Buffer Preparation Protocol

For Vesicular Transport assay and ATPase assay

This protocol describes about buffer preparation for Vesicular Transport assay and ATPase assay.
For your convenience, you do not need complicated reagent preparation by purchasing following products.

- For Vesicular Transport assay
  - Vesicular Transport Assay Reagent Kit for BSEP
    (GenoMembrane, Cat. No. GM3001)
  - Vesicular Transport Assay Reagent Kit for MRPs and BCRP
    (GenoMembrane, Cat. No. GM3010)
  - Vesicular Transport Assay Reagent Kit for MDR1
    (GenoMembrane, Cat. No. GM3030)

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1. Preparation of Vesicular Transport Assay Reagent for BSEP

1.1. Stock solution

Solution	Preparation	Storage
1.7 M Tris	Dissolve 20.594 g of Tris (hydroxymethyl) aminomethane (MW; 121.14) in Ultra pure water to make 100 mL.	4˚C
100 mM Hepes-Tris	Dissolve 2.383 g of HEPES [2-[4-(2-Hydroxyethyl) -1-piperazinyl]ethanesulfonic Acid] (MW; 238.31) in 90 mL of Ultra pure water, and adjust the pH to 7.4 with 1.7 M Tris. Add Ultra pure water to make a final volume of 100 mL.	4˚C
1 M KNO_3	Dissolve 10.11 g of Potassium Nitrate (MW; 101.10) in Ultra pure water to make 100 mL.	4˚C
100 mM $Mg(NO_3)_2$	Dissolve 2.564 g of Magnesium Nitrate Hexahydrate (FW; 256.41) in Ultra pure water to make 100 mL.	4˚C
1 M $MgCl_2$	Dissolve 10.165 g of Magnesium Chloride Hexahydrate (FW; 203.3) in Ultra pure water to make 50 mL.	Room temperature
200 mM MgATP	Dissolve 2.21 g of Na_2ATP (MW; 551.1) in 10 mL of Ultra pure water, and add 4 mL of 1 M $MgCl_2$. After adjustment of the pH of this solution to 7.0 with 1.7 M Tris, finally adjust the volume to 20 mL by adding Ultra pure water.	After subdivision into small portions, store at −20˚C
250 mM Na_2AMP	Dissolve 1.97 g of Na_2AMP (MW; 391.2) in 10 mL of Ultra pure water. After adjustment of the pH of this solution to 7.0 with 1.7 M Tris, finally adjust the volume to 20 mL by adding Ultra pure water.	After subdivision into small portions, store at −20˚C
1 M Sucrose	Dissolve 34.23 g of Sucrose (MW; 342.32) in Ultra pure water to make 100 mL.	4 ˚C

1.2. Buffer A1: Reaction Buffer

Solution	Amount added	Final concentration
100 mM Hepes-Tris	1 mL	10 mM
1 M KNO_3	1 mL	100 mM
100 mM $Mg(NO_3)_2$	1 mL	10 mM
1 M Sucrose	0.5 mL	50 mM
Ultra pure water	Sufficient	
Final volume	**10 mL**	

After subdivision into small portions, store at −20˚C.

1.3. Buffer B1: Stopping Buffer

Solution	Amount added	Final concentration
100 mM Hepes-Tris	100 mL	10 mM
1 M KNO_3	100 mL	100 mM
1 M Sucrose	50 mL	50 mM
Ultra pure water	Sufficient	
Final volume	**1000 mL**	

Store at 4˚C.

1.4. Reagent C1: 10 mM MgATP solution

Dilute 200 mM MgATP with Buffer A1 to make a 10 mM solution.

1.5. Reagent D1: 10 mM MgAMP solution

Mix 80 μL of 250 mM Na_2AMP and 20 μL of 1 M $MgCl_2$ to prepare 200 mM MgAMP (prepare just before use). Dilute 200 mM MgAMP with Buffer A1 to make 10 mM MgAMP solution.

2. Preparation of Vesicular Transport Assay Reagent for MRPs and BCRP

2.1. Stock solution

Solution	Preparation	Storage
1.7 M Tris	Dissolve 20.594 g of Tris (hydroxymethyl) aminomethane (MW; 121.14) in Ultra pure water to make 100 mL.	4˚C
100 mM MOPS-Tris	Dissolve 2.093 g of MOPS 〔3-(N-Morpholino) propanesulfonic Acid〕(MW; 209.26) in 90 mL of Ultra pure water, and adjust the pH to 7.0 with 1.7 M Tris. Add Ultra pure water to make a final volume of 100 mL.	4˚C
1 M KCl	Dissolve 7.455 g of Potassium Chloride (MW; 74.55) in Ultra pure water to make 100 mL.	4˚C
1 M $MgCl_2$	Dissolve 10.165 g of Magnesium Chloride Hexahydrate (FW; 203.3) in Ultra pure water to make 50 mL.	Room temperature
200 mM MgATP	Dissolve 2.21 g of Na_2ATP (MW; 551.1) in 10 mL of Ultra pure water, and add 4 mL of 1 M $MgCl_2$. After adjustment of the pH of this solution to 7.0 with 1.7 M Tris, finally adjust the volume to 20 mL by adding Ultra pure water.	After subdivision into small portions, store at −20˚C
250 mM Na_2AMP	Dissolve 1.97 g of Na_2AMP (MW; 391.2) in 10 mL of Ultra pure water. After adjustment of the pH of this solution to 7.0 with 1.7 M Tris, finally adjust the volume to 20 mL by adding Ultra pure water.	After subdivision into small portions, store at −20˚C

2.2. Buffer A2: Reaction Buffer

Solution	Amount added	Final concentration
100 mM MOPS-Tris	5 mL	50 mM
1 M KCl	0.7 mL	70 mM
1 M $MgCl_2$	75 µL	7.5 mM
Ultra pure water	Sufficient	
Final volume	**10 mL**	

After subdivision into small portions, store at −20˚C.

2.3. Buffer B2: Stopping Buffer

Solution	Amount added	Final concentration
100 mM MOPS-Tris	400 mL	40 mM
1 M KCl	70 mL	70 mM
Ultra pure water	Sufficient	
Final volume	**1000 mL**	

Store at 4˚C.

2.4. Reagent C2: 10 mM MgATP solution

Dilute 200 mM MgATP with Buffer A2 to make a 10 mM solution.

2.5. Reagent D2: 10 mM MgAMP solution

Mix 80 µL of 250 mM Na_2AMP and 20 µL of 1 M $MgCl_2$ to prepare 200 mM MgAMP (prepare just before use). Dilute 200 mM MgAMP with Buffer A2 to make 10 mM MgAMP solution.

2.6. Reagent G: 200 mM Glutathione solution

Dissolve 0.615 g of glutathione (MW; 307.32) in 8 mL of Ultra pure water, then adjust to pH6.8 with NaOH and add Ultra pure water to make a final volume of 10 mL. After subdivision into small portions, store at −20˚C.

3. Preparation of Vesicular Transport Assay Reagent for MDR1

3.1. Stock solution

Solution	Preparation	Storage
1.7 M Tris	Dissolve 20.594 g of Tris (hydroxymethyl) aminomethane (MW; 121.14) in Ultra pure water to make 100 mL.	4˚C
100 mM MOPS-Tris	Dissolve 2.093 g of MOPS 〔3-(N-Morpholino) propanesulfonic Acid〕(MW; 209.26) in 90 mL of Ultra pure water, and adjust the pH to 7.0 with 1.7 M Tris. Add Ultra pure water to make a final volume of 100 mL.	4˚C
1 M KCl	Dissolve 7.455 g of Potassium Chloride (MW; 74.55) in Ultra pure water to make 100 mL.	4˚C
1 M $MgCl_2$	Dissolve 10.165 g of Magnesium Chloride Hexahydrate (FW; 203.3) in Ultra pure water to make 50 mL.	Room temperature
200 mM MgATP	Dissolve 2.21 g of Na_2ATP (MW; 551.1) in 10 mL of Ultra pure water, and add 4 mL of 1 M $MgCl_2$. After adjustment of the pH of this solution to 7.0 with 1.7 M Tris, finally adjust the volume to 20 mL by adding Ultra pure water.	After subdivision into small portions, store at −20˚C
250 mM Na_2AMP	Dissolve 1.97 g of Na_2AMP (MW; 391.2) in 10 mL of Ultra pure water. After adjustment of the pH of this solution to 7.0 with 1.7 M Tris, finally adjust the volume to 20 mL by adding Ultra pure water.	After subdivision into small portions, store at −20˚C

3.2. Buffer A2: Reaction Buffer

Solution	Amount added	Final concentration
100 mM MOPS-Tris	5 mL	50 mM
1 M KCl	0.7 mL	70 mM
1 M $MgCl_2$	75 µL	7.5 mM
Ultra pure water	Sufficient	
Final volume	**10 mL**	

After subdivision into small portions, store at −20˚C.

3.3. Buffer B2: Stopping Buffer

Solution	Amount added	Final concentration
100 mM MOPS-Tris	400 mL	40 mM
1 M KCl	70 mL	70 mM
Ultra pure water	Sufficient	
Final volume	**1000 mL**	

Store at 4˚C.

3.4. Reagent C2: 10 mM MgATP solution

Dilute 200 mM MgATP with Buffer A2 to make a 10 mM solution.

3.5. Reagent D2: 10 mM MgAMP solution

Mix 80 µL of 250 mM Na_2AMP and 20 µL of 1 M $MgCl_2$ to prepare 200 mM MgAMP (prepare just before use). Dilute 200 mM MgAMP with Buffer A2 to make 10 mM MgAMP solution.

3.6. Reagent E: 10% SDS solution

Dissolve 10 g of SDS in Ultra pure water to make 100 mL. Store at room temperature.

3.7. Reagent F: 0.1 N H_2SO_4

Store at room temperature.

4. Preparation of ATPase assay Reagent

4.1. Stock solution

Solution	Preparation	Storage
1.7 M Tris	Dissolve 20.594 g of Tris (hydroxymethyl) aminomethane (MW; 121.14) in Ultra pure water to make 100 mL.	4 ℃
100 mM MOPS-Tris	Dissolve 2.093 g of MOPS 〔3-(N-Morpholino) propanesulfonic Acid〕(MW; 209.26) in 90 mL of Ultra pure water, and adjust the pH to 7.0 with 1.7 M Tris. Add Ultra pure water to make a final volume of 100 mL.	4 ℃
1 M KCl	Dissolve 7.455 g of Potassium Chloride (MW; 74.55) in Ultra pure water to make 100 mL.	4 ℃
2 M NaN_3	Dissolve 13.0 g of Sodium Azide (MW; 65.01) in Ultra pure water to make 100 mL.	After subdivision into small portions, store at −20˚C
1 M DTT	Dissolve 3.085 g of Dithiothreitol (MW; 154.25) in Ultra pure water to make 20 mL.	After subdivision into small portions, store at −20˚C
1 M NaH_2PO_4	Dissolve 1.56 g of Sodium Dihydrogen Phosphate Dihydrate (FW; 156.01) in Ultra pure water to make 10 mL.	After subdivision into small portions, store at −20˚C
400 mM $MgCl_2$	Dissolve 4.066 g of Magnesium Chloride Hexahydrate (FW; 203.3) in Ultra pure water to make 50 mL.	Room temperature

4.2. Reaction Buffer

Solution	Amount	Final concentration
100 mM MOPS-Tris	25 mL	50 mM
100 mM EGTA	0.050 mL	0.1 mM
1 M KCl	2.5 mL	50 mM
2 M Sodium azide	0.125 mL	5 mM
1 M DTT	0.1 mL	2 mM
Ouabain	36.4 mg	1 mM
Ultra pure water	Sufficient	
Final volume	**50 mL**	

After subdivision into small portions, store at −20˚C.

4.3. 100 mM Orthovanadate Solution

1) Dissolve 0.3678 g of Sodium Orthovanadate (MW; 183.91) in Ultra pure water to make 16 mL (120 mM Sodium Orthovanadate).
2) Adjust to pH 10 with 1N HCl.
3) Boil at 100˚C for 10 min.
4) Allow to stand to reach room temperature.
5) Adjust to pH 10 with 1N HCl if pH >10, or with 1N NaOH if pH <10.
6) Dilute with Ultra pure water to 20 mL.
7) After subdivision into small portions, store at −20˚C.

4.4. 200 mM MgATP solution

1) Dissolve 2.21 g of Na_2ATP in 10 mL of 400 mM $MgCl_2$, adjust with 1.7 M Tris to pH 7.0, and then add Ultra pure water to make 20 mL.
2) After subdivision into small portions, store at −20˚C.

4.5. 10 mM Phosphate Standard solution

1) Dilute 20 µL of 1 M NaH_2PO_4 with 1,980 µL of Ultra pure water to make 10 mM NaH_2PO_4.
2) After subdivision into small portions, store at −20˚C.

4.6. Stop solution : 10% (w/v) SDS (Sodium Lauryl Sulfate, Sodium Dodecyl Sulfate) solution

1) Dissolve 10 g of SDS in Ultra pure water to make 100 mL.
2) Store at room temperature.

◆ 10% LDS (Lithium Dodecyl Sulfate) also can be used as Stop solution

4.7. Coloring Solution

1) Prepare the following solutions

Solution	Preparation	Storage
10% Ascorbic acid (pH 5.0)	Dissolve 2 g of L(+)-Ascorbic acid (MW: 176.13) in 15 mL of Ultra pure water, adjust with 10 N NaOH (ca. 1 mL) to pH 5.0, then add Ultra pure water to make 20 mL.	Prepare in each experiment
15 mM Zinc acetate	Dissolve 0.165 g of Zinc Acetate Dihydrate (FW: 219.51) in 50 mL of Ultra pure water.	−20 ℃
35 mM Ammonium molybdate−15 mM zinc acetate	Dissolve 0.865 g of Hexaammonium Heptamolybdate Tetrahydrate (FW: 1236) in 20 mL of 15 mM zinc acetate.	Prepare in each experiment

2) Mix 20 mL of 10% Ascorbic acid with 5 mL of 35 mM ammonium molybdate−15 mM zinc acetate.
3) Add 200 µL of mixed solution to all wells at the step 5.15 of ATPase assay protocol.

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