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TRAINING MANUAL

DETERMINATION OF ENDOCRINE DISRUPTING CHEMICALS (EDCS) IN FISH / MUSSEL USING AN *IN-VITRO* YEAST ESTROGENIC SCREENING (YES) TEST

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Introduction

Several wildlife species are exposed to biologically active concentrations of endocrine-disrupting chemicals, (EDCs) in North America, Europe, and other countries; as a result, there is a growing concern on the potential harmful effects of exposing these animal species to EDCs that are capable of modulating or disrupting the endocrine system (Depledge and Billinfhurst, 1999; Canesi *et al*., 2004). The observed abnormalities in the aquatic species may vary from subtle changes to permanent alterations; these phenomenon including disturbed sex differentiation with feminized or masculinized sex organs, changed sexual behaviours, and altered immune function (Vos *et al*., 2000; Colborn *et al*., 1993). Some coastal areas of China are polluted and mussels and seafood contaminated with EDCs (Chiu *et al*., 2018). As a result of environmental pollution and degradation, the biodiversity of freshwater species in Pakistan has been seriously disturbed. Pesticides and related chemicals that are capable of blocking the action of hormones in fish and other aquatic species may lead to reproductive dysfunction and abnormal development (Khan and Law, 2005). The level of EDCs in the wastewater in the environment of the Karachi region was high in Pak colony area since this area is near heavy industries which discharge pollutants into the waters (Khan *et al*., 2007). The Arabian Sea is very rich in marine species, there are about 788 species of marine fish including 70 commercial species of fish (sardine, Hilsa, shark, Mackerel, Butterfish, Pomfret, Sole, Tuna, sea bream, Jew fish, Cat Fish, Shark, and Eel), 769 species of marine molluscs, 287 species of marine crustaceans, and 101 species of marine annelids in the coastal regions of Pakistan alone.

As far as we know, no systematically study has been conducted to evaluate the extent of EDCs contamination in the fish and mussel species of Karachi coast, only some work have been reported by Khan *et al*. (2007, 2009) related to waters. Therefore, we have absolutely no idea about the potential effects of EDC chemicals on the economically important fish, prawn and mussel species. Moreover, no information, expertise and research facility related to research in this area is currently available in the country. This training manual will use a tool for screening bioassay to determine if significant EDCs levels that are present in the economical important fish and mussel species.

This training manual will facilitate the understanding of biological responses of fish and mussel species over a wide range of EDCs levels in the environment. The training results of some studies will also provide information on the EDC concentrations that should be used to conduct toxicological studies in the laboratory and information that are needed to conduct risk assessment studies and protection of aquatic biodiversity especially fish and mussel species. This training manual is prepared in collaboration with Ben, he is working

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in this field and currently participating in Greater Vancouver Research Project at the Toxicology Program, Department of Biological Sciences, Simon Fraser University. I, also thanks to Ben for his experimental and calculation support for research project and this training manual and Jasen Nelson for using of his preparation of reagent techniques. Thanks to Prof. Dr. Francis Law for final review of this manual and Higher Education Commission (HEC) for Post Doctorate fellowship funding support.

Many endocrine disrupting chemicals (EDCs) have deleterious effects on the reproductive organs for both male and female fish. Male fish have shown an increase in vitellogenin production (egg yolk precursor) and have displayed feminization of reproductive organs after exposure to EDCs (Beresford *et al*., 2004; Jobling *et al*., 2003; Routledge and Sumpter, 1996). When female Japanese medaka (*Oryzias latipes*) eggs were exposed to different concentrations of estrogens used in industry, only female medaka and intersexed males (with testis and ova) hatched from the eggs (Metcalfe *et al*., 2001). Another study, Jobling *et al*. (2003) discovered that low concentrations of 17 β-estradiol also increased egg laying in fish and embryo production in snails. Male fish caged near Waste Water Treatment Plants (WWTP) were found to produce vitellogenin (Harries *et al*., 1997). These studies show the serious effects of exposing aquatic wildlife to estrogenic compounds.

The most potent EDCs found in the environment are the natural and synthetic hormones such as 17 β-estradiol (E2), estrone (E1), and 17 α-thynylestradiol (EE2). We use E2 in our bioassay, as it is one of the most potent EDCs; detecting its presence is of the greatest importance because it is capable of causing the most damage to the animals. E2 is produced in the ovaries, gonads, and, to a lesser extent, in the arterial walls, the brain, and adrenal cortex. E2 also acts as a growth hormone for the female reproductive organs (Hertz, 1985).

There are several screening procedures used commonly to screen for EDCs in the aquatic environment. These include chemical analysis, *in vitro*, and *in vivo* bioassays. Chemical analysis is useful for identification and quantification of compounds in complex environmental mixtures. The YES bioassay is an *in vitro* estrogen-receptor bioassay commonly used in the detection of a complex estrogenic chemical mixture in the environment.

In this study, *Saccaromyces cerevisae* strain BJ3505 was transfected. *S. cerevisiae* is a species of budding yeast known as Baker's yeast. It is a model organism due to its small size and fast generation time (doubling time=100 minutes). It is easy and cost efficient to culture, in addition, it can be genetically manipulated, and mutants can be easily isolated. This test will confirm if the YES bioassay can be used to detect and quantify EDCs in fish tissues. This test also will determine the accuracy of the assay from week to week and look at the stability of estrogen in stored samples. It is consequential to monitor the levels of EDCs in exposed organisms, considering the adverse effects these chemicals may have on their endocrine system.

Preparation of Extraction

1. Prepare 2 grams of fish/mussel and place it in 5mL of methanol in a large glass test tube. Allow the tissue to soak in methanol for 30 minutes and homogenize with the Brinkmann Homogenizer (Polytron). Centrifuge the homogenized sample at 30rpm for 20 minutes (Fig. 1). After centrifugation, remove the methanolic extract and place it in a smaller test tube. Extract the tissue sample again with 5mL of methanol and centrifuge at 30rpm for 20 minutes. After the methanolic extract is removed for the second time, add with 2mL of chloroform to the tissue pellet and centrifuge for 15 minutes at 30rpm. Carefully remove the chloroform extract with pressure pipette from the tissue and then centrifuging the combined methanolic and chloroform extract at 30rpm for 8 minutes to precipitate the tissue residues contained in the extract. Measure the final volume of the extract and then evaporate to dryness in a rotator evaporator (Rotavapor, Fig. 2). Reconstitute the extract in 400µL methanol and transfer to small vials to be dried with gentle stream of nitrogen.

Fig. 1. Refrigerated Centrifuge machine.

Fig. 2. Heidolph Micro Spin-Vap Rotary Evaporator.

2. After nitrogen evaporation, reconstitute the extract with 500 µL ethanol and placed in a freezer at -40°C overnight. Sample extract will mixed with ethanol: 100% sample (tube 1), 40/160[40uL sample from tube 1+ 160uL etho], 20/180 [20uL sample from tube 2+180uL etho], 20/180 [20uL sample from tube 3+180uL etho], 20/180 [20uL sample from tube 4+180uL etho] and 20/180 [20uL sample from tube 5+180uL etho] dilutions before being used in the YES assay.

Preparation of Standard Dilution Series

E2 standard curve is prepared by using 11 concentrations of the E2 standard solution. A pure ethanol blank is also prepared (Table 1).

Stock solution
The E2 stock solution (0.01 M) is prepared by Dissolving 27.24 mg of estradiol in 10 ml of absolute ethanol.

Substock solution
To prepare the substock solution, add 20µL aliquot of the stock solution to 980 µL of ethanol.

Working solution
To prepare 100 nM working solution, add 10 µL aliquot of the substock to 990 µL of ethanol. The 200 µL aliquot of the working solution is used as the highest concentration for the standard dilution series.

Table 1. Standard Dilution Series.

	A1	A2	A3	A4	A5	A6	A7	A8	A9	A10	A11	A12
E2 (micro-liter)	200	60	20	12	20	40	40	40	40	20	20	0
EtOH (micro-liter)	0	140	180	188	180	160	160	160	160	180	180	200
		From A1	From A1	From A1	From A2	From A3	From A4	From A5	From A6	From A8	From A9	
(nM)	100	30	10	6	3	2	1.2	0.6	0.04	0.06	0.04	0

Preparation of Yeast (Gaido yeast assay)

Before Five days the start of an experiment, spread transgenic yeast on an agar plate and incubated at 30°C for three days. After the yeast colony appears to be growing on the agar plate, parafilmed and stored in a fridge at -10˚C. This procedure is adopted from Gaido *et al*. (1997) and Lorenzen *et al*. (2004).

Day One

1) At 10.00 am, first select a single colony of yeast from a streaked plate and put in 5mL of selective media in a 50mL polypropylene tube (Fig. 3).
2) Grow at 30 degrees Celsius with shaking overnight.

Fig. 3. 50mL Polypropylene tube.

Day Two
At 2.00 pm, the yeast will be diluted 1:10 by adding 45mL of media to the 5 mL.

Day Three
At 10.00 am, overnight culture of yeast are diluted 1:1 by re-suspending the yeast and transferring half the yeast to another 50 mL propylene tube and adding 25 ml of media.

Preparation of 96 well Plate

10µL of the standard dilution series and the sample series will be pipetted onto a 96 well plate (Fig. 4). This is repeated twice for each standard concentration and three times for each sample dilution. The plate is left to dry for 30 minutes.

At 2:00 pm the yeast is exposed to the standard/samples on the plate by re-suspending the yeast and mixing 20mL of yeast with 200 µL of copper sulphate. The copper sulphate stimulates the production of hER in the yeast cells.

200 µL of this mixture is then added to each well by using a multi channel pipette (Fig. 5). The plate is slightly shaken and parafilmed and to be kept in the incubation room at 30 °C overnight.

To prepare the 2-Nitrophenyl-1-β-D galactopyranoside (ONPG) solution, dissolve 0.022 grams of ONPG in 10.9 ml of Z-buffer and left in the shaker overnight.

Day Four
Start assay at 9:00 am, the yeast in each well is re-suspended and transfer 100 µL to a new 96 well plate.

Ten percent SDS (110µL) and 11µL of oxalyticase, and 29.7µL of mercaptoethanol are added to the ONPG solution prepared the day before; then add 100µL of the mixture to each of the 96 wells. After adding the ONPG solution allow the plate to sit for 40 minutes before to be read by a UV/Vis spectrophotometer (Fig. 6) at 415nm (colorimetric change) and 595 nm (for turbidity caused by growing of yeast cells).

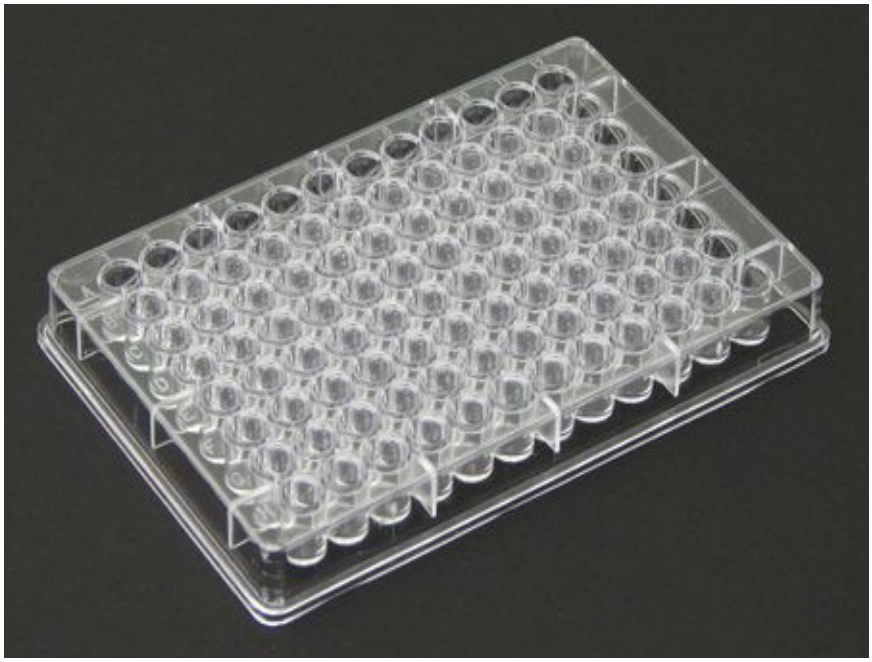

Fig. 4. 96-well microplates

Fig. 5. A Multi channel pipette.

Fig. 6. The SPECTRAmax 340PC microplate spectrophotometer (Microplate Reader).

Data Calculation and Statistical Analysis

This section explains how to use pre-designed Excel template to calculate the amount of E2 equivalents in the environmental samples analyzed through YES bioassay.

Following is the step by step walk through on how to use the template in addition to some explanations of the calculation method.

The UV reading at 415 nm represents the over all colorimetric change of the sample and the reading at 595 nm represents the turbidity caused due to yeast growth. In order to obtain the colorimetric change specifically due to the reaction between ONPG and ortho-Nitrophenyl-β-galactoside, we subtract the reading at 595 nm by 415 nm. On the template, simply copy and paste readings at directed area shown in Figure 7 and the result of subtraction along with the average of each sample replications will shown in the table underneath where the readings are supposed to be placed (Fig. 8).

2. The second step involving plotting the dose response curve for both standard and samples. In addition, we are also interested to acquiring the EC50 and the hill slope for both of our standard and samples. In order to obtain a curve from our data, the UV readings are normalized and the dilution factors are log-transformed for better data analysis with Prism 4.

The Excel template organizes the UV readings as the variables for y-axis and concentration factors as the variables for x-axis (Fig. 9). From the organized table on the Excel template we can copy and paste the table onto Prism 4 graphing program directly for further data analysis. Once our data have been transferred onto Prism 4 we will be able to obtain our dose response curves by following the steps listed below

- Click on “Analyze”
- Then go to “Data Manipulation” and choose “Transform”
- In Transform, click on the box says “Transform X Value Using” then choose “X = Log [X]”, finally press “OK” at lower right to exit the window.

- Again click on "Analyze"
- Go to "Data Manipulation" and choose "Normalize"
- In "Normalize" section if we are plotting our standard then simply click on "OK" at lower right to normalize our data. On the other hand, if we are plotting for our samples we need to click on "Y = _____ Becomes_100% for all data sets" and input the average of the top 2-3 points of our standard curve. This is because we are plotting our sample dose response curves by comparing to the maximum of our standard curve.
- After finishing the above steps our data now is log transformed and normalized; from this point we click on "Analyze", go to "Curves & Regression" then choose "Nonlinear Regression [Curve Fit]"
- In "Nonlinear Regression [Curve Fit]" window we choose "Sigmoidal Dose-Response [Variable Slope]" option then click "Unknowns From Standard Curve". Finally Press "OK" on the lower right to obtain our curve and data summary.

After plotting the dose response curves for both the standard and samples, we move on to calculate the E2 equivalence in our samples. The calculation is done by using the following formula suggested by Lorenzen *et al*. (2004): E2 equivalent = [standard EC50(ng/ml) / extract EC50] * [volume of assay medium (ml) / (volume of extract tested (µl)] * [volume of stock extract (µl) / ww of sample (g)]. "Volume of the assay medium" is the amount of extract you have before rotator evaporation; "Volume of extract tested" is the amount of sample we put into each well on the 96-well plate; "Volume of stock extract" is the amount of solvent we reconstitute our sample after nitrogen evaporation; as for "ww of the sample" is the wet weight of our sample.

To further broaden the accuracy of our calculation we also calculate our results by using EC20 and EC 30 in the formula mentioned above instead of just calculating with EC50. Since Prism 4 only shows us EC50 from our curves, we have to covert EC50 to EC 20 and EC 30 by using the following formula: ECX = [X/(100-X)^(1/H)] * EC50, where X is 20 or 30 depending on the variable we want and H is the hill slope of the dose response curve.

It is easy to use the template to calculate our E2 equivalence since all the formula for calculation have been already set up and will automatically calculate our result once we input the required information into the template. Input the information of our samples into Table 1 (Fig. 10); after doing so we input the EC50/hill slope we obtained for our standard and samples from Prism 4's dose response curve summary into Table 2 (Fig. 11). As soon as we finished filling the information into Table 1 and 2 on the template the E2 equivalence of our samples will be shown in Table 3 (Fig. 12). Important to note that in Table 3 we are obtaining the results calculated by using EC 50, 30 and 20 along with the average of all three results for each of our samples.

415nm	2.283	1.96	3.126	2.955	1.999	2.662	1.734	2.713	2.673	1.028	0.951	0.612
	3.063	2.586	2.96	2.779	2.734	2.772	2.627	2.73	2.967	0.936	1.183	0.713
	1.839	1.138	0.995	0.825	0.78	0.808	0.935	0.927	0.753	0.68	0.742	0.698
	1.877	1.097	0.839	0.723	0.748	0.704	1.169	0.846	0.903	0.673	0.74	0.841
	1.57	1.161	0.8	0.853	0.751	0.615	1.063	0.979	0.626	0.854	0.711	0.653
	1.224	1.253	0.932	0.772	0.748	0.76	0.836	0.872	0.797	0.788	0.754	0.712
	1.121	1.228	0.8	0.912	0.833	0.733	1.075	0.865	0.837	0.731	0.792	0.799
	1.41	1.354	0.917	0.682	0.758	0.99	1.031	0.995	0.842	0.812	0.732	0.743
595nm	0.261	0.375	0.372	0.285	0.346	0.405	0.356	0.337	0.26	0.299	0.314	0.271
	0.351	0.296	0.36	0.271	0.228	0.267	0.333	0.365	0.379	0.297	0.376	0.28
	0.489	0.387	0.442	0.353	0.337	0.315	0.329	0.314	0.311	0.302	0.273	0.312
	0.468	0.349	0.287	0.29	0.305	0.281	0.454	0.281	0.435	0.31	0.337	0.414
	0.418	0.432	0.285	0.394	0.315	0.268	0.44	0.448	0.273	0.442	0.34	0.264
	0.385	0.504	0.384	0.333	0.332	0.349	0.358	0.416	0.376	0.397	0.371	0.352
	0.382	0.492	0.337	0.44	0.376	0.352	0.513	0.39	0.429	0.367	0.399	0.389
	0.444	0.559	0.365	0.34	0.369	0.547	0.44	0.423	0.409	0.406	0.35	0.351

Fig. 7. Table for input UV readings.

	Standard	1.00E-07	3.00E-08	1.00E-08	6.00E-09	3.00E-09	2.00E-09	1.20E-09	6.00E-10	4.00E-10	6.00E-11	4.00E-11	0
		2.022	1.585	2.754	2.67	1.653	2.257	1.378	2.376	2.413	0.729	0.637	0.341
		2.712	2.29	2.6	2.508	2.506	2.505	2.294	2.365	2.588	0.639	0.807	0.433
	Average	2.367	1.9375	2.677	2.589	2.0795	2.381	1.836	2.3705	2.5005	0.684	0.722	0.387
		13						14					
	Sample	1	0.2	0.002	0.0002	0.00002	0.0002	1	0.2	0.002	0.0002	0.00002	0.0002
		1.35	0.751	0.553	0.472	0.443	0.493	0.606	0.613	0.442	0.378	0.469	0.386
		1.409	0.748	0.552	0.433	0.443	0.423	0.715	0.565	0.468	0.363	0.403	0.427
		1.152	0.729	0.515	0.459	0.436	0.347	0.623	0.531	0.353	0.412	0.371	0.389
	Average	1.30366667	0.74266667	0.54	0.454666667	0.440666667	0.421	0.648	0.569667	0.421	0.3843333	0.414333	0.400667
Standard Deviation		0.13461922	0.01193035	0.021656	0.019857828	0.004041452	0.0730205	0.058643	0.041199	0.060308	0.0251064	0.049973	0.022855
		15						16					
	Sample	1	0.2	0.002	0.0002	0.00002	0.0002	1	0.2	0.002	0.0002	0.00002	0.0002
		0.839	0.749	0.548	0.439	0.416	0.411	0.478	0.456	0.421	0.391	0.383	0.36
		0.739	0.736	0.463	0.472	0.457	0.381	0.562	0.475	0.408	0.364	0.393	0.41
		0.966	0.795	0.552	0.342	0.389	0.443	0.591	0.572	0.433	0.406	0.382	0.392
	Average	0.848	0.76	0.521	0.417666667	0.420666667	0.4116667	0.5436667	0.501	0.420667	0.387	0.386	0.387333
Standard Deviation		0.11376731	0.031	0.050269	0.06757465	0.034239354	0.0310054	0.0586884	0.062217	0.012503	0.0212838	0.006083	0.025325

Fig. 8. Result of UV reading subtraction and average.

Std	X	Y1	Y2
	1.00E-07	2.022	2.712
	3.00E-08	1.585	2.29
	1.00E-08	2.754	2.6
	6.00E-09	2.67	2.508
	3.00E-09	1.653	2.506
	2.00E-09	2.257	2.505
	1.20E-09	1.378	2.294
	6.00E-10	2.376	2.365
	4.00E-10	2.413	2.588
	6.00E-11	0.729	0.639
	4.00E-11	0.637	0.807
		0.341	0.433

13	X	Y1	Y2	Y3
	1.00E+00	1.35	1.409	1.152
	1.00E-01	0.751	0.748	1.152
	1.00E-02	0.553	0.552	0.729
	1.00E-03	0.472	0.433	0.515
	1.00E-04	0.443	0.443	0.459
	1.00E-05	0.493	0.423	0.347

14	X	Y1	Y2	Y3
	1.00E+00	0.606	0.715	0.623
	1.00E-01	0.613	0.565	0.531
	1.00E-02	0.442	0.468	0.353
	1.00E-03	0.378	0.363	0.412
	1.00E-04	0.469	0.403	0.371
	1.00E-05	0.386	0.427	0.389

15	X	Y1	Y2	Y3
	1.00E+00	0.839	0.739	0.966
	1.00E-01	0.749	0.736	0.795
	1.00E-02	0.548	0.463	0.552
	1.00E-03	0.439	0.472	0.342
	1.00E-04	0.416	0.457	0.389
	1.00E-05	0.411	0.381	0.443

16	X	Y1	Y2	Y3
	1.00E+00	0.478	0.562	0.591
	1.00E-01	0.456	0.475	0.572
	1.00E-02	0.421	0.408	0.433
	1.00E-03	0.391	0.364	0.406
	1.00E-04	0.383	0.393	0.382
	1.00E-05	0.36	0.41	0.392

Fig. 9. The table that organizes dilution factors and UV readings into x-axis and yaxis respectively (For both the standard and samples).

Sample	13	14	15	16
Volume of assay mediur	2	2	2	2
Volume of extract testec	10	10	10	10
Volume of stock extract	500	500	500	500
ww of sample (g)	3	3	3	3

Fig. 10. Table for input sample information.

Table 2: Input EC50 and Hill Slop Obtained from Prism			
Formula for Calculating EC20 and EC 30			
EC20 calculation:			
[(20/100-20)^(1/h)]*EC50			
EC30 calculation:			
[(30/100-30)^(1/h)]*EC50			
*Where h is the hill slope of the graph.			
	Hill Slope	EC50	
Standard	18.7	1.74E-10	
13	0.4942	0.5276	
14	1.389	0.05963	
15	1.058	0.02987	
16	0.9523	0.04085	

Fig. 11. Table for input EC50 and hill slope of the dose response curves.

Table 3: Hormone Equivalent Results				
Formula: Hormone Equivalents (ng/g	[std EC50(ng/ml) / ectrac			
Per Gram EC20				
13	14	15	16	
1.68E-07	2.443E-07	6.67E-07	5.636E-07	
Per Gram EC30				
5.82E-08	1.71E-07	4.12E-07	3.29E-07	
Per Gram EC50				
1.10E-08	9.70E-08	1.94E-07	1.42E-07	
7.913E-08	1.706E-07	4.24E-07	3.449E-07	Average

Fig. 12. Summary of the E2 equivlent results.

Preparation of Growth Medium for Gaido:

1. NaH_2PO_4 (Sodium Phosphate, monobasic) 1.73gm
2. Na_2HPO_4 (Sodium Phosphate, dibasic) 1.77gm
3. $(NH_4)_2SO_4$ (Ammonium Sulphate) 2.5gm
4. Y.N Base (Yeast Nitrogen Base) 0.85gm
5. Dextrose 10.0gm
6. Lysin 5.0ml
7. Histidine 5.0ml
8. DD Water 490ml

Putting in the Autoclave for 1 hour

Preparation of Reagent (Nelson, 2002)

1. 10X Yeast Nitrogen Base without Amino Acids (YNB)

a. Weigh out 67g Yeast Nitrogen Base without Amino Acids.
b. Place in 1000 ml graduated cylinder.
c. Bring up to 1000 ml with distilled water.
d. Mix with magnetic stir bar on stir plate.
e. Filter sterilize with 1000 ml 0.2 µ filter unit. Transfer to 1000 ml sterile glass bottle.

2. 20% Dextrose Stock

a. In 1000 ml beaker, dispense 800ml distilled water, add magnetic stir bar, and place on magnetic stirrer.
b. Weigh out 200g Dextrose
c. Add Dextrose slowly to vigorously stirring distilled water.
d. Filter sterilize with 1000 ml-0.2 µ filter unit. Transfer to 1000 ml sterile glass bottle.

3. 10 mM Copper Sulfate

a. Weigh out 0.25g Copper Sulfate pentahydrate. Place in 100 ml graduated cylinder.
b. Bring up to 100 ml with distilled water.
c. Filter sterilize with 100 ml-0.2 µ filter unit. Transfer to 100 ml sterile glass bottle.

4. 10% SDS

a. Weigh out 10g Lauryl Sulfate. Place in 100 ml graduated cylinder.
b. Bring up to 100 ml with distilled water. Mix well.
c. Transfer to 100 ml sterile glass bottle.

5. 1 M Sodium Chloride

a. Weigh out 58.44 g NaCl. Place in 1000 ml graduated cylinder.
b. Bring to 1000 ml with distilled water. Mix well.
c. Filter sterilize with 1000 ml-0.2 µ filter unit. Transfer to 1000 ml sterile glass bottle.

6. 50% Glycerol with 100 mM NaCl

a. Put 50 ml glycerol into 100 ml graduated cylinder.
b. Add 10 ml of 1M NaCl solution.
c. Bring up to 100 ml with distilled water. Mix well.
d. Transfer into 100 ml sterile glass bottle.

7. Oxalyticase - Lot no. L187F, 50,000 units/mg, 5 mg = 250000 U total

a. Make a 200U/µl solution by adding 1.25 ml of 0.1 M NaCl/50% Glycerol solution to vial
b. Mix well. Store at 4°C.

8. Z Buffer

a. Weigh out:
16.1 g Na_2HPO_4
5.5 g NaH_2PO_4
0.75 g KCl
0.25 g $MgSO_4$
b. Place in 1000 ml graduated cylinder.
c. Bring up to 800 ml with distilled water.
d. Adjust pH to 7.0 while stirring with stir bar on stir plate.
e. Bring up to 1000 ml with distilled water.
f. Filter sterilize with 1000 ml-0.2 µ filter unit. Transfer to 1000 ml sterile glass bottle.

9. Amino Acids

a. **LYS**-1.8g L-lysine-HCl in 500 ml of distilled water. Autoclave.

b. **HIS**-1.2 g L-histidine-HCl in 500 ml of distilled water. Sterilize with 500 ml-0.2 µ filter unit.

10. M Sodium Phosphate Buffer, pH 6.8

weigh: 6.90 g monbasic soidum phsophate
7.10 g dibasic sodium phosphate
- dissolve in 500 ml milli-Q water, check pH, filter sterilize and store at 4 °C

11. Growth Media for Gaido ER Transformed Yeast - buffered

a. Measure out: 50 ml 10X YNB
50 ml 20% Dextrose
5 ml Lysine
5 ml Histidine
250 ml 0.1 M sodium phosphate buffer, pH 6.8

b. Bring up to 500 ml with distilled water. Mix well.

c. Filter sterilize with 500 ml-0.2 µ filter unit. Transfer to 500 ml sterile glass bottle.

d. Store at room temperature.

Growth of Yeast

1. Take a small scraping of yeast from frozen culture and streak onto selective medium plate. If this does not work, thaw a 100µL aliquot of stock yeast and add 50µL to 5 ml Gold media (non-selective) in a 50 ml polypropylene tube. Incubate at 30°C at 300 rpm until culture becomes cloudy - usually overnight. Innoculate a selective media plate with a loop full of culture. Spread in quadrants so that yeast become sufficiently dispersed to produce well separated colonies.
2. Place the plate at 30°C and allow growing until colonies are visible. This may take a few days.
3. Once colonies are visible, seal plate and store at 4°C.

Preservation of Yeast ER Cultures (method used by Burnison Lab)

1. Innoculate 10 ml of ER selective media with a single colony.
2. Incubate o/n with shaking at 30C
3. Centrifuge at 2000g for 2 minutes to pellet yeast
4. Remove supernatant and resuspend yeast in 5 ml of medium containing 15% glycerol
5. Pipet 1 ml of the cell suspension into cryovials
6. Cool slowly by placing vials at 4C for 30 min, -20 C for 30 min then transfer to -70C freezer

Preparation of 15% glycerol/ER sel:
- add 0.75 ml sterile glycerol to 4.25 ml ER selective medium
2000g = 3400 rpm on Thermo IEC cell culture centrifuge.

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