E2 Embryo Media with Methylene Blue (Revised May 2013 JLM)

At ZIRC 0.5X E2 with 0.5 mg/L Methylene Blue is used as a working solution (Conductivity≈1000-1100 µS; Osmolality≈28 mmol/kg). We prepare three concentrated stock solutions, E2A, E2B and E2C, and 0.1% Methylene Blue which are then used to make a large volume (20L) of the 0.5X E2 working solution. This protocol is a modification (half strength) of the E2 Embryo Medium described in C. Nüsslein-Volhard and R. Dahm (2002) ZEBRAFISH, Oxford University Press, A Practical Approach.

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E2A (100X) contains:

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<tbody>
<tr>
<td>1.5</td>
<td>M</td>
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<tr>
<td>50</td>
<td>mM</td>
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<td>100</td>
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<td>15</td>
<td>mM</td>
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<td>5</td>
<td>mM</td>
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To prepare 2 L of E2A (100X) combine the following according to the instructions below:

- In a 2 L beaker, add a large stir bar and Nanopure dH₂O to 1600 ml
- Add the above dry reagents in the order listed; stir to dissolve each one completely before adding the next reagent
- Add 40 mL E2A Buffer Mix (see recipe below), continue stirring to mix
- Transfer solution to a 2 L graduated cylinder and adjust final volume to 2.0 L with Nanopure dH₂O
- Cover the graduated cylinder with Parafilm and invert 2-3 times to mix completely
- Filter sterilize into two 1 L bottles
- Store in refrigerator at 4°C

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E2A Buffer Mix contains:

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<table>
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<tbody>
<tr>
<td>750</td>
<td>mM</td>
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<tr>
<td>250</td>
<td>mM</td>
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To prepare 1L of E2A Buffer Mix combine the following according to the instructions below:

- In a 1 L beaker, add a large stir bar and Nanopure dH₂O to 750 mL
- Add the above dry reagents in the order listed; stir to dissolve each one completely before adding the next reagent
- Transfer solution to a 1 L graduated cylinder and adjust final volume to 1.0 L with Nanopure dH₂O
- Cover the graduated cylinder with Parafilm and invert 2-3 times to mix completely
- Filter sterilize
- Store in refrigerator at 4°C

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E2B (500X) contains:

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<tbody>
<tr>
<td>500</td>
<td>mM</td>
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To prepare 1 L of E2B (500X) combine the following according to the instructions below:

- In a 1 L beaker, add a large stir bar and Nanopure dH₂O to 800 mL
- Add the above dry reagent and stir to dissolve completely
- Transfer solution to a 1 L graduated cylinder and adjust final volume to 1.0 L with Nanopure dH₂O
- Cover the graduated cylinder with Parafilm and invert 2-3 times to mix completely
- Filter sterilize
- Store in refrigerator at 4°C
E2C (500X) contains: 350 mM NaHCO₃

To prepare 500 mL of E2C (500X) combine the following according to the instructions below:

14.7 g Sodium Bicarbonate (NaHCO₃)
- In a 1L beaker, add a medium stir bar and Nanopure dH₂O to 350 mL.
- Add the above dry reagent and stir to dissolve completely
- Transfer solution to a 500 mL graduated cylinder and adjust final volume to 500 mL with Nanopure dH₂O
- Cover the graduated cylinder with Parafilm and invert 2-3 times to mix completely
- Filter sterilize
- Aliquot into 20 ml portions (in 50 ml Falcon tubes)
- Store in lab freezer at -20°C
- Thaw 20 mL aliquot just prior to use

0.1% Methylene Blue

To prepare 1L of 0.1% Methylene Blue, combine the following according to the instructions below:

1.0 g Methylene Blue
- Add 1.0 L Nanopure dH₂O to a 1 L bottle
- Add the above dry reagent to the bottle and shake to dissolve completely
- Store at room temperature (28°C)

FINAL WORKING SOLUTION: 0.5X E2 with Methylene Blue:

To prepare 20L of 0.5X E2 with Methylene Blue

- Fill 20 L carboy with Reverse Osmosis water to 19 L, aerate until ready to mix
- Add the following to the carboy in the order listed (shake/stir all solutions before measuring)

100 mL 100x E2A
20 mL 500x E2B
20 mL 500x E2C
10 mL 0.1% Methylene Blue

- Adjust final volume to 20 L with Reverse Osmosis water
- Aerate and stir to mix
- Flush carboy spout by flowing out 200-400 mL and pouring back into top of carboy
- Remove a small aliquot (~50 mL) to check conductivity and pH
- Adjust pH to 7.2-7.6 (with concentrated HCl or concentrated NaOH) if necessary
- Store and use at room temperature (28°C), make fresh weekly

E2 Embryo Media Final Working Solution Molar Concentrations:

<table>
<thead>
<tr>
<th>0.5X E2</th>
<th>1.0X E2</th>
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<tbody>
<tr>
<td>7.5 mM NaCl</td>
<td>15 mM NaCl</td>
</tr>
<tr>
<td>0.25 mM KCl</td>
<td>0.5 mM KCl</td>
</tr>
<tr>
<td>0.5 mM MgSO₄</td>
<td>1.0 mM MgSO₄</td>
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<tr>
<td>75 µM KH₂PO₄</td>
<td>150 µM KH₂PO₄</td>
</tr>
<tr>
<td>25 µM Na₂HPO₄</td>
<td>50 µM Na₂HPO₄</td>
</tr>
<tr>
<td>0.5 mM CaCl₂</td>
<td>1.0 mM CaCl₂</td>
</tr>
<tr>
<td>0.35 mM NaHCO₃</td>
<td>0.7 mM NaHCO₃</td>
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<tr>
<td>0.5 mg/L Methylene Blue</td>
<td>0.5 mg/L Methylene Blue</td>
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