**DAB Detection of Biocytin Labeled Tissue**

1. **Scope and Applicability:** This SOP describes the process for diaminobenzidine (DAB) detection of biocytin filled cells. This protocol is optimized for use with brain slices cut at 350 µm thick, in which cells are first filled with biocytin (i.e., post-electrophysiological recording), fixed in 4% PFA/2.5% glutaraldehyde, and transferred to PBS until ready to stain.
2. **Materials:**
	1. 24-well tissue culture plates containing PFA/Glutaraldehyde fixed brain sections containing biocytin filled cells, in PBS (prepared using SOP PF0282)
	2. 5 µM DAPI solution (SOP RP0193)
	3. PBS (-) and PBS (+) (SOP RP0001)
	4. 30% Hydrogen peroxide (H2O2) (Calbiochem 386790 or equivalent)
	5. 10% Triton X-100 (SOP RP0195)
	6. Vectastain ABC Kit (Vector Laboratories PK-4000)
	7. 0.1% Triton X-100 in PBS (SOP RP0195)
	8. DAB (2 options are available)
		1. DAB Solution (SOP RP0168)
		2. Biocare Betazoid DAB Chromogen Solution (SOP RP0266)
	9. Aqua-Poly/Mount Medium (Polyscience, Inc 8606-20)
	10. MilliQ water
	11. Serological pipettes (5 mL, 10 mL, 25 mL, 50 mL)
	12. P20, P200, P1000 pipette tips (Rainin GP-L10F, GP-L200F, GP-L1000F or equivalent)
	13. Kimwipes or paper towels
	14. Petri Dish (Falcon 351029 or equivalent)
	15. Single-well Receiver Trays (Millipore PSSW010R5 or equivalent)
	16. 24 Well plates (Costar 3738 or equivalent)
	17. 15 mL conical tube (VWR 21008-670 or equivalent).
	18. 25 mm square spacers with 19 mm diameter aperture. Die-cut from ISC Racers Tape Helicopter-OG Surface Guard Tape (8 mil Outdoor Grade, part no HT1308) with custom die (Custom Converting, via quote 111471-01).
	19. 1x3 Gelatin Coated Slides (SOP PF0246)Use appropriate project-specific barcoded, labeled slides
	20. Coverslips; 22x22 mm, #1.5 (Electron Microscopy Sciences 72204-01 or equivalent)
	21. Small Paintbrush (Ted Pella Red Sable #1 or equivalent)
	22. Parafilm
3. **Equipment:**
	1. PELCO Prep-Eze 24-wellplate insert (Ted Pella 36172 or equivalent), also referred to as “insert plates”
	2. PELCO Prep-Eze 12-wellplate insert (Ted Pella 36170 or equivalent).
	3. PELCO Prep-Eze 6-wellplate insert (Ted Pella 36168 or equivalent).
	4. Low-depth trays, 12” Pyrex dishes or equivalent
	5. Automatic pipette (Pipette Aid)
	6. Pipettemen (P20, P200, P1000)
	7. Dissecting Microscope (Leica M60 or equivalent)
	8. Orbital Rotator at Room Temp (Thermo Fisher 2314 or equivalent)
	9. 4ºC Refrigerator
	10. Orbital Rotator set up in 4ºC Refrigerator (Thermo Fisher 2314 or equivalent)
	11. Balance
4. **Safety:**
	1. Nitrile gloves
	2. Lab coat
	3. Safety glasses
	4. Fume hood

**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. **Output:**
	1. DAB labeled cells in brain slices stained with DAPI and mounted on 1x3 gelatin coated slides.
2. **Reference Documents:**
	1. PF0246 Coating Slides with Gelatin
		1. <https://www.protocols.io/view/coating-slides-with-gelatin-bf64jrgw>
	2. PF0289 Post Patch Clamp Slice Fixation
		1. <https://www.protocols.io/view/post-patch-clamp-slice-fixation-bg5tjy6n>
	3. PF0301 Patch-Seq Recording and Extraction
		1. <https://www.protocols.io/view/patch-seq-recording-and-extraction-bepyjdpw>
	4. RP0001 Phosphate Buffered Saline (PBS)
		1. <https://www.protocols.io/view/phosphate-buffered-saline-pbs-ddsg26bw>
	5. RP0007 10% Tween 20
		1. <https://www.protocols.io/view/10-tween-20-ddsd26a6>
	6. RP0168 DAB Solutions
		1. <https://www.protocols.io/view/dab-solutions-bgndjva6>
	7. RP0193 5 µM DAPI cell staining solution
		1. <https://www.protocols.io/view/5-um-dapi-cell-staining-solution-bg47jyzn>
	8. RP0195 10% Triton X-100
		1. <https://www.protocols.io/view/10-triton-x-100-bezhjf36>
	9. RP0266 Biocare Betazoid DAB Solution
		1. To be Published
3. **Setup:**
	1. Obtain 24 wellplate with insert containing fixed slices with biocytin filled cells in PBS (-) from 4°C storage.
		1. Transfer sections into a PELCO 24 wellplate insert if they are currently in a 24 wellplate:
			1. Fill a single well receiver tray with 40-50 mL of PBS (-).
			2. Place an empty PELCO 24-well plate insert into the tray. Make sure that the PBS fills all of the wells in the insert at least halfway.
			3. Using a paintbrush or transfer pipet, transfer sections to be stained into insert plates, one section per well, into the same well location on insert as original plate.
	2. Obtain a low-depth tray and line with Kimwipes or paper towels for blotting plate inserts.
	3. Obtain single well receiver trays for use with 24 wellplate inserts.
	4. 5 µM DAPI in PBS, 1% H2O2 in PBS, ABC solution, and DAB need to be made fresh prior to use (same-day of use).
		1. 5 µM DAPI, 1% H2O2 in PBS and ABC solution are required on day 1 of protocol.
		2. DAB solution is used on day 2.
	5. See Table 1 for protocol details. 40-50 mL of reagent is required for each Single-well Receiver Tray. For incubations in a 24 wellplate, 1 mL/well required. For incubations in a 12 wellplate, 2 mL/well required. For incubations in a 6 wellplate, 3 mL/well required make appropriate amounts for the number of slices being stained.
	6. Obtain spacers from stock of previously-cut material.
4. **Methodology/Procedures:**
	1. **Biocytin Staining**
		1. The biocytin protocol is outlined in **Table 1**. ***All incubations unless otherwise stated should be done on a shaker set to low-medium speed.***
		2. Begin staining protocol at step 1 by placing insert plates containing fixed biocytin filled slices into single-well receiver trays filled with 40-50 mL of 5 µM DAPI in PBS (to be performed in a fume hood). See **Table 1** for incubation times.
			1. *DAPI is an eye, skin and respiratory irritant, mutagen and corrosive hazard. Avoid contact or inhaled exposure. If eye or skin contact occurs, wash affected areas with water for 15 minutes and notify EHS. See MSDS (section 10.3.1) for precautions.*
		3. When finished with an incubation step, remove insert plates from Single-well Receiver Trays and blot 2-3 times on Kimwipes or paper towels in a low-depth tray or Pyrex dish to remove excess reagent before placing the insert into the next tray containing fresh solution.
			1. *Single well trays used for non-wash steps should be rinsed out after use. Trays used for PBS do not need to be rinsed in between wash steps.*
		4. Follow the detection steps as outlined in **Table 1, Steps 1-8**. Use 40-50 mL of each reagent per tray unless otherwise stated.
		5. While slices are in first PBS (+) washes (*steps 2-4*), prepare 1% H2O2 in PBS (-) used to block peroxidase activity.
			1. *Prepare* *45 mL of 1% H2O2 in PBS (-) for each insert containing sections being stained.* *See section* ***11.1.1*** *for reagent prep instructions.*
		6. Once the sample plates are in 1% H2O2 in PBS, discard used PBS (+) from previous wash steps and refill single well receiver trays with fresh PBS (+).

**Table 1: DAB Detection of Biocytin Labeled Tissue**

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| **Step** | **Reagent** | **Time (min)** | **Staining Plate** | **Incubation conditions** | **Exact?**(N indicates step is flexible) |
| **1** | 5 µM DAPI in PBS | 15 | *Single Well* | Fume Hood, Room temp  | **Y** |
| **2** | PBS (+) | 10+ | *Single Well* | Shaker, Room temp | N |
| **3** | PBS (+) | 10+ | *Single Well* | Shaker, Room temp | N |
| **4** | PBS (+) | 10+ | *Single Well* | Shaker, Room temp | N |
| **5** | 1% H2O2 in PBS | 30 | *Single Well* | Shaker, Room temp | **Y** |
| **6** | PBS (+) | 10+ | *Single Well* | Shaker, Room temp | N |
| **7** | PBS (+) | 10+ | *Single Well* | Shaker, Room temp | N |
| **8** | PBS (+) | 10+ | *Single Well* | Shaker, Room temp | N |
| **9** | ABC Reagent *(Room Temp)* | 60 | *24 Well plate* | Shaker, Room temp | **Y** |
| **10** | ABC Reagent *(4°C)* | 2 days (40+ hrs) | *24 Well plate* | Shaker, 4°C | N |
| **11** | ABC Reagent *(Room Temp)* | 60 | *24 Well plate* | Shaker, Room temp | **Y** |
| **12** | PBS (+) | 10+ | *Single Well* | Shaker, Room temp | N |
| **13** | PBS (+) | 10+ | *Single Well* | Shaker, Room temp | N |
| **14** | PBS (+) | 10+ | *Single Well* | Shaker, Room temp | N |
| **15** | DAB  | *Mouse:* 3:00*Human:* 4:30 | *Single Well* | Room Temp | **Y** |
| **16** | PBS (-) | 10+ | *Single Well* | Shaker, Room temp | N |
| **17** | PBS (-) | 10+ | *Single Well* | Shaker, Room temp | N |
| **18** | PBS (-) | 10+ | *Single Well* | Shaker, Room temp | N |

* + 1. After peroxidase block, perform PBS (+) washes (*steps 6-8*) and prepare ABC reagent.
			1. ***ABC reagent must be prepared 30 minutes prior to use. Do not vortex.***
			2. Volume Required (mL) = (# slices in 24 well \* 1 mL ABC solution) + (# slices in 12 well \* 2 mL ABC solution) + (# slices in 6 well \* 3 mL ABC solution). *See section* ***11.1.2*** *for reagent prep instructions.*
			3. Once ABC has been prepared and had a chance to stand for 30 minutes, swirl container/tube to mix and then dispense 1 mL of solution into each well of a fresh 24 wellplate that will contain a tissue slice (same wells that contain slices in inserts being stained). Dispense 2 mL to each 12 well plate being used, and 3 mL to each 6 well plate.
			4. Prepare one 24 wellplate of ABC reagent for each 24 well insert containing tissue sections being processed. Repeat for each 6 well and 12 well inserts.
		2. When slices have completed PBS washes after peroxidase block, blot inserts 2-3 times on Kimwipe or paper towel, and transfer into appropriate multi-well plate(s) containing fresh ABC reagent.
		3. Incubate slices in ABC for 60 minutes on a shaker at room temp.
		4. After 60 minutes at room temp, parafilm the plates and transfer to shaker set up in 4°C refrigerator and leave to incubate for two days.
			1. *Slices should incubate at least 40 hours in ABC solution at 4°C.*
		5. The following day, remove plates from 4°C and incubate in ABC solution for an additional 60 minutes at room temperature on a shaker.
		6. Blot inserts 2-3 times on Kimwipe or paper towel and continue with PBS (+) wash steps outlined in **Table 1, steps 12-14**, using 45-50 mL of reagent per tray.
		7. While slices are in PBS washes, prepare DAB solution.
			1. *Prepare* *50 mL DAB working solution for each insert containing sections being stained.* *See section* ***11.1.3*** *for reagent prep instructions.*
				1. *If using alternative DAB please see 11.1.4, Table 7 for prep instructions*
			2. Do not add the H2O2 to working solution until immediately prior to use.
		8. ***To perform DAB reaction using plate inserts:***
			1. When time for DAB incubation (step 15), pour 40-50 mL of DAB working solution prepared in previous step into a single well receiver tray.
			2. Prepare a separate fresh single well receiver tray with PBS (-).
			3. Remove insert plate containing sections from PBS wash and blot 2-3 times on Kimwipe before transferring into single well receiver tray filled with DAB working solution.
			4. Develop sections using standardized DAB incubation times:
				1. *Mouse brain slices:* **3 min DAB incubation**
				2. *Human tissue slices:* **4 min 30 sec DAB incubation**
			5. After DAB incubation complete, immediately transfer plate insert containing sections from DAB into tray of fresh PBS (-) to stop DAB reaction.
			6. Perform final wash steps using fresh PBS (-) as outlined in **Table 1, Steps 16-18**.
		9. ***To perform DAB reaction using alternate DAB solution:***
			1. When time for DAB incubation, using the repeat pipettor, pipette the appropriate volume of the alternate DAB solution into the respective welled plates. If using a 24 well plate, pipette 1 mL of solution into each well containing a slice of tissue, 2 mL of solution into each well for a 12 well plate, and 3 mL of solution for 6 mL plate.
			2. Prepare a separate fresh single well receiver tray with PBS (-).
			3. Remove insert plate containing sections from PBS wash and blot 2-3 times on Kimwipe before transferring into single well receiver tray filled with DAB working solution.
			4. Develop sections using standardized DAB incubation times:
				1. *Mouse brain slices:* **3 min DAB incubation**
				2. *Human tissue slices:* **4 min 30 sec DAB incubation**
		10. After DAB incubation complete, immediately transfer plate insert containing sections from DAB into tray of fresh PBS (-) to stop DAB reaction.
			1. Perform final wash steps using fresh PBS (-) as outlined in **Table 1, Steps 16-18**.
		11. ***To perform DAB reaction manually:***
			1. When time for DAB incubation (step 15), pour 20 mL of DAB working solution into a petri dish and bring to microscope.
			2. Prepare a fresh single well receiver tray with PBS (-) and add a fresh insert plate. Keep next to microscope.
			3. Bring trays/inserts containing sections to microscope, and using a paintbrush, transfer one section at a time to petri dish containing DAB working solution.
			4. Develop one section at a time under microscopic visualization until target cell has reached desired stain intensity
				1. Target cell should become visible in section quickly after entering DAB solution. Flip section over using paintbrush to check both surfaces for target cell as it will only be visible from one side.
				2. DAB incubation is standardized to 3 mins for mouse tissue and 4 mins minutes and 30 seconds for human tissue (see Section 8.1.14.4). Stop reaction when background staining near target cell becomes visible or when target cell has reached desired intensity.
				3. Transfer stained slices from DAB solution into empty insert in fresh PBS (-) to stop DAB reaction. Slices should be transferred to the same well location across inserts to keep track of sections. Plate insert should also be labeled identically to the previous insert for tracking purposes.
				4. Proceed until all slices have been developed in DAB.
			5. After all slices have been developed, transfer insert to a fresh tray of PBS and perform washes as outlined in **Table 1, Steps 16-18.**
	1. **Mounting Sections and Coverslipping with Aqua-Poly/Mount**
		1. Allow tube to come to room temperature prior to use (wait a minimum of 30 minutes) before making any adjustments for flow rate as viscosity will change. Follow manufacturers expiration date for usage. A new lot of Aqua-Poly/Mount mounting media needs QC testing prior to use in production (refer to Section 10.2: Technical Information for protocol).
		2. Label gelatin coated slides with specimen/cell information for each slice to be mounted.Appropriate labels and barcodes will be project-specific.
		3. Remove Aqua-Polymount Mounting Media from 2-4°C storage and allow tube to come to room temperature prior to coverslipping (30 minutes wait time is adequate).
		4. Mount stained slices onto slides. Slices can be mounted onto labeled gelatin coated slides in two different ways; by mounting slices then adding a spacer, or by applying spacers first then mounting slices.
			1. ***Method 1: Apply spacers first then mount:***
				1. Place a 25 mm x 25 mm, 200 µm spacer adhesive liner removed on center of slide and press down along all four sides of spacer to firmly attach.

*Ensure that there are no wrinkles in spacer and that it lies flat against the slide. If spacer not flat against the slide it can introduce large bubbles once coverslipped, as well as cause issues downstream when scanning.*

*If spacer becomes wrinkled, remove from slide and place a new one on slide being careful to avoid any wrinkles.*

* + - * 1. Using a paintbrush, transfer stained sections from insert plate and mount a single section onto a gelatin coated slide, placing it as close to center of spacer as possible.
				2. Use a dissecting scope to ensure slices are mounted flat with no folds/wrinkles.
				3. Mount section “cell side up”.

Use dissecting scope to manually determine which face should be mounted up or use orientation information provided from upstream groups.

Do not allow tissue to dry for extended periods of time between mounting and cover-slipping. No more than one minute should elapse between the mounting and cover-slipping step. If necessary, prior to applying coverslip use a Kimwipe to wick away any residual moisture to help slices adhere flat to slide. Use a paintbrush to push down on areas with folds if necessary.

* + - * 1. Mount dorsal side up for mouse, pial surface up for human (towards tab of slide).
				2. Only 1 section per slide should be mounted.
				3. Should the coverslip not sit flush against the spacer surface, due to thickness of the mounted tissue, repeat steps 8.2.4.1.1-8.2.4.1.1.2 and apply a second spacer directly on top of the first.
				4. Add an additional ~130 µL of Aqua-Poly/Mount media into center of spacer, starting below section and moving slowly up. *Depending on slice size and degree of folds, volume of Aqua-Poly/Mount can range from ~110-175 µL/slide. Ensure sufficient medium around tissue and avoid voids in spacer aperture in assembled slide.*
				5. If section is too long, and doesn’t fit within the spacer’s well, contact Electrophysiology.They will assess if the tissue can be resected to fit within the margins of the spacer.
				6. Record the use of a second spacer due to increased section thickness or resection due to specimen length in the “Biocytin\_Version2” electronic documentation tracking form. Under tab 5, “5-Plate\_Map” document which specimenrequired the addition of a spacer or resection.
				7. Set aside to adhere while beginning to mount the next section**.**
			1. ***Method 2: mount first then apply spacers:***
				1. Fill a clean plastic petri dish with 0.1% Triton/PBS and bring to dissecting microscope.

*Prepare a 1:10 dilution using 10% Triton X-100 (SOP RP0195) in PBS (-) for final concentration of 0.1% Triton/PBS.*

* + - * 1. Using a paintbrush, transfer stained sections from insert plate into petri dish and mount a single section onto a gelatin coated slide, placing it as close to center as possible.
				2. Use a dissecting scope to ensure slices are mounted flat with no folds/wrinkles.

Mount section “cell side up”.

Use dissecting scope to manually determine which face should be mounted up, or use orientation information provided from upstream groups.

Mount dorsal side up for mouse, pial surface up for human (towards tab of slide).

Slices should be mounted in center of slide.

* + - * 1. Do not allow tissue to dry for extended periods of time between mounting and cover-slipping. No more than one minute should elapse between the mounting and cover-slipping step. If necessary, prior to applying coverslip use a Kimwipe to wick away any residual moisture to help slices adhere flat to slide. Use a paintbrush to push down on areas with folds if necessary.
				2. Place a 25 mm x 25 mm, 200 µm spacer with adhesive liner removed in center of slide and press down along all four sides of spacer to firmly attach.

*Ensure that there are no wrinkles in spacer and that it lies flat against the slide. If spacer not flat against the slide it can introduce large bubbles once coverslipped, as well as cause issues downstream when scanning*.

*If spacer becomes wrinkled, remove from slide and place new one around section being careful to avoid any wrinkles***.**

* + - 1. Set aside to adhere while beginning to mount the next section**.**
		1. When ready to coverslip mounted slices, ensure stock bottle has reached ambient temperature prior to cover-slipping and dispensing liquid. Invert stock bottle of Aqua-Poly/Mount and apply two to three drops to section -or- use a P200 pipet to dispense ~130 µL of Aqua-Poly/Mount media into center of spacer, starting below section and moving slowly up. *Depending on slice size and degree of folds, volume of Aqua-Poly/Mount can range from ~110-175 µL/slide. Ensure sufficient medium around tissue and avoid voids in spacer aperture in assembled slide.*
		2. Coverslip using 22x22 mm coverslips.
			1. Using a paintbrush or pipette tip, carefully lower the top edge of the coverslip at an angle while gently applying pressure to force any excess medium and air bubbles away from tissue and out of the coverslip. Gently tilt the slide to remove any medium at the edges of the slide and coverslip.
			2. *Bubbles and artifacts (wrinkles, etc.) must be avoided at the site of stained cell. Remove/Reposition tissue as necessary to achieve this.*
			3. Press down on opposite corners of coverslip (top left and bottom right, bottom left and top right) applying gentle pressure over the spacer to ensure coverslip isn’t “floating” and is flat mounted to spacer.
		3. Repeat steps **8.2.3 to 8.2.6** until all stained sections have been mounted and cover-slipped.
		4. After all slices have been cover-slipped, slides can be stored overnight at room temperature remaining horizontal while drying and protected from light or stored at 4°C. Drying at 4°C will increase drying times. Do not heat the slides as this can damage or fade some stains or reactions. Slides containing fluorescent chromogens should be stored in the dark.
		5. After drying the slides for 24-48 hours (depending on volume of media used) slides can be transferred to slide boxes or imaged following SOP MC0135: *Z Stack Image Capture with Zeiss AxioImager.* Keep slides protected from light unless they are being cleaned/imaged.
1. **Take Down:**
	1. Excess/unused 5 µM DAPI solution should be disposed of in the DAPI waste container.
	2. Retain used and left over DAB solution in appropriately labeled container for disposal by licensed waste hauler.
	3. All other reagents used in protocol can be disposed of as non-hazardous waste.
2. **Technical Information:**
	1. **Staining setup**
		1. Designate Single-well trays (**Figure 1**) for each reagent (setup shown in **Figure 2**). The same PBS trays can be reused throughout the staining process (with fresh reagent).
		2. Low-depth tray with Kimwipes can be replaced with any shallow container large enough to hold two tissue culture plates (i.e. 12” square Pyrex dish).



**Figure 1:** PELCO Staining Insert Plate and Tray

* 1. **QC of Aqua-Poly/Mount:**
		1. Prior to using a new lot of Aqua-Poly/Mount (minimum of one week prior to use in Production), remove Aqua-Poly/Mount from 4°C, allow to come to room temperature and prepare one test slide from each new lot of Aqua-Poly/Mount by placing a spacer onto a gel coated slide, adding the Aqua-Poly/Mount, and coverslipping slide. Each slide should be labeled with the date and the new lot number being tested.
		2. The test slides need to dry a minimum of 24-48 hours and can then be sent to imaging for review. Imaging will pass or fail the new lot/batch based on the amount of debris seen on the test slides.
		3. Mark QC Pass/Fail on each tube after receiving results back from imaging
		4. Put a checkmark on label next to QC Pass on all tubes that passed Imaging QC.
		5. ***QC Fail*** – Remove all tubes that failed and dispose of appropriately.
	2. **Reagent Shelf Life/Expiration Dates**
		1. **5 µM DAPI** staining solution is always prepared fresh the day of use.
		2. **1% H2O2 in PBS (-)** needs to be prepared fresh the day of staining.
		3. **ABC Solution** needs to be prepared fresh the day of staining.
		4. **DAB:** DAB Solution (SOP RP0168)needs to be prepared fresh the day of staining. Biocare Betazoid DAB Working solution is stable for 5 days if stored at 2-8°C.
		5. **Aqua-Poly/Mount Mounting Media** can be stored in 2-4°C per the manufacturer’s expiration date.
	3. **Product Information:** Reagent product inserts and safety information may be found at the manufacturer’s sites linked below.
		1. MSDS for DAPI: <https://www.thermofisher.com/order/catalog/product/D1306>

***DAPI is an eye, skin and respiratory irritant, mutagen and corrosive hazard. Avoid contact or inhaled exposure. If eye or skin contact occurs, wash affected areas with water for 15 minutes.***

* + 1. DAB Product Information and MSDS: <http://www.sigmaaldrich.com/catalog/product/sigma/d5637?lang=en&region=US>
		2. **VECTASTAIN ABC Kit:** <http://www.vectorlabs.com/catalog.aspx?prodID=384>
1. **Appendix:**
	1. **Reagent Prep**

**Table 2: *1% H2O2 in PBS (-) Preparation***

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| ***1% H2O2 in PBS*** | **Final Vol.****45 mL** | **Final Vol.****90 mL** | **Final Vol.****135 mL** | **Final Vol.****180 mL** |
| **PBS (-)** | 43.5 mL | 87 mL | 130.5 mL | 174 mL |
| **30% H2O2** | 1.5 mL | 3 mL | 4.5 mL | 6 mL |

* + 1. **Table 3: ABC reagent preparation**

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| **ABC Reagent** | **Final Vol.****5 mL** | **Final Vol.****10 mL** | **Final Vol.****20 mL** | **Final Vol.****25 mL** | **Final Vol.****50 mL** |
| **PBS (-)** | 2.4 mL | 4.8 mL | 9.6 mL | 12 mL | 24 mL |
| **10% Triton X-100** | 2.5 mL | 5 mL | 10 mL | 12.5 mL | 25 mL |
| **ABC Standard Kit Reagent A** | 50 µL | 100 µL | 200 µL | 250 µL | 500 µL |
| **ABC Standard Kit Reagent B** | 50 µL | 100 µL | 200 µL | 250 µL | 1. µL
 |

* 1. **DAB reagent preparation (RP0168)**
		1. Prepare 0.05% DAB and 0.3% H2O2 as detailed in SOP RP0168- *DAB Solutions.*

**Table 4: 0.05% DAB in PBS**

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **0.05% DAB** | **Final Vol.****20 mL** | **Final Vol.****50 mL** | **Final Vol.****100 mL** | **Final Vol.****200 mL** |
| **1x PBS (-)** | 20 mL | 50 mL | 100 mL | 200 mL |
| **DAB** | 10 mg | 25 mg | 50 mg | 100 mg |

**Table 5: 0.3% H2O2**

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **0.3% H2O2** | **Final Vol.****1 mL** | **Final Vol.****2 mL** | **Final Vol.****5 mL** | **Final Vol.****10 mL** |
| **MilliQ H2O** | 1 mL | 2 mL | 5 mL | 10 mL |
| **30% H2O2** | 10 µL | 20 µL | 50 µL | 1. L
 |

* + 1. Referencing **Table 6** below, combine 0.05% DAB and 0.3% H2O2 to prepare DAB working solution; stir to mix.Do not add the H2O2 to working solution until immediately prior to use.

**Table 6: DAB working Solution (RP0168)**

|  |  |  |  |
| --- | --- | --- | --- |
| **DAB Working Solution** | **Final Vol.****20 mL** | **Final Vol.****50 mL** | **Final Vol.****100 mL** |
| **0.05% DAB in PBS** | 20 mL | 50 mL | 100 mL |
| **0.3% H2O2** | 200 µL | 500 µL | 1 mL |

* + 1. **Alternate DAB reagent preparation (RP0266)**
			1. In event of Sigma DAB shortage, Biocare Betazoid DAB Chromogen Kit can substituted.
			2. Prepare Betazoid DAB Working Solution according to Table 7, below, and as detailed in SOP RP0266 Biocare Betazoid DAB Solution
			3. **Table 7: Biocare Betazoid DAB Working Solution Preparation**

|  |  |  |  |
| --- | --- | --- | --- |
| **Reagent** |  **Volume Needed****1 mL** |  **Volume Needed****25 mL** |  **Volume Needed****50 mL** |
| **Betazoid DAB Chromogen** | 32 µL (~1 drop) | 800 µL | 1600 µL |
| **Betazoid DAB Substrate Buffer** | 1 mL | 25 mL | 50 mL |