**Patch-Seq Internal Solution with Biocytin**

1. **Scope and Applicability:** Internal solution used for patch clamp electrophysiology, modified for cleanliness and mRNA capture and sustainability
2. **Materials:**
   1. Nuclease-free water (Ambion AM9938-100ml)
   2. RNase Zap or RNase Away Decontaminant (Invitrogen AM9780, Thermo Scientific 53225-514 or equivalent)
   3. Potassium Gluconate (K-Gluconate) (Sigma G4500)
   4. Potassium Chloride (2 M *KCl*, molecular biology grade) (Thermo Fisher Scientific AM9640G)
   5. 4-(2-Hydroxyethyl) piperazine-1-ethanesulfonic acid, N-(2-Hydroxyethyl) piperazine-N′-(2-ethanesulfonic acid) (1 M HEPES, BioPerformance certified) *(C8H18N2O4S)* (Sigma H3537 or equivalent)
   6. Adenosine 5′-triphosphate magnesium salt (ATP-Mg)(Sigma A9187)
   7. Guanosine 5’-triphosphate sodium salt hydrate (GTP-Na) (Sigma **G8877**)
   8. Sodium Phosphocreatine (Sigma P7936)
   9. Ethylene Glycol-bis (2-aminoethylether)-N,N,N’,N’-Tetraacetic Acid (EGTA, molecular biology grade) *([-CH2OCH2CH2N(CH2CO2H)2]2*) (Sigma E3889 or equivalent)
   10. Glycogen, RNA grade (Thermo Fisher Scientific R0551)
   11. Potassium Hydroxide *(KOH)* Pellets (Thermo Fisher P250-500)
   12. Biocytin (Sigma B4261)
   13. RNase Inhibitor 40 U/µl (Takara 2313B)
   14. Dye of choice from the following list:
       1. Alexa Fluor 488 Hydrazide (Thermo A10436)
       2. Alexa Fluor 594 Hydrazide (Thermo A10438)
       3. Alexa Fluor 405 Cadaverine (Thermo A30675)
       4. Cascade Blue Hydrazide, Trisodium Salt (Thermo C687)
   15. Argos Sterilized 22um PVDF Syringe-Driven Filters (FV32S)
   16. BD 10 ml syringes w/ luer-lok tips (VWR 75846-756)
   17. 1.5 ml DNA/RNA LoBind microcentrifuge tubes (VWR 80077-230)
   18. 15 x 5 ml tubes (VWR 89429-316)
   19. 50 ml conical tube (VWR 21008-951)
   20. 15 ml conical tube (VWR 21008-670)
   21. Disposable plastic spatulas (VWR 80081-188)
   22. P20 pipette tips (Rainin GPL20F)
   23. P200 pipette tips (Rainin GPL200F)
   24. P1000 pipette tips (Rainin GPL1000F)
   25. Steriflip 0.22 µm (Thermo Fisher SCGP00525 or Millipore SE1M179M6)
   26. Weigh boats
   27. Weigh paper
3. **Equipment:** 
   1. RNase-free stir bar
   2. Magnetic stir bar remover
   3. Microcentrifuge
   4. Osmometer (Vapro 5600)
   5. pH meter
   6. RNase-free hood
   7. Stir plate
   8. Vacuum pump (Gast DOA-P104-AA) with Tygon tubing
   9. P20, P200, P1000 pipettor
   10. 8-channel P10 or P20 pipettor.
   11. 100 ml glass beaker designated for Patch-Seq Internal Solution preparation
   12. 100 ml graduated cylinder designated for Patch-Seq Internal Solution preparation
4. **Safety:**
   1. Eye protection
   2. Gloves
   3. Lab coat
5. **Output:**
   1. 80 ml of Patch-Seq Internal Solution with Biocytin, pH 7.3, Osmolarity 305-320 mOsm containing:
      1. 110 mM K-Gluconate
      2. 4 mM KCl
      3. 10.0 mM HEPES
      4. 1 mM ATP-Mg
      5. 0.3 mM GTP-Na
      6. 10 mM Sodium Phosphocreatine
      7. 0.2 mM EGTA
      8. 20 µg/ml Glycogen
      9. 0.5% Biocytin
      10. 0.5 U/µl RNase Inhibitor
      11. ~50 µM of desired dye
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

**Warning: Personal Protective Equipment (PPE) and cleanliness for RNase-free conditions 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. These should be prepared in an RNase-free hood and used for Patch-Seq Internal Solution only. Label the stock bottles with contents, preparer’s initials, preparation date, storage requirement and expiration date.
      1. **EGTA**: Prepare a 192 mM stock solution by first dissolving 3.65 g of EGTA in 32 ml nuclease-free water in a labeled 50 ml conical tube. Add 4M KOH dropwise until the EGTA goes into solution (this may take up to 6 ml). Bring the final volume up to 50 ml with nuclease-free water. Store the stock solution at 4oC for up to 60 days.
      2. **Fluor Dye Stock:** Prepare a ~10 mM stock by adding 175 µl of nuclease-free water into 1 mg unit of desired dye in 1.5 ml lo-bind tube. Aliquot 25 µl of stock into PCR strips and store at -20°C for up to 6 months (Reference Appendix 11.2)
         1. Label each tube with its expiration date.
   2. Obtain an RNase-free stir bar and place it in the glass beaker designated for Patch-Seq Internal Solution preparation.
   3. Set up the stir plate in the hood.
2. **Methodology/Procedures** (record all lot numbers and volumes used in the appropriate reagent prep notebook):
   1. **To make 80 ml of Internal Solution: All steps below are done in RNase-free hood**
      1. Gather all materials in an RNase-free hood and wipe down with RNase zap or RNase Away Decontaminant and then 70% EtOH.
      2. Using a graduated cylinder, measure 60 ml of nuclease-free water. Add the water to an appropriately labeled RNase-free glass beaker containing an RNase-free stir bar.
      3. Obtain a glass dish or tray and place a mixture of wet and dry ice onto it. Place the beaker down into the ice mixture for the duration of the process. Place the glass beaker in the glass dish on a stir plate and begin stirring at a high setting (300 RPM or higher).
      4. Using a calibrated balance, weigh out 2.06 g of K-Gluconate. Slowly add weighed K-Gluconate to the glass beaker while stirring.
      5. Using a P200 pipettor, add 160 µl of the 2 M KCl solution to the glass beaker while stirring.
      6. Using a P1000 pipettor, add 800 µl of the 1 M HEPES to the glass beaker while stirring.
      7. Using a calibrated balance, weigh out 40.56 mg of ATP-Mg. Slowly add weighed ATP-Mg to the glass beaker while stirring.
      8. Using a calibrated balance, weigh out 12.56 mg of GTP-Na. Slowly add weighed GTP-Na to the glass beaker while stirring.
      9. Using a calibrated balance, weigh out 204.08 mg of Sodium Phosphocreatine. Slowly add weighed Sodium Phosphocreatine to the glass beaker while stirring.
      10. Using a calibrated balance, weigh out 400 mg of biocytin. Slowly add weighed biocytin to the glass beaker while stirring.
      11. Using a P200 pipettor, add 82 µl of the 192 mM EGTA stock solution to the glass beaker while stirring.
      12. Using a P200 pipettor, add 80 µl of the 20 mg/ml Glycogen solution to the glass beaker while stirring.
      13. Continue stirring at a high speed (300 RPM or higher) until all the chemicals are fully in solution. If necessary, brief periods of vortexing can be used to help dissolve the biocytin.
      14. Using a calibrated pH meter in a fume hood, measure the pH of the internal solution. Adjust the pH to 7.3 by adding 4N KOH in 20 µl increments. Caution, use small incremental volumes to avoid overshooting the 7.3 target pH. If this happens, the prep must be discarded.
      15. Remove stir bar using a magnetic stir bar remover.
      16. Adjust final volume to 80 ml with nuclease-free water using the graduation marks on the side of the glass beaker (if calibrated) or a graduated cylinder.

|  |  |  |
| --- | --- | --- |
| Reagent | Amount | Concentration |
| K-Gluconate | 2.03 g | 110 mM |
| KCl solution (2M) | 160 µl | 4 mM |
| HEPES Solution (1M) | 800 µl | 10 mM |
| ATP-Mg | 40.56 mg | 1 mM |
| GTP-Na | 12.56 mg | 0.3 mM |
| Sodium Phosphocreatine | 204.08 mg | 10 mM |
| EGTA; 192 mM stock | 82 µl | 0.2 mM |
| Glycogen solution (20 mg/ml) | 80 µl | 20 µg/ml |
| Biocytin | 400 mg | 13.4 mM |
| Bring up to this final volume with nuclease-free water (after adjusting pH) | 80 ml |  |

Table 1: Reagents Required for Preparation of 80 ml Patch-Seq Internal Solution with Biocytin

* + 1. Measure the osmolarity of a 10 µl sample of the solution using a Vapro osmometer. If the osmolarity is not 220-230 mOsm, adjust as needed:
       1. If the osmolarity is too high, add nuclease-free water.
       2. If the osmolarity is too low, add a volume (X) of 1 mg/ml K-Gluconate solution determined by the following equation:



D = desired osmolarity

O = observed osmolarity

X = volume of solution to add (in ml)

* + 1. Confirm that the correct osmolarity has been reached after addition of K-Gluconate or water.
    2. Record volume of water and/or K-Gluconate solution required to adjust osmolarity.
  1. Filter the internal solution through a Steriflip 0.22 µm filter system.
     + 1. Using Tygon tubing, attach the filter system to the Gast vacuum pump that sits directly outside of the fume hood (or lab bench vacuum). Turn on the pump.
  2. Aliquot 5 ml quantities of filtered internal solution into 5 ml microcentrifuge tubes.
     1. Expect approximately 15 x 5 ml tubes per batch.
  3. Label the tubes with the Internal solution with Biocytin printed labels.
  4. Store 5 ml aliquots at -80°C for up to 60 days.

|  |  |  |  |
| --- | --- | --- | --- |
| **Reagent** | **Stock Concentration** | **Volume** | **Final Concentration** |
| RNase Inhibitor | 40 U/µl | 62.5 µl | 0.5 U/µl |
| Fluor stock (Variant Dependent on Request) | ~10mM | 25 µl | 50uM |

Table 2: Reagents added to each 5 ml aliquot to make final solution

* 1. Every six weeks, five to eight 5 ml aliquots will be thawed and the following will be added and filtered:
     1. Using a P200 pipettor, add 62.5 µl of RNase Inhibitor (40 U/µl) to 5 ml of internal solution.
     2. Using a P200 pipettor, add 25 µl of the requested Fluor stock (~10 mM) to the same 10 ml of internal solution.
     3. Set up a Agros PVDF filter and a BD 10ml syringe complex to filter the combined internal solution.
     4. Transfer 5000 µl internal solution into the syringe/filter complex and filter the solution into a new 15 ml BD Falcon tube.
     5. Repeat this step with a fresh set of filters to ensure solution is free of particles.
     6. Measure and record the osmolarity of a 10 µl sample of the solution using the Vapro osmometer. The acceptable osmolarity should be in the range of 305-320 mOsm.
     7. Test for clogging and/or contamination. **Load 1 µl into glass capillary pipette and place in IVSCC rig and monitor, under 40x guidance, for any blockage of pipette opening. Repeat 10-15 times for 2 minutes each. \*critical step\***
     8. Aliquot 200 µl into 1.5 ml LoBind tubes (37-39 tubes per 10 ml), using a P1000 pipette.
     9. Store filled PCR strips in a freezer box in the -80°C freezer.
     10. Label box with reagent name, date, preparer’s initials, and expiration date.
     11. 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 nuclease-free water.
   3. Clean balance.
   4. Rinse all beakers, graduated cylinders, and other equipment used to prepare internal solution with nuclease-free water only **(No Detergent)**. Invert all beakers and graduated cylinders and leave on clean absorbent pad to air dry.
   5. Wipe down RNase-free hood with RNase Zap or RNase Away Decontaminant followed by 70% EtOH. Remove tip waste bucket from hood, dispose of tips, then re-wipe down with RNase Zap or RNase Away Decontaminant followed by 70% EtOH then return bucket to hood.
2. **Technical Information:**
   1. Storage Conditions:

|  |  |  |
| --- | --- | --- |
| **Component** | **Storage Temperature** | **Shelf Life** |
| EGTA; 192 mM | 4ºC | 60 days |
| Alexa Fluor 488 Hydrazide 10 mM | -20°C | 6 months |
| Alexa Fluor 594 Hydrazide 7.5 mM | -20°C | 6 months |
| Alexa Fluor 405 Cadaverine 8.6 mM | -20°C | 6 months |
| Cascade Blue Hydrazide 9.6 mM | -20°C | 6 months |
| 4N KOH Stock | Rt (base cabinet) | 2 years |
| Internal Solution | -80ºC | 60 days |

1. **Appendix**
   1. Materials required for preparation of 80 ml of Internal Solution

|  |  |  |
| --- | --- | --- |
| **Material** | **Supplier,**  **Part Number** | **Amount**  **Required**  **for 80 ml** |
| 50 ml filter system, 0.22 µm | Thermo Fisher SCGP00525 or Millipore SE1M179M6 | 1 ea |
| K-Gluconate | Sigma G4500 | 2.06 g |
| KCl | Sigma P9541 | 23.88 mg |
| HEPES | Sigma H3537 | 800 µl |
| ATP-Mg | Sigma A9187 | 40.56 mg |
| GTP-Na | Sigma G8877 | 12.56 mg |
| Sodium Phosphocreatine | Sigma P7936 | 204.08 mg |
| EGTA | Sigma E3889 | 5.98 mg |
| Glycogen | Thermo Scientific R0551 | 80 µl |
| 4N KOH | BDH Chemical BDH3212-1 | 20 ml |
| Biocytin | Sigma B4261 | 400 mg |
| RNase Inhibitor | Takara 2313A | 1.0 ml |
| Alexa Fluor 488 Hydrazide | Thermo A10436 | 2.28 mg |
| Alexa Fluor 594 Hydrazide | Thermo A10438 | 3.04 mg |
| Alexa Fluor 405 Cadaverine | Thermo A30675 | 2.67 mg |
| Cascade Blue Hydrazine | Thermo C687 | 2.38 mg |

* 1. Concentration required for 25 µl aliquots of desired dye stock solution

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Material** | **Molecular Weight** | **Stock Concentration** | **Aliquots** | **Concentration**  **for 5 ml** |
| Alexa Fluor 405 Cadaverine | 666 g/mol | 8.6 mM | 25 µl | 42 µM |
| Alexa Fluor 488 Hydrazide | 570 g/mol | 10 mM | 25 µl | 49 µM |
| Alexa Fluor 594 Hydrazide | 758 g/mol | 7.5 mM | 25 µl | 37 µM |
| Cascade Blue Hydrazide | 596 g/mol | 9.6 mM | 25 µl | 47 µM |