

CYTOTROPHOBLAST PREPARATION

EXPERIMENT _____ DATE _____ PERFORMED BY _____

A. EQUIPMENT AND MATERIALS NEEDED:

0.1% Nonidet P-40 (Sigma, N-0896)	Histology Staining Cont (Baxter, S7652-2)
6-Well Dishes (Thomas Scientific, 152795)	L-glutamine (Sigma, G5763)
10X Bicarbonate Free Hanks' Balanced Salt Solution in 1 ml aliquots (Gibco, 14060-024)	Metzenbaum Scissors
12x75 Test Tubes (Borosilicate Disposable Culture Tubes; Fisher Scientific, 14-961-26)	MgSO ₄ (Sigma, M2643)
15 ml/50 ml Polystyrene Centrifuge Tubes [Corning Sterile Disposable with Plug Seal Cap; Baxter C3973-15 (15 ml) C3974-50 (50 ml)]	NaCl (Sigma, S-5886)
100X Pen-Strep-Neomycin (PSN) in 1 ml aliquots (Gibco BRL, 600-5640AG)	Nalgene 0.1 or 0.2 μ Filter Assemblies, Type TC (Baxter, T4216-6 or T4216-7)
Absorbent Pads (LabMat Bench Cover; Macalaster Bicknell, 32238-6206)	NaN ₃ (Sigma, S2002)
Bouin Fixative (Poly Scientific, S129)	NaOH, 10N (Fisher Scientific, SS277)
Bowl, 6 x 2.5 in., stainless steel	Neutral Buffered Formalin (NBF)
CaCl ₂ (Sigma, C7902)	Paper Towels
Ca-Mg free Hank's with 25 mM Hepes (CMF-Hank's)	Paraformaldehyde (Electron Microscopy Sciences, 19208)
Calf Serum, see Step C5 for preparation (Gibco BRL, 200-6170AJ)	Pasteur Pipets/Tips/Sterile, Disposable, Glass Pipets, Pipettors
Peristaltic Pump, three channel (Pharmacia, P-3, or equivalent). Must be able to move liquid at 1 ml/min rate.	PBS (pH 7.4), see step B6 for preparation: Monobasic Sodium Phosphate (Sigma, S-5011). Dibasic Sodium Phosphate (Sigma, S-5136). NaCl
Cutting Board	Percoll (Sigma, P-1644)
DMEM-High Glucose with 25 mM Hepes (DMEM-HG)	Coverslips, 22 mm ² (Fisher Scientific, 12-541-B, 22x22-1.5D)
DNase I, Type IV (Sigma, D5025)	Plastic Weigh Boats
Erlenmeyer Flasks (250 ml, 500 ml)	Razor Blades, single edge safety
Fetal Calf Serum (FCS) heat inactivated at 56°C for 30 mins. (Gibco BRL, 240-6000AJ)	Specimen Cups
Forceps	Trizma Base (Sigma, T-1503)
HCl, conc. (Fisher Scientific, A144SI-212)	Trypsin, Type I (Sigma, 8003)
Hemocytometer (Fisher Scientific, 02-671-5)	Shaking Water Bath (Precision Model 25)
Trypsinization Flasks (Bellco)	Water Bath, 37°C

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B. INITIAL PREPARATORY STEPS (Can be done before placental receipt is confirmed.)

1. Turn on shaking water bath to 37°C.
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3. Take trypsin (Sigma T-8003) and DNase (Sigma D-5025, 1,500-2,500 Kunitz units/mg) out of freezer ("Placental Enzymes") to warm up.
4. Place CMF Hank's and DMEM-HG in water bath.
5. Make 4 L of saline (36 g NaCl in 4 L deionized H₂O).
6. Filter ~50 ml PBS into a 50 ml centrifuge tube using a 0.2µ syringe filter. For preparation of PBS stock:
 - A. Sodium Phosphate Buffer
 - i. Prepare 0.2M NaH₂PO₄ (Monobasic)
 - 2.4 g Monobasic, anhydrous
 - 100 ml DDW
 - ii. Prepare 0.2M Na₂HPO₄ (Dibasic)
 - 5.68 g Dibasic, anhydrous
 - 200 ml DDW
 - iii. Prepare 200 ml of buffer
 - Start volume of Dibasic: approx. 160 ml
 - pH to 7.4 with Monobasic
 - B. PBS (Phosphate Buffered Saline)
 - i. 10 mM Sodium Phosphate Buffer
 - 20 ml Sodium Phosphate Buffer
 - ii. 150 mM NaCl
 - 3.52 g
 - iii. Bring up to 400 ml with DDW
7. Fill specimen cup with NBF.
8. Prepare counter with absorbent pads, paper towels, specimen cup with 5-10 ml bleach, cutting board, razor blades, Metzenbaum scissors, forceps.

C. FINAL PREPARATORY STEPS (Only done if placenta is to arrive.)

1. Weigh out digesting enzymes into 250 ml Erlenmeyer flasks as follows:

FLASK	HANK'S (ml)	TRYPsin(mg)	DNase (mg)	TIME (min.)
1	150	228	30	30
2	100	150	20	30
3	75	120	15	30
4	75	120	15	30

•Add 1 µl 100 mM CaCl₂ and 1 µl 800 mM MgSO₄ per 1 ml digest solution (stock solutions kept in refrigerator).

2. Pour 400 ml of Hank's media sterilely into a clean 500 cc Erlenmeyer flask (for each prep to be done) and place back into water bath.
3. Thaw out three 1 ml Eppendorfs of 100X Pen-Strep-Neomycin (PSN).
4. Pour about 100 ml of DMEM-HG sterilely into a 250 ml Erlenmeyer flask, add 2 ml PSN concentrate, keep at 37°C.
5. Thaw out 1 tube of calf serum per prep. This tube of calf serum is adjusted based on calculations done each time a new 500 ml bottle of calf serum is opened and aliquoted. The

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goal is to be able to adjust each aliquot of calf serum to a density of 1.017 g/ml with sterile PBS (pH 7.4). Using the following procedure for each new calf serum bottle:

- a. Weigh (to at least 3 decimal places) 0.5 ml calf serum with a volumetric pipet (weigh empty, tare scale, fill, weigh), multiply by 2. This equals the density (d_1). d_2 =desired density (in this case **1.017**). V_1 =starting volume of calf serum. Use the following formula to calculate the amount of PBS to add to each aliquot when performing a trophoblast prep. Since a total volume of at least 33 ml is needed of calf serum adjusted to the desired density, you may need to try different aliquot sizes in the equation below.

$$d_2 = \frac{d_1 V_1 + \text{ml PBS}}{V_1 + \text{ml PBS}}$$

6. In preparation for plating isolated trophoblasts, thaw out one 15 ml tube of FCS per 150 ml of media, if needed.
7. Thaw out two 1 ml 10X bicarbonate free Hank's Eppendorfs per Percoll gradient.
8. Pipet 1.5 ml of calf serum (adjusted to desired density with PBS) into 22 sterile 15 ml polystyrene centrifuge tubes. Place in rack in two sets of six, then one set of ten.
9. Make each Percoll gradient fresh as follows:
 - a. 18 ml Percoll (stored sterilely at 4°C, allow all to drain from pipet) plus 2.0 ml 10X Hank's-Bicarb free (rinse Eppendorf with solution) 90% Percoll.
 - b. Place 14 12x75 mm glass test tubes in a rack.
 - c. Add 90% Percoll and Ca-Mg free Hank's (CMF Hank's) as follows:

% Percoll	ml 90% Percoll	ml Hank's
70	2.33	0.67
65	2.17	0.83
60	2.00	1.00
55	1.83	1.17
50	1.67	1.33
45	1.50	1.50
40	1.33	1.67
35	1.17	1.83
30	1.00	2.00
25	0.83	2.17
20	0.67	2.33
15	0.50	2.50
10	0.33	2.67
5	0.17	2.83

- d. Beginning at the 70% tube, mix each solution well with a glass Pasteur pipet.
- e. With either a clean Pasteur pipet or a slow peristaltic pump (1 ml/min.), beginning with 70% Percoll, layer Percoll solutions into a 50 ml polystyrene (clear) conical centrifuge tube using side of tube to add solutions.

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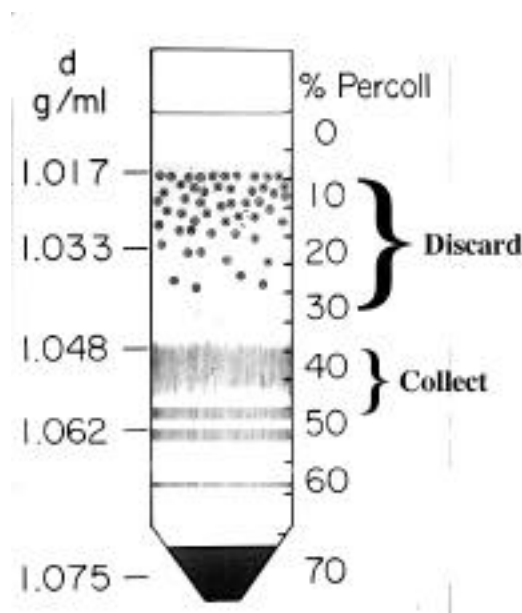
D. PLACENTAL PREPARATION (All spins performed at 25°C)

Birth time _____ Time prep began _____

1. Begin preparation as soon as possible after delivery.
2. Note any infectious precautions from Labor Floor staff.
3. Place placenta on cutting board, maternal side up.
4. Using new razor blades, first cut a thin piece of tissue from maternal to fetal surface, a piece of umbilical cord, and a section of the membranes. Fix in NBF.
5. Cut away membranes. Cut up about one third of placenta into ~1 inch cubes, selecting the softest, thickest portions. Make sure that all pieces go from maternal to fetal surface. Place in 6 x 2.5 inch stainless steel bowl.
6. Thoroughly rinse tissue with 4 liters of saline (~30 s/rinse) by agitating tissue in solution like a clothes washing machine, do not squeeze tissue, only agitate solution. Drain carefully between rinses. Tissue should be light pink when done, with no red.
7. Place one piece of placental tissue at a time on several paper towels. Using a Metzenbaum scissors, first remove and discard the entire decidual layer, then cut small pieces of soft villous tissue from core of placental chunk, avoiding fibrous tissue and vessels. Collect between 30 to 35 g into a plastic weigh boat.
8. Mince tissue until pieces are about ~0.2 to 0.3 cm across (takes about 1 min).
9. Pour 150 ml of warmed CMF Hank's into the first Erlenmeyer flask containing enzymes, mix well, then pour solution into a 250 ml trypsinization flask. Add 1 ml of 100X PSN. Add CaCl₂ and MgSO₄ solutions. Add minced placental tissue, mix and place into shaking water bath. Water in bath should be at least as high as solution in flask to insure uniform warming. Speed should be no more than 50 RPM.
10. Remove digestion flask from bath every 5 minutes and swirl vigorously to resuspend tissue.
11. At the end of 30 minutes, remove digestion flask and place on a slant in a small glass histology staining container (3x1.5 inches). Allow tissue fragments to settle for 1 min. With a clean 10 ml pipet and hand held or motor driven pipetter, slowly remove supernatant from just below the surface at a point which is farthest away from tissue fragments. Transfer ~81 ml of supernatant to a specimen cup containing bleach. Discard the specimen cup contents.
12. Add 100 ml of CMF Hank's to enzymes in flask #2, mix well. Add CaCl₂ and MgSO₄ solutions. Add solution to digestion flask containing tissue fragments, return to shaking water bath for 30 min. Repeat step 10 above.
13. Remove digestion flask and place on glass histology staining container. Allow tissue fragments to settle for 1 min.
14. With a clean 10 ml pipet and hand held or motor driven pipetter, slowly remove supernatant from just below surface at a point which is farthest away from tissue fragments.
15. While tilting a 15 ml centrifuge tube containing 1.5 ml of calf serum, place filled 10 ml pipet into tube along side to about half way down. Very **slowly** begin to layer digest supernatant onto calf serum. If mixing occurs, stop, and then begin again more slowly. As layer develops, one can increase speed of pipetting. Repeat for next five tubes. Top off all six tubes to the 15 ml mark. Spin in RC3B at 2,200 RPM (padded buckets) for 15 min at 25°C. (Resultant pellet should reveal a lower RBC band then a white upper band. The white band contains the trophoblasts.)
16. Add 75 ml warm CMF Hank's to enzymes in flask # 3, mix well. Add CaCl₂ and MgSO₄ solutions. Add solution to digestion flask, return to shaking water bath for 30 minutes (see instruction in step 10 above).
17. Remove supernatants from the six tubes just spun, taking care not to aspirate off the white band. Try to remove all the white fluffy material at the calf serum interface, this material is precipitated trypsin and DNase. Resuspend each pellet with 1 ml of warm DMEM-HG which contains PSN. Pool, set aside, lightly capped, at room temperature.

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18. Repeat steps 13 to 15 and 17 above (adding 75 ml of CMF-Hank's to last flask of enzymes). Try to collect as much of the supernatant as is possible. This usually means ten 15 ml tubes. Pool last digestion into two sets of 5 tubes each. Result is four tubes containing pooled calf serum pellets.
19. Centrifuge four pooled specimens in RC3B for 10 mins at **2,200 RPM**. Remove supers, taking care not to remove any of the white upper bands. Resuspend with 1 ml warm DMEM-HG containing PSN. Pool suspensions.
20. Very carefully layer pooled digests onto Percoll gradient. Depending on how many RBCs are in the suspensions, more DMEM-HG may need to be added to allow the solution to float on the top layer of the Percoll gradient. An additional 1 ml should be sufficient. Keep adding DMEM-HG until suspension floats as it is added to the top of the Percoll gradient. Spin at $1,200 \times g$ for 20 minutes **without the brake** (**1,400 RPM** in RC3B with an HG-4L rotor). (Use this time to prepare and warm plating media. See Step 23 below). Very carefully remove tube from centrifuge and place in styrofoam support in completely upright position. While looking at gradient from below (you need to actually kneel down on floor in front of gradient which is on bench top) with good florescent backlighting, remove top fluffy white band with pasteur pipet connected to vacuum flask (see figure below). Do not remove next distinct band! With clean pasteur pipet and bulb, while still kneeling in front of gradient, collect next large band and thin band below this which both contain the trophoblasts. The ideal way to remove trophoblast bands is as follows: depress bulb completely, place tip of pipet at top of solution above band, slowly aspirate while moving tip around circumference of centrifuge tube and slowly moving tip down to stay at top of solution. Do not stop aspiration once started, do not push solution back down once started, do not place tip of pipet much below the top of gradient solution. Lift pipet cleanly away from gradient solution while still applying some vacuum to pull solution into pipet. Pipet material into clean 50 ml polystyrene centrifuge tube. Repeat aspirations as above until all of these two bands are removed. Make sure you do not collect material below this band, this will lead to increased contamination with other cell types (in order: macrophages, neutrophils, lymphocytes, RBCs).



21. Dilute the collected middle bands with DMEM-HG containing PSN to the 50 ml mark. Spin in the RC3B for 10 min at 2,200 RPM (turn centrifuge brake back on). Work with trophoblasts under sterile conditions from this point on. Resuspend pellet with 10 ml of the final plating media. If a combined serum-free/serum experiment is planned, resuspend

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- first pellet in serum-free media, count cells as below, aliquot suspension as needed, pellet, resuspend individual pellets into final media.
22. With sterile pipet tip, dilute 50 μ l of the cell suspension into 0.95 ml of DMEM-HG-PSN. Count solution twice with hemacytometer. Count all large cells (do not count RBCs or cells the size of RBCs) in middle large square (twenty-five boxes subdivided into 16 fine boxes). Average counts, multiply by 20, then by 10,000 to yield the number of cells/ml present in the 10 ml suspension. Dilute suspension to desired concentration with plating media (which has been previously warmed).
 23. Standard plating media:
 - a. With fetal calf serum (10% FCS):
 - 135 ml DMEM-HG
 - 15 ml FCS
 - ~88 mg L-glutamine
 - 1.5 ml 100X PSN (Gibco)
 - Filter in Nalgene filter assembly (0.2 μ).
 - b. Serum Free:
 - ~148.5 ml DMEM-HG
 - ~88 mg L-glutamine
 - 1.5 ml PSN
 - Filter in Nalgene filter assembly (0.2 μ).
 24. Incubate cells at 37°C in 5 % CO₂ atmosphere. Change media daily. Media is only good for five days due to L-glutamine breakdown.

E. FIXATION

1. To fix cells, remove from incubator, work on bench top. Wash cells 2 times with PBS, fix for 5-10 min with fixative (NBF, Bouin's, or 4% paraformaldehyde), wash 3-4 times with PBS. Store at 4°C in PBS-0.1% NaN₃ (for as long as 2 years). See the following page for 4% paraformaldehyde fixative.

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PREPARATION OF 4%-PARAFORMALDEHYDE FIXATIVE

NOTE: This procedure should be performed under a **fume hood**. Paraformaldehyde produces strong fumes, and may be a reproductive toxin or carcinogen. Wear **gloves**.

1. Heat ~100 ml DDW to 60-70°C.
2. While heating, weigh out 4g p-formaldehyde for a conc. of 8% (2X) in 50 ml DDW. Place in a 150 ml beaker and set aside.
3. Prepare 50 ml of 2X TBS (20 mM Trizma Base, 300 mM NaCl):
 - a. Add ~876.6 mg NaCl to a 100 ml beaker.
 - b. Add 5 ml of 20X Tris Buffer, pH 7.5.
 - c. Add DDW to 50 ml.
 - d. Mix on stir plate and set aside.
4. When DDW has been heated to 60-70°C, pour ~40 ml into the beaker containing the p-formaldehyde. Mix on stir plate with continued heating.
5. After 2-3 minutes, slowly add 10N NaOH in drops until the p-form. solution clears. This should take no more than 3 or 4 drops.
6. With continued stirring, add the 50 ml of 2X TBS prepared earlier.
7. pH to 7.5 using conc. and 1N HCl. Add DDW up to 100 ml.
8. Fixative should be used at room temperature.
9. Store at 4°C up to 2 wks.

4% PARAFORMALDEHYDE FIXATION (6-Well Plates)

1. Wash wells X2 with TBS, pH 7.5.
2. Add ~1 ml/well 4% p-form. fixative. Incubate at room temperature for 10 min.
3. Wash X1 with TBS.
3. Add ~1 ml/well 0.1% Nonidet P-40 (NP-40). Incubate at room temperature for 2 min.
4. Wash X2 with TBS.
5. Add 3-4 ml TBS-0.1% Azide to each well. Store plate at 4°C until further use.
6. Check occasionally to make sure the wells have not dried out.