 mM sodium chloride
10 mM sodium phosphate buffer Na_xPO_4

To prepare 1x PBS: NaCl 130 mM = 7.6 g/l

Na_xPO_4 10 mM = 50 ml Na_xPO_4 stock solution /l

Aqua dest. ad 1000 ml

3) Sterile 3 x phosphate buffered saline solution (PBS); pH 7,2

390 mM sodium chloride
30 mM sodium phosphate buffer Na_xPO_4

To prepare 3x PBS: NaCl 390 mM = 22.8 g/l

Na_xPO_4 30 mM = 150 ml Na_xPO_4 stock solution /l

Aqua dest. ad 1000 ml

4) Paraformaldehyde solution (PFA) usually within the concentration range 4-8 %: Use high-grade, fresh paraformaldehyde (powder), e.g. from Sigma (# P-6148) and store under proper conditions – dark, at +4 ° C (or under argon/ liquid nitrogen). **Wear a lab coat, use protective mask, eye glasses, gloves, work in the fume-hood!** *Note, the concentration of the PFA can be modified (lower/higher concentration). The fixation protocol must then be adapted accordingly.* For pH adjustments: 2 M NaOH (prepare 100 ml); 2 M HCl

Procedure for preparation of PFA (Important: Use protective mask, eye glasses, gloves, work in the fume-hood!):

1) If you wish to prepare a 100 ml fixation solution: heat 65 ml ddH2O to 60° C (note, it is important with deionized water). If you wish to prepare a larger stock solution, scale up accordingly. For example, for a 300 ml fixation solution, heat 3x65 ml ddH2O).
2) If a 4 % PFA solution is desired, add 4 g PFA (or more if you wish a higher concentration). If you wish to prepare a larger stock solution, scale up accordingly. For example, for a 300 ml fixation solution, add 3x4 gram PFA). Comment: The solution becomes “milky”.
3) To dissolve the milky appearance, add one drop of 2 M NaOH solution and stir rapidly until the solution has nearly clarified (should take 1-2 min). If the solution has not yet clarified, add carefully more drops (slowly, drop by drop) until the solution becomes clear (no particles should be discernable). However, avoid adding too much NaOH because otherwise you must spend more time on adjusting the pH.
4) Remove from heat source and add 33 ml of 3xPBS (or scale up if you wish to prepare a larger volume, e.g for 300 ml fixation solution, add 3x33 ml 3xPBS). Check that the pH is neutral (between pH 7-7,5, use a pH indicator strip if you prefer to protect your pH electrode from PFA). If you use a pH indicator strip, add a drop of the PFA solution onto the strip, avoid dipping the strip into the solution because otherwise your PFA solution may get colored).
5) Filtrate solution slowly through a 0.2 µm filter into a new tube (e.g. a 15-50 ml falcon tube). Use gloves, face mask and watch out for spills! After a while the filter has become clogged, do not press anymore. Take a new filter. (approximately 50-100 ml solution can be used per sterile filter). Collect all PFA waste in special designed PFA waste bins stored in the fume hood. Check up how PFA waste must be handled in your institute.
6) Quickly cool down solution to 4° C and store it at this temperature up to 1 day (in worse case 1 week, however it is better to aliquot (e.g. 2 ml, 10 ml, 50 ml in eppendorf or falcon tubes) the fixation solution and store at – 20° C for longer periods (at least for months). It is also possible to store PFA under liquid nitrogen, Thaw frozen aliquots just before usage and use within 24 hours. Avoid too frequent freezing and thawing. Place a mark on your tube for each freezing-thawing cycle. Place the frozen aliquots in a plastic bag with a safety label.

A comment on your sampling and sample treatment, prior to fixation:

- Check appropriate sampling time and shipping of your sample. If possible, fixation should be done on freshly taken samples.
- Check if your sample needs to be washed, diluted or concentrated prior to fixation.
- If your sample is solid (or pelleted), dissolve in an appropriate buffer (e.g. 1xPBS).
- If your cells have been filtered onto a membrane filter, consider if you can do the fixation on your filtration unit, or if you must remove your filter and do the fixations in a separate tray(s), thereby moving your filter with a forceps during the various steps.

Standard procedure for PFA fixation (note, modifications may be necessary, depending on the nature of the sample and sampling technology):

Procedure:

1) Add 3 volumes of x % (e.g. 4 %) PFA fixative to one volume of sample* and fix for minimum 0,5 h up to 3 h (general recommended time) at 4° C. If necessary or unavoidable, samples can also be fixed for longer periods (e.g. over night). However, it is recommended to explore the optimal fixation time for unknown samples. Avoid too weak fixation or too strong fixation of your samples. Furthermore, for systematic comparison of different samples, the same fixation time should be used. **Ratio of fixative versus biomass depends on the PFA concentration.*

2) Pellet sample by centrifugation (approx 1-2 min, 5000 rpm, modify harvest conditions where ever appropriate, the main goal is just to produce a pellet of your fixed samples) and remove fixative (supernatant).

3) Wash cells in 1xPBS (or other suitable buffer) via centrifugation as described above, and then resuspend in one volume of 1x PBS.

4) If necessary, repeat steps 2-3. Residues of PFA may produce autofluorescening particles, so it is important to remove all PFA.

5) For end-fixation (storage at longer periods), add one volume (in relation to the volume used for resuspension) of ice-cold 100% EtOH and mix (final concentration of EtOH should be 50 %).

6) For short term storage, store at room temperature or at +4 ° C. For long term storage, store fixed samples at – 20° C.

Fixation protocol II (generally recommended for gram-positive prokaryotes but may also work occasionally on certain gram-negative prokaryotes, Archaea and Eukarya). Modified from Amann, 1995:

Chemicals: Ice-cold 96-100 % EtOH, molecular grade.

Procedure:

1) Harvest your sample (where appropriate wash it via centrifugation with a suitable buffer) and dissolvein a suitable buffer (e.g. 1xPBS).

2) Add 1 (better ≥1,1!) volume of ice-cold 96-100 % EtOH (molecular grade) to one volume* of environmental sample, mix (final concentration of EtOH should be minimum 50 %).

3) For short term storage, store at room temperature or at +4 ° C. For long term storage, store at – 20° C.