**Fresh 4% Paraformaldehyde in PBS for Perfusion**

1. **Scope:** Freshly prepared 4% Paraformaldehyde in PBS is used to fix adult and developing mouse brains during the transcardial perfusion process, as well as for immersion fixation. This solution can be used for no more than one week post-prep date.
2. **Materials:**
	1. Paraformaldehyde powder (Sigma 441244 or equivalent)
	2. MilliQ Water
	3. 5M NaOH (VWR BDH3225-1 or equivalent)
	4. 10X PBS (Ambion 9625 or equivalent)
3. **Equipment:**
	1. Heated stir plate
	2. Stir bar
	3. Magnetic stir bar remover
	4. Calibrated pH meter
	5. Balance (in hood)
	6. Fume hood
	7. Vacuum pump (Gast DOA-P104-AA or equivalent)
	8. 500 mL filter flask
	9. Corning 500 mL filter system (0.45 µm), including Tygon tubing
		1. If 0.45 µm filters are unavailable, 0.22 µm filters are an acceptable substitute.
	10. Glass Pyrex bottle (sufficient size for amount desired; minimum of 750 mL volume)
	11. P1000 Pipettor with tips
	12. Transfer pipettes, 3 mL plastic
4. **Safety:**
	1. Gloves
	2. Lab coat
	3. Eye protection
	4. Sleeve protectors
	5. Fume hood
5. **Output:**
	1. Fresh 4% paraformaldehyde in 1X PBS, for perfusion and immersion fixation
6. **Reference Documents:**
	1. EQ0006 pH Meter Calibration and Usage
		1. To be Published
	2. EQ0020 Balance Calibration Validation
		1. To be Published
7. **Setup:**
	1. Verify that the pH meter is calibrated.
8. **Methodology**
	1. Record all lot numbers and volumes used in the appropriate reagent prep notebook.
	2. **To make 1000 mL of 4% PFA: (see chart below for reagent amounts for greater volumes of 4% PFA).**
		1. Weigh out 42 g of PFA powder in the fume hood. Take care not to inhale any PFA dust, and clean up any spills immediately.
		2. Add the PFA powder to 500 mL of MilliQ H2O in a **glass** bottle of appropriate size for the final solution volume (e.g. 1 L volume for 1 L total of 4% PFA in PBS).
		3. Add 1 mL of 5 M NaOH.
		4. Add stir bar, set to medium speed, and set plate temperature to 65°C.
		5. Dissolve PFA powder with heat and stirring. Solution will be nearly clear or clear once the PFA has dissolved – which takes approximately 30 minutes.
		6. Add 100 mL of 10X PBS.
		7. Add MilliQ H2O up to the 1 L mark (approximately 400 mL).
		8. After adding the 10X PBS and MilliQ H2O, the bottle should be cool enough to handle easily. If not, allow the bottle to cool in the hood until it is cool enough to handle (~20-27°C). Do not pH a hot solution.
		9. While stirring, add HCl dropwise using a transfer pipette to adjust pH to 7.4. Do not “back-pH” by readjusting with a base if the desired pH is overshot; solution will need to be re-prepared.
		10. When the correct pH is attained, remove the stir bar using a magnetic stir bar remover.
		11. In the fume hood, using the Corning 500 mL filter system, filter the PFA through the 0.45 µm filter system.
			1. Using Tygon tubing, attach the Corning filter system to a 500 mL filter flask, and then attach the filter flask to the Gast vacuum pump that sits directly outside of the fume hood. Turn on the pump.
			2. Filter 500 mL at a time, stirring the remaining PFA yet to be filtered constantly on a stir plate.
			3. Use one filter system for every 500 mL of PFA.
			4. If 0.45 µm filters are unavailable, 0.22 µm filters are an acceptable substitute.
		12. Label the bottle, “**4% PFA in 1X PBS**” with **date/initials**.
		13. Store at 4°C for up to one week.

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| **PFA Powder\*** | **MilliQ H2O** | **5M NaOH** | **10X PBS** | **MilliQ H2O** | **Total** |
| 42 grams | 500 mL | 1 mL | 100 mL | ~400 mL | **1 L** |
| 84 grams | 1000 mL | 2 mL | 200 mL | ~800 mL | **2 L** |
| 126 grams | 1500 mL | 3 mL | 300 mL | ~1200 mL | **3 L** |
| 168 grams | 2000 mL | 4 mL | 400 mL | ~1600 mL | **4 L** |
| 210 grams | 2500 mL | 5 mL | 500 mL | ~2000 mL | **5 L** |
| *\* A small percent excess of PFA is measured, to ensure that the final reagent concentration post-filtration is ≥4%* |

1. **Take Down:**
	1. Dispose unused reagent in the designated PFA waste barrel, and throw filtration container away in standard nonhazardous waste stream.
	2. Thoroughly rinse glass bottle with dH2O and allow to air dry. Do not use soap.
2. **Technical Information:**
	1. **QC:**
		1. Final solution should have a pH = 7.4 when measured around room temperature, or when glass bottle can be handled by hand without insulated gloves (~20-27°C).
		2. There should not be particulate matter in the solution post-filtration.
	2. **Storage:**
		1. Store 4% PFA at 4°C. Do not freeze.
		2. May be used for up to 7 days when stored at 4°C. Any 4% PFA older than seven days should be disposed of appropriately.
	3. **Reagent Information:**
		1. 4% paraformaldehyde (PFA) is a cross linking aldehyde-based fixative. Its primary mode of action is to cross link adjacent polypeptides by forming methylene bridges with lysine residues and arginine residues. PFA powder consists of very long chain, highly polymerized formaldehyde, which dissolves in basic solutions with heat, by slowly depolymerizing into smaller chain polymers. Following dissolving, aggregates of certain size threshold left in solution are removed by filtration.
3. **Disposal:**
	1. All waste container/Tecan reservoirs containing 4% PFA solution must be emptied into the PFA waste barrel daily.
	2. Full PFA waste barrels are disposed of off-site by contracted waste management.