EMSER 70-03/ 4842

STUDY PROTOCOL

ACCELERATED BIODEGRADATION OF 8-2 TELOMER B ALCOHOL

SPONSOR	E. I. du Pont de Nemours and Company DuPont Chemical Solutions Enterprise Wilmington, DE 19898, USA
TESTING FACILITIES:	E. I. du Pont de Nemours and Company Environmental and Microbiological Sciences & Engineering Corporate Center for Engineering Research Central Research & Development Glasgow, Building 300, P.O. Box 6101 Newark, DE 19714-6101 USA AND E. I. du Pont de Nemours and Company Haskell Laboratory for Health and Environmental Sciences Newark, DE 19714 USA
STUDY DIRECTOR:	Ning Wang, Ph.D.
PROPOSED STARTING DATE:	30-June-2003
PROPOSED COMPLETION DATE:	September-2003
STUDY NUMBER:	EMSER 70-03 / 4842

STUDY PROTOCOL APPROVAL

Study Director: _____ _____
Ning Wang, Ph.D. Date
DuPont Central Research & Development

Research Manager: _____ _____
John T. Gannon, Ph.D. Date
DuPont Central Research & Development

Analytical Director: _____ _____
S. Mark Kennedy Date
DuPont Haskell Laboratory for Health
and Environmental Sciences

1.0 SUMMARY

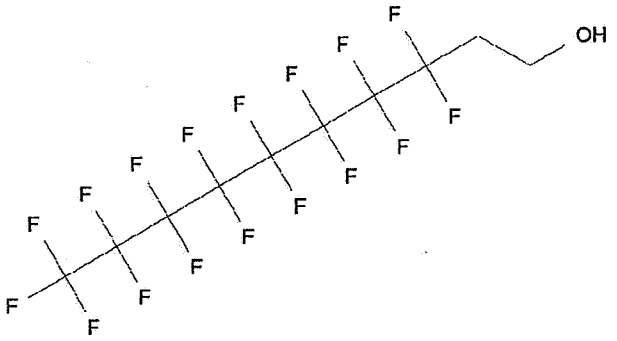
The biodegradability of the test substance will be determined with activated sludge or sludge culture originated from POTW or industrial waste treatment facilities. The solution of the 8-2 telomer B alcohol (8-2 TBA) in E2-BSMYE cell growth medium is inoculated with sludge or sludge culture and kept in closed bottles in the dark at room temperature. Degradation is followed by analysis of the concentration of the test chemical and its possible metabolites (biotransformation products) during the test (28-90 days). The extent of biodegradation of the test chemical can be expressed as the percentage of the test chemical that has been converted and the accumulation of its major metabolites, including Fluoride.

2.0 OBJECTIVES

1. Determine the biodegradation potential of 8-2 Telomer B Alcohol.
2. Identify possible degradation products.
3. Determine the degree of defluorination.

3.0 TEST SUBSTANCE

Name:	8-2 Telomer B Alcohol
Synonym:	(Perfluorooctyl)ethanol, 8-2 TBA
Active substance(s)	8-2 Telomer B Alcohol, + 99%
CAS Name:	1-Decanol, 3,3,4,4,5,5,6,6,7,7,8,8,9,9,10,10,10-heptadecafluoro-
CAS Number(s):	678-39-7
Structure:	



H Number	H-24691
Lot Number:	P.00/001

EMSE Sample Number:	E93386-80
Concentration of a.s., nominal:	99%
Concentration of a.s., analyzed:	99.2%
Certificate of Analysis Date:	13-Sept-2001
Expiration Date:	Unknown
Date Received:	26-Mar-2002
Solubility at 25°C.:	~140 $\mu g\ L^{-1}$
Vapor pressure:	0.023 mm Hg
Stability:	Stable at ambient room temperature
Appearance/Color:	White solid
Storage Conditions:	Room temperature; keep tightly closed
Safety Precautions:	Wear lab coat, protective gloves, and safety glasses
Supplier:	Clariant Co.

4.0 TEST SYSTEM

4.1 Test System Justification

The test system incorporated published EPA guideline OPPTS 835.3120 and OECD guideline 301D & 302D.

4.2 Description of Test System

The test system consists of individually capped test vessels made of material that does not significantly sorb the test substance and the sludge culture. All experiments will be conducted in triplicate (unless otherwise noted) in the test vessels at room temperature. A calibrated temperature chart-recorder or thermometer will be placed in proximity to the samples to monitor the temperature. All test samples will be uniquely identified and documented in the study records.

4.3 Sources of Inoculum

1. The inoculum used in this study will come either from Wilmington (DE) municipal waste treatment plant or an industrial waste treatment facility or other appropriate sources.

5.0 ANALYTICIAL REFERENCE STANDARDS, REAGENTS, AND SOLVENTS

Analytical reference standards that are used to aid the identification of the test substance and its potential degradation products will be documented in the study records. All chemicals and solvents will be reagent grade or purer. Sources will be documented in the

study records. Deionized water produced at the testing facility will be used throughout in the study.

6.0 APPARATUS AND BOTTLES AND CONTAINERS

6.1 Normal laboratory apparatus and:

(a) Biological safety cabinet or equivalent

(b) Water bath, incubator or equivalent, for keeping test vessels or bottles at constant temperature with the exclusion of light.

6.2 Bottles or Containers

(a) Test vessels: 50-300 ml with stoppers or caps

(b) 0.001 - 8 L Bottles for general purpose such as medium and sample preparations, centrifugation etc.

7.0 PREPARATION OF STOCK SOLUTIONS OF ORGANIC NUTRIENTS

7.1 If needed, dissolve in water of 20-400g/L of an organic nutrient (e.g. yeast extract or glucose) for microorganism growth. Sterilize the medium and store it at room temperature or refrigerated.

8.0 PREPARATION OF E2-BSMYE GROWTH MEDIUM

8.1 E2 basal salts medium + yeast extract (E2-BSMYE)

8.1.1 Mix 5 ml of 200 x P (phosphate) salts stock (pH to 7.2), 100 ml of Non-P salts stock solution, 5 ml of E2 trace elements, and 100 ml of 20 % w/v $(NH_4)_2SO_4$ stock solution with 500 mL of deionized water. (See Attachment 2-5 for stock solution preparations.)

8.1.2 Adjust pH to 6.5~7.5 with NaOH or HCl, add 2.5 - 100 mL of organic nutrient stock solution or ethanol and bring the volume to 1.0 L with water.

8.1.3 Filter-sterilize the solution using a 0.2 µm Nalgene filter unit or equivalent.

8.1.4 Store at room temperature.

9.0 PREPARATION OF 8-2 TELOMER B ALCOHOL SOLUTION

9.1 If needed, dissolve appropriate amount of 8-2 TBA in a suitable solvent (Ethanol or methanol. Store the stock solution properly.

10.0 PREPARATION OF SODIUM CYANIDE STOCK SOLUTION

10.1 If necessary, weigh appropriate amount of NaCN in a container and add appropriate volume of sterile water to a final concentration of 50 – 500 mM. Store the NaCN stock solution at room temperature or refrigerated.

11.0 INOCULUM PREPARATION

11.1 Collect a fresh sample of activated sludge from a POWT or from other microbial sources. If necessary, settle the sludge for 5- 30 min and collect upper aqueous phase of the sludge as the inoculum. Remove coarse particles if necessary by filtration with Whatman #1 filter paper and keep the sludge aerobic thereafter. The sludge or sludge filtrate will be used as an inoculum for sludge culture preparation.

11.2 If sludge has to be taken from a high rate treatment plant, or is thought to contain inhibitors, it should be washed with E2-BSMYE medium. Settle or centrifuge the re-suspended sludge after thorough mixing, discard the supernatant and again re-suspend the washed sludge in a further volume of E2-BSMYE medium. Repeat this procedure until the sludge is considered to be free from excess substrate or inhibitor. The cleaned-up sludge will be used as an inoculum for sludge culture preparation.

12.0 PREPARATION OF SLUDGE CULTURE

12.1 Add 0.050-5 mL of sludge or sludge filtrate (inoculum) to 50-500 mL volume of sterilized test vessels that contained 4-100 mL of E2-BSMYE growth medium. Add 8-2 TBA to a final concentration of 0.3-50 mg/L if necessary (Solvent concentration < 0.1% in test vessels).

12.2 Incubate the vessels at room temperature or controlled temperature (20-30 °C) for 1 – 15 days.

12.3 If needed, transfer 0.050-5 mL of cell culture from step *12.2* to sterilized test vessels that contained 4-100 mL of E2-BSMYE growth medium. Add test chemical to a final concentration of 0.3-50 mg/L if necessary.

12.4 Repeat steps *12.2* and *12.3* as needed to acclimate the culture to 8-2 TBA.

12.5 Centrifuge the sludge culture at 2000-20000g for 5-15 min and decant the supernatant. Resuspend the pellet with E2-BSMYE growth medium.

12.6 Repeat the step *12.5* 2-3 times.

12.7 Read Optical O.D. (600 nm) of the sludge or sludge culture.

13.0 PROCEDURES FOR DIFFERENT EXPERIMENTAL TREATMENTS

Note: Add appropriate amount of different solutions according to different experimental treatments. The following table is provided as an example for filling 120 mL serum bottles with 40ml of different test medium. The actual medium volume per bottle and bottle size may be varied and will be recorded in the study records.

Treatment	n**	Solutions to be added (mL)						
		14C-Aniline. HCl (0.5-2g/L)	E2-BSMYE medium	3 g 8-2 TBA /l (in ethanol)	Sludge or culture	Autoclaved sludge or culture	0.2 M NaCN in water	Spike with 3 g/L of 8-2 TBA (in ethanol)
#1	4		40	0.002 - 0.012	0.05 - 0.5	0	0	0
#2	4		40	0.002 - 0.012	0	0.05 - 0.5	0.1	0
#3	3		40	0	0.05 - 0.5	0	0	0.002 - 0.012
#4	2		40	0	0.05 - 0.5	0	0	0
#5	2	0.02 - 0.06	40	0	0.05 - 0.5	0	0	0

Note: The glass serum bottles used in different treatments are not coated. The volume calculation is on a per bottle (40 mL) basis. The volume per bottle of the test medium can be varied to suit the study conditions.
** Number of replicates for each sampling time point.

13.1 Treatment 1 – Biodegradation Test Vessels

13.1a Prepare enough solution to fill a total of 16 – 24 bottles (4 replicates × 4 - 6 sampling time points) or appropriate number of bottles. For example, to make 1 L of the treatment solution, mix 1.25 – 12.5 mL of the sludge or sludge culture and 987.5 - 998.8 mL of E2-BSMYE medium. Make appropriate adjustments when making less than one liter or more than one liter of the treatment solution.

13.1b Transfer 40 mL of the mixed solution to each of the individual serum bottles. Add 2 – 12 uL of 3g/L of 8-2 TBA stock solution to each of the serum bottles. Seal the bottles with pre-washed (with methanol and sterile water) septa or butyl rubber stoppers, or equivalent. If needed, cover the mouth of each of the serum bottles with a piece of pre-washed (with

methanol and sterile water) aluminum foil before sealing the bottles with septa or stoppers.

13.2 Treatment 2 – Abiotic Control

13.2a Prepare enough solution to fill a total of 16 – 24 bottles (4 replicates × 4 - 6 sampling time points) or appropriate number of bottles. For example, to make 1 L of the treatment solution, mix 1.25 – 12.5 mL of autoclaved sludge or sludge culture, 2.5 mL of 0.2 M NaCN stock solution, and 985 - 996.3 mL of E2-BSMYE medium. Make appropriate adjustments when making less than one liter or more than one liter of the treatment solution.

13.2b Transfer 40 mL of the mixed solution to each of the individual serum bottles. Add 2 – 12 uL of 3g/L of 8-2 TBA stock solution to each of the serum bottles. Seal the bottles with pre-washed (with methanol and sterile water) septa or butyl rubber stoppers, or equivalent. If needed, cover the mouth of each of the serum bottles with a piece of pre-washed (with methanol and sterile water) aluminum foil before sealing the bottles with septa or stoppers.

13.3 Treatment 3- Test Substance Spike Recovery Control

13.3a Prepare enough solution to fill a total of 12 – 18 bottles (3 replicates × 4 - 6 sampling time points) or appropriate number of bottles. For example, to make 1 L of the treatment solution, mix 1.25 – 12.5 mL of the sludge or sludge culture and 987.5 - 998.8 mL of E2-BSMYE medium. Make appropriate adjustments when making less than one liter or more than one liter of the treatment solution.

13.3b Transfer 40 mL of the mixed solution to each of the individual serum bottles. Seal the bottles with pre-washed (with methanol and sterile water) septa or butyl rubber stoppers, or equivalent. If needed, cover the mouth of each of the serum bottles with a piece of pre-washed (with methanol and sterile water) aluminum foil before sealing the bottles with septa or stoppers.

The bottles will be spiked with 8-2 TBA stock solution 3g/L in ethanol) at each sampling time point for 8-2 TBA spike recovery analysis, after 10-15 mL of medium is withdrawn from the bottles to other containers.. If necessary, the 10-15 mL of sample medium will be spiked with standard fluorinated acids stock solution at each sampling time point for fluorinated acids spike recovery analysis.

13.4 Treatment 4 Intrinsic (Growth Medium Matrix) Control

13.4a Prepare enough solution to fill a total of 8 – 12 bottles (2 replicates × 4 - 6 sampling time points) or appropriate number of bottles. For example, to make 1 L of the treatment solution, mix 1.25 – 12.5 mL of the sludge or sludge culture and 987.5 - 998.8 mL of E2-BSMYE medium. Make appropriate adjustments when making less than one liter or more than one liter of the treatment solution.

13.4b Transfer 40 mL of the mixed solution to each of the individual serum bottles. Seal the bottles with pre-washed (with methanol and sterile water) septa or butyl rubber stoppers, or equivalent. If needed, cover the mouth of each of the serum bottles with a piece of pre-washed (with methanol and sterile water) aluminum foil before sealing the bottles with septa or stoppers.

13.5 Treatment # 5- Reference Compound Biodegradation Control (Positive Control)

13.5a Prepare enough solution to fill a total of 8 – 12 bottles (2 replicates × 4 - 6 sampling time points) or appropriate number of bottles. For example, to make 1 L of the treatment solution, mix 1.25 – 12.5 mL of the sludge or sludge culture, 987.5 - 998.8 mL of E2-BSMYE medium, and 1.0 mL of ^{14}C-aniline.HCl stock solution. Make appropriate adjustments when making less than one liter or more than one liter of the treatment solution.

13.5b Transfer 40 mL of the mixed solution to each of the individual serum bottles. Seal the bottles with pre-washed (with methanol and sterile water) septa or butyl rubber stoppers, or equivalent.

13.6 At day zero, withdraw approximately 5 mL aliquot (or the appropriate volume) of test medium from each of the four serum bottles for ***Treatments 1 & 2*** and from the two bottles for ***Treatment 4*** into appropriate containers for fluoride analysis. If necessary, freeze the sample containers and sample bottles until extraction and analysis. Also withdraw 5-10 mL aliquot of the test medium from each of the 4 serum bottles for ***Treatments 1 & 2*** and from the two bottles for ***Treatment 4*** into appropriate containers for extraction of biotransformation products (fluorinated acids) with acetonitrile. If necessary, freeze the 5-10 mL sample medium until extraction and analysis. After the medium withdrawing, Inject MTBE-H_2SO_4 solution into each of the sample bottles for extraction of 8-2 TBA.

For ***Treatment 3***, withdraw 10-15 mL (or the appropriate volume) of the test medium from the three or four serum bottles into appropriate containers and spike the 10-15 mL solution with standard fluorinated acids and incubate for 10-120 min; followed by adding acetonitrile for extraction of fluorinated acids. Spike the rest of 25-30 mL of medium left in the serum bottles with 2 – 12 µL (or appropriate volume) of 8-2 TBA stock solution (3g/L in ethanol) and incubate for 10-120 min; Inject MTBE-H_2SO_4 solution into each of the sample bottles for extraction of 8-2 TBA.

For ***Treatment 5***, withdraw approximately 0.01 – 0.5 mL (or appropriate volume) of the ^{14}C-aniline.HCl medium from the two serum bottles into each of the scintillation vials. Alternatively, withdrawn 0.5 – 2 mL of the air with a gas-tight syringe from inside the sealed serum bottles and inject the air slowly into 1-5 mL of 0.5-1.5 Normality of NaOH or $Ba(OH)_2$ in scintillation vials (or other container) for ^{14}C radioactivity measurement. Freeze the vials, if necessary, until analysis of ^{14}C radioactivity. Freeze the serum bottles after sampling, if necessary.

13.7 Incubate the rest of sealed serum bottles at room temperature in an incubator/shaker or equivalent with shaking (approximately 50-300 RPM).

13.8 Periodically (approximately at day 7, day 14, and day 28, day 56, and day 90 sampling time points), repeat the ***Step 13.6*** and ***Step 13.7***, as needed.

13.9 Inject organic nutrient stock solution or sludge or sludge culture to each of the sealed test vessels containing 40 mL of test solution at ~ day 30 and day 60, if the test lasts beyond 28 days.

14.0 ANALYTICAL METHODS

GC/MS and LC/MS/MS analysis will be conducted in Haskell laboratory. The samples will be submitted for analysis only when the test vessels (**Treatment 1**) showed significant defluorination and at least 50% of ^{14}C-aniline.HCl was mineralized in reference (positive) control vessels (**Treatment 5**) within 28 days of the test period. The experiment will be terminated and repeated if above two criteria are not met.

Total samples to be submitted for GC/MS and LC/MS/MS analysis: 1) GC/MS quantification of 8-2 TBA: up to 78 samples (13 samples at each sampling time point – Days 0, 7, 14, 28, 56, and 90); 2) LC/MS/MS analysis of fluorinated acids such as 8-2 acid, 8-2 unsaturated acid, PFOA, and PFHA: up to 78 samples (13 samples at each sampling time point – Days 0, 7, 14, 28, 56, and 90).

14.1 Fluoride (See Attachment 1)

For ***Treatment 1 & 2*** and/or for other ***Treatments*** as appropriate, NaOH (or equivalent) will be added to the sample medium withdrawn from the serum bottles at each sampling time point (approximately at days 0, day7, day 14, day 28, day 56, and day 90). After incubation at room temperature for 0.2 – 4 h, the solution will be neutralized with H_2SO_4 (or equivalent). If necessary, centrifuge the sample solution after the neutralization. The neutralized solution will be used for F^- ion analysis by an appropriate analytical method or can be frozen until the analysis. The analytical method used will be documented in the study records and the final report.

14.2 ***Biotransformation products (Fluorinated acids)***

Appropriate volume of test medium (5-15 mL) withdrawn from the test vessels at each sampling time point will be extracted with 10-40 mL of acetonitrile for 1- 6 h. The clear upper phase after the extraction will be filtered with a nylon filter and subject to LC/MS/MS identification and quantification.

14.3 ***8-2 TBA***

For ***Treatment 1 - 4***, 25-50 mL of MTBE-H_2SO_4 (50 mM final in MTBE) will be injected into each of the sample bottles after the medium withdrawing (for Fluoride and fluorinated acid analysis) at each sampling time point (approximately at days 0, day7, day 14, day28, day 56, and day 90). After 1-6 h incubation, the settled upper MTBE phase will be transferred to 50-mL polypropylene tubes and centrifuged at ~2000 rpm for 5-20 min in a clinical centrifuge. After centrifugation, the clean upper MTBE phase will be used for GC/MS quantification of 8-2 TBA.

14.4 ***^{14}C-aniline.HCl***

For ***Treatment 5***, concentrated HCl (or equivalent) will be added to the sample medium (0.01 – 0.5 mL) in scintillation vials from ***Step 13.6***. The final concentration of HCl in the vials is approximately 10-150 mM. The vials are incubated for 0.1- 6 h at room temperature before adding scintillation cocktail for ^{14}C radioactivity measurement with a scintillation counter.

15.0 DATA ANALYSIS

15.1 ***Time course of the concentration changes of parent compound 8-2 BA.***

15.2 ***Time course of the accumulation of the major stable metabolites.***

15.3 Mass Balance (M_b) Calculation (theoretical M_b equals 1.0)

$$M_b = [\Sigma(D_1 + D_2 + \cdots + D_n)]/P_c$$

D_1 – Concentration of degradation product 1; D_2 – concentration of degradation product 2; D_n – concentration of degradation product n.

P_c - Concentration of parent compound.

15.4 Time course of the accumulation of the F^- ion.

15.5 Tabulate all the stable metabolites identified during the course of the experiment.

16.0 STATISTICAL METHODS AND CONTROL OF BIAS

Statistical methods including means, standard deviations, and regression lines will be used as appropriate. Bias will be effectively controlled through duplicate sampling, replicate analysis, sample spiking, and maintenance of material balance.

17.0 REPORTING OF DATA

Interim reports may be written and submitted to the study sponsor during the various phases of the preliminary and definitive studies. A draft of the final report will be submitted to the sponsor approximately one month after the conclusion of all of the definitive studies. The final report will include, but not be limited to:

a. Study information containing the identification of testing facility, location of the raw data, study dates, and name of the study director.

b. Names of principal study personnel and the management.

c. Information on the test substance and information on analytical standards. Method of preparation of dosing solution, the amount and identity of any cosolvents or extractants used, and the amount of test substance used.

d. Description of experimental design and experimental procedures and any deviations from the procedures stated in the protocol.

e. Description of analytical methods and recoveries.

f. Results and discussion to include, but not be limited to:

   1) Time course of the concentration changes of parent compound 8-2 BA.

   2) Time course of the accumulation of the major stable metabolites.

   3) Mass balance (M_b) calculation of stable metabolites versus parent compound.

   4) Time course of the accumulation of the F^- ion.

   5) A list of all stable metabolites identified during the course of the test.

   6) Possible biodegradation pathways of 8-2 Telomer B Alcohol.

18.0 STUDY RECORD MAINTENANCE AND RETENTION

All original paper data generated during the study, the original signed final report and all original facility-specific raw data will be retained by in the DuPont CR&D Environmental & Microbiological Sciences and Engineering Laboratory (EMSE) archive, excluding the original analytical data and related original facility-specific raw data regarding the analysis of 8-2 TBA and possible biotransformation products conducted by participating analytical facility. The original analytical data for 8-2 TBA and metabolite analysis and all original facility-specific raw data regarding the 8-2 TBA quantification and biotransformation products analysis by the participating analytical facility will be retained in DuPont Haskell laboratory for Health and Environmental Sciences.

Alternatively, the original data may be retained by Iron Mountain Records Management, Wilmington, DE 19802, U.S.A. At least one copy of the signed final report will be provided to the sponsor.

19.0 GLP COMPLIANCE

This study will be Non-GLP.

20.0 REFERENCES

1. Organization for Economic and Cooperative Development (OECD) Guidelines for Testing of Chemicals; Section 3: Degradation and Accumulation; Ready Biodegradability: 301 A Doc Die-Away Test; 301 D Closed Bottle Test (1993).
2. Organization for Economic and Cooperative Development (OECD) Guidelines for Testing of Chemicals; Section 3: Degradation and Accumulation; Proposed 302D Inherent Biodegradability - Concawe Test (October, 2001)
3. U.S. Environmental Protection Agency, Office of Prevention, Pesticides, and Toxic Substances (OPPTS), Fate, Transport and Transformation Test Guidelines, OPPTS 835.3120, Sealed Vessel CO2 Production Test. EPA 712-C-98-311, January 1998.
4. Ning Wang, DuPont EMSE Report No. 15-03, Accelerated Biodegradation of 8-2 Telomer B Alcohol – A Preliminary Screening Study (2003).
5. Ning Wang, DuPont EMSER 14-03 study protocol: Aerobic Ready Biodegradation of ^{14}C-labeled 8-2 Telomer B alcohol (2003).

ATTACHMENT 1

F^- Ion Extraction

1. Withdraw 5 mL from the sealed serum bottles at each sampling time points into individual 15-mL polypropylene tubes that already contained 50 µL of 5N sodium hydroxide; shake the bottles at about 300 rpm for 0.2-4 h.

2. Add 41.7 µL of concentrated (6 N) sulfuric acid to the 15-mL tubes and mix well and store the samples refrigerated for several days or in a freezer for further treatments in **Step 3**.

3. If necessary, centrifuge the sample solution from **Step 2** after thawing the samples and transfer 4-5 mL supernatant into 50-mL polypropylene tubes and add 4-5mL of TISAB II® (Thermo Orion; cat#: 940909) solution.

4. Measure F^- ion concentration with F^- selective electrode.

Fluoride Ion Analysis with Ion-Selective Electrode

1. Turn on the Thermo Orion 710A plus pH/ISE meter (Serial #_____) at least 15 min before the measurement.
2. Flush out old reference electrode filling solution and fill the reference chamber of the F- ion selective electrode (Thermo Orion cat#: 9609BN; Lot # _____; Received _____) with the fresh solution (Thermo Orion Cat# 90061; Lot #_____; Received_____).
3. Insert the F- ion selective electrode into each sample solution to be measured or into F- standard solution made in ½ strength TISAB II® buffer.
4. Periodically, gently shake the measurement solution.
5. Record the conductivity in mV over time: _____ min after the incubation (usually 3-7 min).
6. Rinse the electrode with deionized water (mega Ω-cm = 17-18) and wipe the electrode tip to absorb the water with soft tissue between each measurement.

ATTACHMENT 2

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E2 Basal Media P-Salts Stock Solution

P-Salts	1x Stock	200x Stock
KH_2PO_4	0.70 g/l	140 g/l
$NaH_2PO_4*H_2O$	0.395g/l	79g/l

Make a 100x stock of phosphate salts (P-Salts) and adjust to pH 7.2 with NaOH
Record Lot numbers of chemicals and weights used in making solution.
Label as P-Salts 100x Stock with Lot # and store at room temp. non sterile.

KH_2PO_4 Potassium Phosphate Monobasic Crystals
FW 136.09 / CAS # 7778-77-0

$NaH_2PO_4*H_2O$ Sodium Phosphate Monobasic Monohydrate Crystals
FW 137.99 / CAS # 10049-21-5

Additional Comments / Notes : _____

_____

_____

_____

Witnessed by : _____ **Date** : _____

ATTACHMENT 3

CCER / EMSE **Notebook: E100633-AC** **Page:** _____

E2 Basal Media Non P-Salts Stock Solution

Non P-Salts	1x Stock	10x Stock
KCl	0.5 g/l	5.0 g/l
$MgSO_4*7H_2O$	0.5 g/l	5.0 g/l
$CaCL_2*2H_2O$	0.025 g/l	0.25 g/l
NaCl	1.0 g/l	10.0 g/l

Make a 10x stock of non-phosphate salts (Non P-Salts) with no pH adjustment.
Record Lot numbers of chemicals and weights used in making solution.
Label as Non P-Salts 10x Stock with Lot # and store at room temp.
Store as non-sterile stock.

KCL	Potassium Chloride Crystals FW 74.56 / CAS # 7447-40-7
$MgSO_4*7H_2O$	Magnesium Sulfate 7-Hydrate Crystals FW 246.48 / CAS # 10034-99-8
$CaCl_2*2H_2O$	Calcium Chloride Dihydrate Crystals FW 147.02 / CAS # 10035-04-8
NaCl	Sodium Chloride FW 58.45 / CAS # 7647-14-5

Additional Comments / Notes : _____

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_____

Witnessed by : _____ **Date** :

ATTACHMENT 4

CCER / EMSE **Notebook: E100633-AC** **Page:** _____

E2 Basal Media Trace Element Stock Solution (200x)

Non P-Salts	gms / 100 mls
$FeSO_4*7H_2O$	1.0
$CuSO_4*5H_2O$	0.1
$CoCl_2*6H_2O$	0.2
$MnCl_2*4H_2O$	0.02
$Na_2MoO_2*2H_2O$	0.05
$C_6H_5Na_3O_7*2H_20$	2.0
$NiCl_2*6H_2O$	0.2
$ZnCl_2$	0.01

Dissolve sodium citrate first and adjust to pH 5.0 with HCl, then dissolve metals.
Label as Trace Element 200x Stock with Lot # and store at room temp.
Store as non-sterile stock.

$FeSO_4*7H_2O$	Ferrous Sulfate Heptahydrate CAS #7782-63-0
$CuSO_4*5H_2O$	Cupric Sulfate Pentahydrate CAS # 7758-99-8
$CoCl_2*6H_2O$	Cobalt Chloride Hexahydrate CAS # 7791-13-1
$MnCl_2*4H_2O$	Manganese Chloride Tetrahydrate CAS # 13446-34-9
$Na_2MoO_2*2H_2O$	Sodium Molybdate Dihydrate CAS # 10102-40-6
$C_6H_5Na_3O_7*2H_20$	Sodium Citrate Dihydrate Tribasic CAS # 6132-04-03
$NiCl_2*6H_2O$	Nickel Chloride Hexahydrate CAS # 7791-20-0
$ZnCl_2$	Zinc Chloride CAS # 7646-85-7

Additional Comments / Notes : _____

_____

Witnessed by : _____ **Date :** _____

ATTACHMENT 5

CCER / EMSE **Notebook: E100633-AC** **Page:** _____

E2 Basal Salts Media + Yeast Extract (E2-BSMYE)

Lot ID # _____ Date : _____

Amount Made _____ Made By :_____

Component Stock Solutions	mls / liter
200x P Salts Stock (pH 7.2)	5 mls
10x Non P-Salts Stock	100 mls
E2 Trace Elements Solution 200x stock	5 mls
20 % $(NH_4)_2$ SO_4 Stock Solution	100 mls
10 40% Yeast Extract Stock	*Variable

Combine stock solutions an adjust pH to 7.2 with NaOH
* Yeast Extract added as need to reach specific final concentration.
Filter sterilize using 0.2 um Nalgene filter unit or equivalent.
Label with lot ID and store sterile at room temperature.

100x P Salts Stock (pH 7.2) Lot # _____
mls added _____

10x Non P-Salts Stock Lot # _____
mls added _____

E2 Trace Elements Solution 200x stock Lot # _____
mls added _____

20% $(NH_4)_2$ SO_4 Lot # _____
mls added _____

10 % Yeast Extract Stock Lot # _____
final conc. of YE _____ mls added _____

Final pH ____ Adjusted with _____ mls NaOH Lot # _____

Additional Comments / Notes : _____ _____
_____ _____
_____