**Artificial Cerebrospinal Fluid VIII (ACSF.VIII)**

1. **Scope and Applicability:** Artificial Cerebrospinal Fluid VIII (ACSF.VIII) is used for multiple applications including incubation of fresh human brain slices prior to electrophysiological recording.
2. **Materials:**
	1. Calcium Chloride, dihydrate (*CaCl2·2 H2O*) (Sigma C3881, C3306 or equivalent)
	2. D-Glucose *(C6H12O6; aka Dextrose)* (Sigma G7021, Sigma G8270 or equivalent)
	3. HEPES *(C8H18N2O4S)* (Sigma H3375 or equivalent)
	4. Magnesium Sulfate, Heptahydrate (*MgSO4·7 H2O*) (Sigma M1880 or equivalent)
	5. Sodium Phosphate monobasic monohydrate (*NaH2PO4 • H2O*) (Sigma S9638 or equivalent)
	6. Potassium Chloride *(KCl)* (Sigma P9333, P5405, P4504 or equivalent)
	7. Sodium Pyruvate *(CH3COCOONa)* (JT Baker 3354 purchased as VWR JT3354 or equivalent)
	8. Sodium L-Ascorbate *(C6H7NaO6 )* (Sigma A7631, A4034 or equivalent)
	9. Sodium Bicarbonate *(NaHCO3)* (Sigma S5761, VWR MK739604 or equivalent)
	10. Sodium Chloride *(NaCl)* (JT Baker 4058 purchased as VWR JT4058 or equivalent)
	11. Thiourea *(NH2CSNH2)* (Sigma T7875 or equivalent)
	12. 1N NaOH (VWR BDH3222-1 or equivalent)
	13. MilliQ Water
	14. OMNI 15 mL conical tubes (VWR 00-2015-25)
	15. 1 L polystyrene bottle (VWR 28199-762 or equivalent)
	16. 500 mL polystyrene bottle (VWR 28199-760 or equivalent)
	17. 250 mL polystyrene bottle (VWR 28199-758 or equivalent)
	18. 1000 mL graduated cylinder
	19. 100 mL graduated cylinder
	20. Pipettor
	21. 50 mL serological pipette (VWR 53106-441 or equivalent)
	22. 25 mL serological pipette (VWR 53300-567 or equivalent)
	23. 10 mL serological pipette (VWR 53300-523 or equivalent)
	24. P200 Pipetteman
	25. P200 pipette tips (Rainin GPL200F or equivalent)
	26. P1000 Pipetteman
	27. P1000 pipette tips (Rainin GPL1000F or equivalent)
	28. 1000 mL beaker
	29. 100 mL beaker
	30. 1 L filter system, 0.45 µm (Corning 430516 purchased as VWR 28199-814, 28199-818 or equivalent)
	31. 3 mL transfer pipette
	32. Weigh paper
	33. Weigh boats
	34. Disposable plastic spatulas
	35. RNase-free stir bar
3. **Equipment:**
	1. Vacuum pump (Gast DOA-P104-AA or equivalent) with Tygon tubing
	2. Osmometer (Fiske 210 or equivalent) or Freezing point osmometer (Advanced Model 3250 or equivalent)
	3. Osmometer sample tubes (Advanced Instruments 3LA825 or equivalent)
	4. pH Meter
	5. Stir plate
	6. Magnetic stir bar remover
	7. Calibrated balance
4. **Safety:**
	1. Gloves
	2. Eye protection
	3. Lab coat
	4. Fume hood
5. **Output:**
	1. 1 L of Artificial Cerebrospinal Fluid VIII (ACSF.VIII) pH 7.3, Osmolarity 305-315 mOsm containing:
		1. 2 mM Calcium Chloride, dihydrate
		2. 25 mM D-Glucose
		3. 20 mM HEPES
		4. 2 mM Magnesium Sulfate, Heptahydrate
		5. 1.2 mM Sodium Phosphate monobasic monohydrate
		6. 2.5 mM Potassium Chloride
		7. 3 mM Sodium Pyruvate
		8. 5 mM Sodium L-Ascorbate
		9. 30 mM Sodium Bicarbonate
		10. 92 mM Sodium Chloride
		11. 2 mM Thiourea
6. **Reference Documents:**
	1. EQ0006 pH Meter Calibration and Usage
		1. To be Published
	2. EQ0020 Balance Calibration Validation
		1. To be Published
	3. PF0284 Measuring Osmolarity with the Vapro 5600 Osmometer
		1. To be Published
	4. PF0302 Measuring Osmolarity with the Advanced Instruments 3250 Osmometer
		1. To be Published

**Warning: Personal Protective Equipment (PPE) should be used at all times while operating this protocol. If you are unsure what PPE you should be using, see your immediate supervisor.**

1. **Setup:**
	1. Prepare working stocks of reagents, as necessary. Label the stock bottles with contents, preparer’s initials, preparation date, storage requirement and expiration date.
		1. **Calcium Chloride, dihydrate**: Prepare a 200 mM stock solution by dissolving 29.4 g of CaCl2·2 H2O in 900 mL of MilliQ Water in a labelled 1 L polystyrene bottle. Top off solution to a final volume of 1 L with MilliQ Water. Store the stock solution at room temperature for up to 60 days.
		2. **1M HEPES**: Prepare a 1 M stock solution by dissolving 238.3 g of HEPES in 900 mL of MilliQ Water in a labelled 1 L polystyrene bottle. Top off solution to a final volume of 1 L with MilliQ Water. Store the stock solution at room temperature for up to 60 days.
		3. **1M Magnesium Sulfate, heptahydrate**: Prepare a 1 M stock solution by dissolving 246 g of MgSO4.7 H2O 1 M in 900 mL of MilliQ Water in a labelled 1 L polystyrene bottle. Top off solution to a final volume of 1 L with MilliQ Water. Store the stock solution at room temperature for up to 60 days.
		4. **Sodium Chloride**: Prepare a 4 M stock solution by dissolving 233.8 g of NaCl in 900 mL of MilliQ Water in a labelled 1 L polystyrene bottle. Top off solution to a final volume of 1 L with MilliQ Water. Store the stock solution at room temperature for up to 60 days.
		5. **Sodium Pyruvate**: Prepare a 500 mM stock solution by dissolving 55 g of CH3COCOONa in 900 mL of MilliQ Water in a labelled 1 L polystyrene bottle. Top off solution to a final volume of 1 L with MilliQ Water. Store the stock solution at 4°C for up to 60 days.
		6. **Thiourea**: Prepare a 1.6M stock solution by dissolving 6.08 g of Thiourea in 40 mL of MilliQ Water. Top off solution to a final volume of 50 mL with MilliQ Water. Aliquot 5 to 7 mL of Thiourea into 15 mL conical tubes. Store the aliquots at -20°C for up to 60 days. The solution must be warmed to room temperature prior to use.
	2. Label a sterile 1 L polystyrene bottle with the reagent name, date and preparer’s initials.
	3. Obtain an RNase free stir bar and place it in the container.
	4. Set up the stir plate in the fume hood.
2. **Methodology/Procedures** (record all lot numbers and volumes used in the appropriate reagent prep notebook):
	1. Consult the reagent request form to determine the volume of ACSF to prepare. Table 1 lists the reagent quantities required to make either 500 mL or 1 L of ACSF.
	2. **To make 1000 ml of Artificial Cerebrospinal Fluid (ACSF.VIII):**
		1. Using a graduated cylinder, measure 600 mL of MilliQ Water. Add the water to an appropriately labeled RNase free container containing an RNase free stir bar.
		2. Place the container on a stir plate and begin stirring at a high setting (7 or higher).
		3. Using a sterile serological pipette or graduated cylinder, add 20 mL of the 1 M HEPES stock solution.
		4. Using a calibrated balance, weigh out 186 mg of Potassium Chloride. Slowly add weighed Potassium Chloride to the container while stirring.
		5. Using a calibrated balance, weigh out 166 mg of Sodium Phosphate monobasic monohydrate. Slowly add weighed Sodium Phosphate monobasic monohydrate to the container while stirring.
		6. Using a sterile serological pipette or graduated cylinder, add 23 mL of the 4 M Sodium Chloride stock solution.
		7. Using a sterile serological pipette or graduated cylinder, add 6 mL of the 500 mM Sodium Pyruvate stock solution.
		8. Using a sterile serological pipette, add 1.25 mL of the 1.6 M Thiourea stock solution. Note: the solution must be at room temperature to fully dissolve.
		9. Using a calibrated balance, weigh out 4.5 g of D-Glucose. Slowly add weighed D-Glucose to the container while stirring.
		10. Weigh out 990 mg of Sodium L-Ascorbate. Slowly add weighed Sodium L-Ascorbate to the container while stirring.
		11. Weigh out 2.52 g of Sodium Bicarbonate. Slowly add weighed Sodium Bicarbonate to the container while stirring.

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| Reagent | 500 mL  | 1000 mL  |
| HEPES; 1 M | 10 mL | 20 mL |
| Magnesium Sulfate, Heptahydrate; 1 M | 1 mL | 2 mL |
| Potassium Chloride | 93 mg | 186 mg |
| Sodium Phosphate monobasic monohydrate | 83 mg | 166 mg |
| Sodium Chloride; 4 M | 11.5 mL | 23 mL |
| Sodium Pyruvate; 500 mM | 3 mL | 6 mL |
| Thiourea; 1.6 M | 625 µL | 1.25 mL |
| D-Glucose | 2.25 g | 4.5 g |
| Sodium L-Ascorbate | 495 mg | 990 mg |
| Sodium Bicarbonate | 1.26 g | 2.52 g |
| Calcium Chloride; 200 mM (add after adjusting pH) | 5 mL | 10 mL |
| Bring up to this final volume with MilliQ water (after adjusting pH) | 500 mL | 1000 mL |

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| **Table 1: Reagents Required for Preparation of 500 mL or 1 L of ACSF.VIII** |

* + 1. Continue stirring at a high speed (setting 7 or higher) until all of the chemicals are fully in solution.
		2. Using a calibrated pH meter, adjust the pH to 7.3 with NaOH.
		3. Using a sterile serological pipette, add 10 mL of the 200 mM Calcium Chloride stock solution.
		4. Using a sterile serological pipette, add 2 mL of the 1 M Magnesium Sulfate stock solution.
		5. Remove stir bar using a magnetic stir bar remover.
		6. Adjust final volume to 1000 mL with MilliQ Water using the graduation marks on the side of the container (if calibrated) or a graduated cylinder.
	1. Measure the osmolarity of the solution.
		1. If using a 3250 Freezing Point Osmometer, measure the osmolarity of a 250 µL sample of the solution; if the osmolarity is not 305-315 mOsm (305-315 mmol/kg), adjust as needed.
		2. If utilizing a Vapro 5600 Osmometer, measure the osmolarity of a 10 µL sample of the solution; if the osmolarity is not 300-310 mOsm (300-310 mmol/kg), adjust as needed.
	2. If the osmolarity is too high, add MilliQ water.
	3. If the osmolarity is too low, add a volume (X) of 1 mg/mL NaCl solution determined by the following equation



* + 1. D=desired osmolarity
		2. O= observed osmolarity
		3. X=volume of 1 mg/mL NaCl solution to add (in mL)
	1. Confirm that the correct osmolarity has been reached after addition of NaCl or water.
	2. Filter the ACSF through a Corning 1 L 0.45 µm filter system.
		1. Using Tygon tubing, attach the Corning filter system to the Gast vacuum pump. Turn on the pump.
		2. Filter 500 mL at a time, stirring the remaining ACSF constantly on a stir plate.
	3. Label the bottle with reagent name, date, time and preparer’s initials.
	4. Record the date of preparation, lot numbers of all components and osmolarity in the reagent prep notebook.
1. **Take Down:**
	1. Dispose of excess reagent directly into municipal sewer system.
	2. Wash the stir bar with RNase Away and rinse with MilliQ water.
	3. Clean balance.
	4. Rinse all beakers, graduated cylinders, and other equipment used to prepare ACSF solutions with MilliQ Water only **(No Detergent)**. Invert all beakers and graduated cylinders and leave on clean absorbent pad to air dry.
2. **Technical Information:**
	1. Artificial Cerebrospinal Fluid (ACSF) for human surgical tissue collections should be made within 24 hours of use. Store at 4°C until use.
	2. The osmolarity of the ACSF normally should not need to be adjusted if all the components were added correctly. If the osmolarity does need to be adjusted, add MilliQ Water to lower or Sodium Chloride to raise the value.
	3. Stock Solution Storage Conditions:

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| **Component** | **Storage Temperature** | **Shelf Life** |
| Calcium Chloride; 200 mM | RT | 60 days |
| HEPES; 1 M | RT | 60 days |
| Magnesium Sulfate; 1M | RT | 60 days |
| Sodium Chloride; 4 M | RT | 60 days |
| Sodium Pyruvate; 500 mM | 4°C | 60 days |
| Thiourea; 1.6M | -20°C | 60 days |

1. **Appendix**
	1. **Materials required for preparation of 1 L of ACSF.VIII**

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| **Material** | **Supplier,** **Part Number** | **Amount Required for 1 L** |
| 1 L polystyrene bottle | VWR 28199-762 | 1 ea |
| 1 L filter system, 0.45 µm  | VWR 28199-814 | 1 ea |
| Calcium Chloride, dihydrate | Sigma C3881 | 294 mg (10 mL of 200 mM stock) |
| D-Glucose | Sigma G7021,G8270 | 4.505 g |
| HEPES | Sigma H3375 | 4.8 g (20 mL of 1 M stock) |
| Magnesium Sulfate, Heptahydrate | Sigma M1880 | 492 mg (2 mL of 1 M stock)  |
| Sodium Phosphate monobasic monohydrate | Sigma S9638 | 166 mg  |
| Potassium Chloride | Sigma P5405, P4504 | 186 mg  |
| Sodium Pyruvate | VWR JT3354 | 330 mg (6 mL of 500 mM stock) |
| Sodium L-Ascorbate | Sigma A7631 | 990 mg |
| Sodium Bicarbonate | Sigma S5761 | 2.52 g |
| Sodium Chloride | VWR 4058 | 5.5 g (23.6 mL of 4 M stock) |
| Thiourea  | Sigma T7875 | 152 mg (1.25 mL of 1.6 M stock) |

* 1. **Rapid Prep method for preparation of 1000 mL ACSF.VIII**
		1. **Setup**
			1. Prepare stock solutions as needed:
			2. 2M Calcium chloride, dihydrate: Prepare a 2M stock by dissolving 14.7 g CaCl2·2 H2O in 30 mL of MilliQ water in a 100 mL beaker. Add MilliQ water to bring the final volume up to 50 mL, then transfer to a labeled 250 mL polystyrene bottle and store at room temperature for up to 60 days.
			3. 2M Magnesium Sulfate, heptahydrate: Prepare a 2M stock by dissolving 24.65 g MgSO4.7 H2O in 30 mL of MilliQ water in a 100 mL beaker. Add MilliQ water to bring the final volume up to 50 mL, then transfer to a labeled 250 mL polystyrene bottle and store at room temperature for up to 60 days.
			4. Label a sterile 1 L polystyrene bottle with the reagent name, date and the preparer’s initials.
			5. Obtain a stir bar and place it in the container.
		2. **Preparation of Stock Materials**
			1. Add approximately 500 mL MilliQ water to a 1000 mL beaker.
			2. Place a stir bar into beaker and place the beaker on a stir plate with a medium setting (4-7).
			3. Weigh out and add 4.77 g HEPES to beaker.
			4. Using a disposable transfer pipette, carefully add 13-14 drops of 5N NaOH to beaker.
			5. Using a P1000 pipette, add 1 mL 2M magnesium sulfate to beaker.
			6. Using a P1000 pipette, add 1 mL 2M calcium chloride to beaker.
			7. Remove stir bar and adjust volume to 950 mL. Pour contents into a sterile 1000 mL bottle and set aside as the liquid stock.
			8. Obtain a 50 mL conical tube and place in a tube holder.
			9. Weigh out and add the contents of Table 2 to conical tube. Note: use new weigh boat for each reagent. Be sure to make any adjustments with new disposable spatula to maintain accuracy and avoid cross-contamination.

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| **Reagent** | **Amount (in grams)** |
| Potassium Chloride KCL | 0.19 |
| Sodium Phosphate monobasic monohydrate NaH2PO4 | 0.17 |
| Sodium Bicarbonate NaHCO3 | 2.52 |
| Glucose | 4.51 |
| Sodium Ascorbate | 0.99 |
| Sodium Pyruvate | 0.33 |
| Thiourea | 0.15 |
| Sodium Chloride NaCl | 5.38 |

Table 2. Solid Stock (contents of conical tube) for rapid prep ACSF.VIII

* + - 1. Set tube aside and obtain a 1 L 0.45 µm filter system.
			2. Attach vacuum pump to filter system and filter the liquid stock.
			3. Once liquid has been filtered into a fresh 1000 mL bottle, label both solid and liquid stock containers with their respective contents, preparation date, expiration date, and preparer’s initials. Tape the conical tube to the side of the bottle and store at 4ºC for up to 60 days.
		1. **Preparation of Reagent (day of use)**
			1. Pour contents of conical tube into bottle of liquid stock, secure cap, and shake vigorously to mix.
			2. Once all powder has dissolved, pour ~40-45 mL ACSF.VIII into the conical tube, then back into the bottle. Repeat 2-3 times or as necessary until no powder residue remains inside tube. Shake bottle again to ensure complete dissolution.
			3. Add a stir bar the bottle and using a calibrated pH meter, measure the pH of the ACSF solution while stirring on stir plate. Acceptable pH is between 7.3 and 7.4.
				1. If pH is too high, add 5N HCl dropwise to lower to desired reading. Remove stir bar when finished.
				2. If pH is too low, add 5N NaOH dropwise to increase to desired reading. Remove stir bar when finished.
				3. Adjust final volume to 1000 mL with MilliQ Water using the graduation marks on the side of the container (if calibrated) or a graduated cylinder.
			4. Using a calibrated freezing point osmometer, measure the osmolarity of the solution. Acceptable osmolarity range is 305-315 mOsm.
				1. Obtain a clean sample tube. Using a P1000 pipette, add 250 µL of ACSF.VIII to bottom of tube.
				2. Gently place tube in sample receptacle.
				3. Press “start” button. Osmometer probe will automatically lower and an alarm will sound when reading is complete.
				4. Once probe has returned to its original position, remove sample tube, rinse with MilliQ water and dry completely with a kimwipe. Between tests and once again after final reading, be sure to gently clean probe with a wet kimwipe (MilliQ water only), and dry completely.

If osmolarity is too high, add a small amount of MilliQ water to ACSF solution, then confirm that the correct osmolarity has been reached. Repeat as necessary.

If the osmolarity is too low, add a volume (X) of 1 mg/mL NaCl solution determined by the following equation:



D=desired osmolarity

O= observed osmolarity

X=volume of 1 mg/mL NaCl solution to add (in mL)

* + - * 1. Confirm that the correct osmolarity has been reached after addition of NaCl or water.
			1. Filter the ACSF through a Corning 1 L 0.45 µm filter system.
				1. ***Sterile filter using 0.22 µm filter if tissue will be used for slice culture.***
	1. **ACSF Formulation Comparison**

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| **Component** | **ACSF.I** | **ACSF.II** | **ACSF.III** | **ACSF.IV** | **ACSF.V** | **ACSF.VI** | **ACSF.VII** | **ACSF.VIII** | **ACSF.IX** |
| Calcium Chloride, dihydrate | 0.5 mM | 2 mM | 2 mM | 2 mM | 1.8 mM | 2 mM | 0.5 mM | 2 mM | 1.3/2.0 mM |
| D-Glucose (Dextrose) | 25 mM | 25 mM | 12.5 mM | 25 mM | 0 | 20 mM | 25 mM | 25 mM | 25 mM |
| DL-AP5 | 0 | 0 | 0 | 0 | 0 | 50 µM | 0 | 0 | 0 |
| DNQX | 0 | 0 | 0 | 0 | 0 | 20 µM | 0 | 0 | 0 |
| HCl | 98 mM | 0 | 0 | 0 | 0 | 0 | 92 mM | 0 | 0 |
| HEPES | 20 mM | 16 mM | 0 | 20 mM | 5 mM | 0 | 20 mM | 20 mM | 0 |
| Kynurenic Acid | 0 | 0 | 1 mM | 0 | 0 | 0 | 0 | 0 | 0 |
| Magnesium Chloride | 0 | 0 | 0 | 0 | 1.0 mM | 2 mM | 0 | 0 | 0 |
| Magnesium Sulfate | 10 mM | 2 mM | 1 mM | 2 mM | 0 | 0 | 10 mM | 2 mM | 1.0/2.0 mM |
| Monosodium Phosphate | 1.25 mM | 1.25 mM | 1.25 mM | 1.25 mM | 0 | 1.25 mM | 1.2 mM | 1.2 mM | 1.2 mM |
| Myo inositol | 3 mM | 3 mM | 0 | 3 mM | 0 | 0 | 0 | 0 | 0 |
| N-acetylcysteine | 12 mM | 12 mM | 0 | 12.3 mM | 0 | 0 | 0 | 0 | 0 |
| N-methyl-d-glucamine | 96 mM | 0 | 0 | 0 | 0 | 0 | 92 mM | 0 | 0 |
| Picrotoxin | 0 | 0 | 0.1 mM | 0 | 0 | 0 | 0 | 0 | 0 |
| Potassium Chloride | 2.5 mM | 2.5 mM | 2.5 mM | 2.5 mM | 5.4 mM | 3 mM | 2.5 mM | 2.5 mM | 3mM |
| Sodium Bicarbonate | 25 mM | 25 mM | 26 mM | 25 mM | 0 | 20 mM | 30 mM | 30 mM | 18 mM |
| Sodium Chloride | 0 | 97 mM | 126 mM | 94 mM | 135 mM | 126 mM | 0 | 92 mM | 126 mM |
| Sodium L-Ascorbate | 5 mM | 5 mM | 0 | 5 mM | 0 | 0 | 5 mM | 5 mM | 0.16 mM |
| Sodium Pyruvate | 3 mM | 3 mM | 0 | 3 mM | 0 | 0 | 3 mM | 3 mM | 0 |
| Taurine | 0.01 mM | 0.01 mM | 0 | 0.01 mM | 0 | 0 | 0 | 0 | 0 |
| Thiourea | 2 mM | 2 mM | 0 | 2 mM | 0 | 0 | 2 mM | 2 mM | 0 |
| Tetrodotoxin (TTX) | 0 | 0 | 0 | 0 | 0 | 100 nM | 0 | 0 | 0 |