

Isolation of Primary Cytotrophoblasts From Human Placenta at Term

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[Abstract] The placenta is a multifaceted organ, fulfilling critical functions for the fetus and the mother. Therefore, it is a critical regulator of the pregnancy, and its dysfunction leads to diseases, including fetal growth restriction and preeclampsia. Studying the placenta is a difficult task since its existence is transient, and its structure is specific to our species. *In vitro* differentiation of primary cytotrophoblast isolated from term human placenta has been widely used in the placental research field as it represents a reliable model to study cellular differentiation and function. Direct alternatives include trophoblastic cell lines, explants, and organoids, but this protocol, based on the separation of the cells on a Percoll gradient, presents the advantage of being relatively cheap and easy to perform in every research laboratory. Furthermore, the 2D culture is a flexible method that can be adapted to various experimental conditions (transfection, drug exposure, metabolic study, observations, *etc.*), allowing mechanistic explorations of cellular processes.

Keywords: Trophoblast, Syncytialization, Placenta, Pregnancy, Preeclampsia, Fetal growth restriction

[Background] Traditionally neglected by scientists, the placenta has long been regarded as a temporary organ with basic nutritional functions. Now, it is recognized as a complex and essential organ for mammalian reproduction, as aberrations in its formation and/or function have immediate consequences on pregnancy outcomes, as well as lifelong impacts on the health of the offspring (Malhotra *et al.*, 2019). At term, the placenta has a discoid shape, with one side facing the amniotic cavity (the chorionic plate) and the other in contact with the endometrium (the decidual plate). Its microstructure is formed of villi that could be compared to multiple trees, which come from the branches of the fetal arteries (Turco and Moffett, 2019). Since these villi are directly in contact with the maternal blood, the human placenta is told hemochorial (Foidart *et al.*, 1992). The interface between the maternal blood and the fetal vessels is called the syncytium. This unique layer constitutes the functional unit of the placenta and comes from the fusion of the mononuclear villous cytotrophoblasts (CTBs) to form multinucleated syncytiotrophoblasts (STBs). This process, known as syncytialization, takes place throughout the pregnancy and is critical for the formation and the functions of the placenta (Costa, 2016). On the other hand, the CTBs can also stay mononuclear but acquire an invasive capacity, especially during the first trimester. In this case, they are called extravillous trophoblasts (EVTs) and are crucial to the adaptations of the maternal arteries to pregnancy (Chang *et al.*, 2018). In both cases, the study of CTBs differentiation represents one of the keys to improve our understanding of placental development

and related diseases.

There are no perfect experimental models to investigate the human placenta as the differences between mammals are considerable, even in species with hemochorial placentas (e.g., rodents and non-human primates) (Roberts *et al.*, 2016). Additionally, disorders such as preeclampsia, in which the primary defect is a failure in placentation, are found only in humans (Brosens *et al.*, 2011). For these reasons, fundamental research on placental biology was initially based on *ex vivo* observations of histologic sections (Burton *et al.*, 1999). In the last decades, various *in vitro* models based on human material were developed and characterized. However, each *in vitro* system has its benefits and limitations and should be carefully selected based on the trophoblast subtype, the gestational age of interest, and the type of experiment conducted (Colson *et al.*, 2020b). In this context, primary human trophoblasts isolated from normal placenta at term and cultured in plastic cell culture dishes is an easy, cost-effective, and reproducible method to study specific molecular and genetic regulations in the placenta after the first trimester of gestation. This protocol is based on the method originally described by Kliman (Kliman *et al.*, 1986) but was significantly improved by our team. The main advantages of this technique are its simplicity, its efficiency, and its cost. In addition, the method gives reproducible results as we have been able to characterize many independent cultures of several days (up to 12 days) over the last 10 years (Debieve *et al.*, 2013; Depoix *et al.*, 2013, 2016, 2018 and 2020; Colson *et al.*, 2020a). Furthermore, the 2D culture is a flexible method that can be adapted to various experimental conditions (transfection, drug exposure, metabolic study, observations, etc.), allowing mechanistic explorations of cellular processes.

Limitations of this technique are the need for ethical permission, a good liaison with clinical staff, and the innate variability between samples. However, this latter limitation can be avoided by carefully selecting the placentas (similar gestational age, parity, and maternal condition) and repeated experiments. To overcome these difficulties, many research groups have turned to cell lines, including human embryonic stem cell-derived trophoblast cells (Amita *et al.*, 2013), human trophoblast stem cells (Okoe *et al.*, 2018), and choriocarcinoma cell lines. Even if the first two cell lines require further characterization, their use is justified when the study is focused on early placentation as first-trimester trophoblasts are difficult to obtain. However, the use of choriocarcinoma cell lines such as JAR, JEG3, or BeWo to study CTB differentiation and functions poses real methodological difficulties. There are growing concerns about the purity, identity, and behavior of these common cell lines (Abbas *et al.*, 2020). For instance, cAMP must be added to the culture medium to induce BeWo differentiation, while primary CTBs spontaneously fuse *in vitro*. Finally, genome-wide DNA methylation studies showed significant variability in comparison with primary cells, which is likely to contribute to differential expression profiles (Apps *et al.*, 2011; Novakovic *et al.*, 2011). Therefore, it becomes difficult to draw definitive conclusions when using such cell lines, which highlights the importance of validating findings by using primary trophoblasts.

Another alternative to primary human CTB cell culture is the use of placental explants from human placentas. Their culture is very easy and has the main advantage of keeping the CTBs in their complex tissue environment. It allows the comparison between placentas from normal pregnancies and

pathological placentas (preeclampsia or fetal growth restriction) (Orendi *et al.*, 2011). However, studies have shown that this technique impairs trophoblast viability and induces an unnatural oxidative stress (Di Santo *et al.*, 2003; Goncalves *et al.*, 2016). Since then, the use of explants in the study of placental biology must be restricted to comparisons with pregnancy-related diseases and must be limited in time. Finally, 3D cell culture models of human trophoblast cells that closely mimic the villous placenta were recently developed (Haider *et al.*, 2018; Turco *et al.*, 2018). These organoids, composed of a layer of CTBs surrounding a core of STBs, can also be differentiated into EVT. These models undoubtedly represent a breakthrough in the understanding of placental biology but require specific technical skills and a consequent budget. For these reasons, we are convinced that primary human term CTB cultures present undeniable advantages that still justify their place in the placental field.

Materials and Reagents

Disposable

1. Percoll (1.130 ± 0.005 g/ml) (GE Healthcare Bio-Sciences AB, catalog number: GE17-0891-01). Keep it sterile and conserve at 4°C
2. 10× Hank's Buffered Saline Solution w/o Ca, w/o Mg, w/o Phenol red (Gibco, Thermo Fisher Scientific, catalog number: 14175053). Keep it sterile and conserve at 4°C
3. 1× Hank's Buffered Saline Solution w/o Ca, w/o Mg, w/o Phenol red (Gibco, Thermo Fisher Scientific, catalog number: 14175095). Keep it sterile and conserve at 4°C
4. Dulbecco's modified Eagle medium (Thermo Fisher Scientific, catalog number: 12440061). Keep it sterile and conserve at 4°C
5. Iscove's Modified Dulbecco Medium (Gibco, Thermo Fisher Scientific, catalog number: 12440061). Keep it sterile and conserve at 4°C
6. Gentamicin 50 mg/ml (Gibco, Thermo Fisher Scientific, catalog number: 15750037). Keep it sterile and conserve at RT
7. Sterile heat-inactivated Fetal Bovine Serum (Gibco, Thermo Fisher Scientific, catalog number: 10082147). Keep it sterile. Aliquote and store at -20°C
8. Dulbecco's phosphate-buffered saline (DPBS) (Gibco, Thermo Fisher Scientific, catalog number: 14190359). Keep it sterile and conserve at 4°C
9. Lyophilized dispase II (Roche Diagnostics, catalog number: 4942078001). Store at 4°C
10. DNase I, grade II (Roche Diagnostics, catalog number: 10104159001). Resuspend at 10 mg/ml and store at -20°C
11. 4 L to 8 L NaCl 0.9% (Carl Roth GmbH + Co. KG, catalog number: 9265.2). Keep it sterile and conserve at 4°C
12. Nunc 200 ml conical centrifuge tubes (Thermo Fisher Scientific, catalog number: 376813)
13. 15 ml centrifuge tubes (Greiner Bio-One International GmbH, catalog number: 188261)
14. 50 ml centrifuge tubes (Greiner Bio-One International GmbH, catalog number: 227261)
15. 5 ml serological pipets (Greiner Bio-One International GmbH, catalog number: 606180)

16. 10 ml serological pipets (Greiner Bio-One International GmbH, catalog number: 607180)
17. 20 ml syringes (Greiner Bio-One International GmbH, catalog number: 474203)
18. 50 ml syringes (Greiner Bio-One International GmbH, catalog number: 475203)
19. Blunt-end 16 G needles (VWR International, catalog number: POPR7938)
20. Corning 40 µm disposable cell strainer (Corning, catalog number: 431750)
21. Plastics for cell culture. Coating with collagen or laminin is not necessary, but better results can be obtained by using pretreated plastics such as the Advanced TC products (Greiner Bio-One International GmbH, catalog number: 665980)

Non-Disposable Sterilized Materials

1. One dish stainless-steel (Carl Roth GmbH + Co. KG, catalog number: 0376.1)
2. One pair of big scissors (Carl Roth GmbH + Co. KG, catalog number: YE80.1)
3. One pair of little scissors (Carl Roth GmbH + Co. KG, catalog number: HEY6.1)
4. One tweezer straight (Carl Roth GmbH + Co. KG, catalog number: 2802.1)
5. One big round sieve (Carl Roth GmbH + Co. KG, catalog number: 8101.1)
6. One little round sieve (Carl Roth GmbH + Co. KG, catalog number: 8096.1)
7. One funnel (Carl Roth GmbH + Co. KG, catalog number: 0166.1)
8. One flat glass recipient (Carl Roth GmbH + Co. KG, catalog number: HX16.1)
9. One 500 ml baffled flasks with screw closure (Carl Roth GmbH + Co. KG, catalog number: PK17.1)
10. One 500 ml baffled flasks, straight neck (Carl Roth GmbH + Co. KG, catalog number: LY96.1)
11. One 1 L beaker (Carl Roth GmbH + Co. KG, catalog number: X695.1)
12. One 2 L beaker (Carl Roth GmbH + Co. KG, catalog number: X696.2)
13. One stainless-steel test sieve, pore size 200 µm (Retsch, catalog number: 60.106.000200)
14. One stainless-steel test sieve, pore size 100 µm (Retsch, catalog number: 60.106.000100)
15. One stainless-steel test sieve, pore size 40 µm (Retsch, catalog number: 60.106.000040)

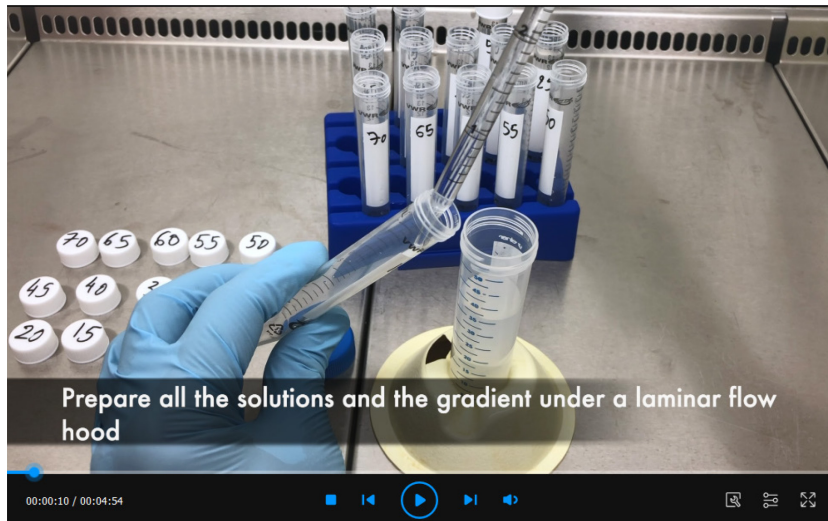
Equipment

1. Pipet controller
2. Vacuum machine
3. Bucket
4. Refrigerated centrifuge at 4°C with adaptors for 15 ml tubes, 50 ml tubes, and 200 ml centrifugation tubes
5. Stand with a clamp
6. Surgical lamp (cold light)
7. Laminar flow hood
8. Atmosphere-controlled humidified incubator
9. Scale

10. Shaking water bath at 37°C
11. Fluorescence microscope and camera

Procedure

See the video 1 joined to this protocol to get a complete overview of the procedure.



Video 1. How to isolate of primary cytotrophoblasts from human placenta at term: an overview of the procedure accompanied by Summer vibes

A. Discontinuous Percoll Gradient Preparation

1. Prepare all the solutions and the gradient under a laminar flow hood.
2. Prepare one solution of 90% Percoll by mixing 19.8 ml of stock Percoll (100%) with 2.2 ml of 10× HBSS.
3. Prepare the following dilutions in 15 ml tubes as indicated in **Table 1**. Briefly centrifuge the solutions and store them at 4°C. The Percoll dilutions can be prepared several days before the isolation procedure.

Table 1. Preparation of the Percoll dilutions for the discontinuous gradient

%	90% Percoll (ml)	1× HBSS (ml)
70	2.8	0.8
65	2.6	1
60	2.4	1.2
55	2.2	1.4
50	2	1.6
45	1.8	1.8
40	1.6	2

35	1.4	2.2
30	1.2	2.4
25	1	2.6
20	0.8	2.8
15	0.6	3
10	0.4	3.2
5	0.2	3.4

4. In a 50 ml tube, delicately drop off 3 ml of each dilution, layer-by-layer, starting with the 70% dilution. To facilitate the process, use a 5 ml serological pipet with the pipet controller set on the passive mode and let the solution descend very slowly.
5. Keep the gradient at 4°C for optimal separation of the cells. It must be freshly prepared the day of the isolation.

B. Placenta Collection

1. Only include placentas retrieved from strictly normal singleton pregnancies at term (37-41 weeks of gestation), without any fetal (growth restriction, malformations, genomic abnormalities, or infection), placental (unique umbilical artery, placenta praevia, or placenta accreta), or maternal (chronic diseases, gestational diabetes, preeclampsia, medications, alcohol, or drugs) complications.
2. Collect material on ice immediately after cesarean section and proceed with digestion within one hour.

C. Tissue Preparation

1. Put the placenta in the stainless steel container and roughly remove the amniotic membranes with the pair of big scissors.
2. Cut the placenta into 2 × 2 cm pieces and put them in the 2 L beaker (Figure 1A).
3. Wash the tissue by adding 500 ml of NaCl 0.9%. Mix well and drain the pieces in the big sieve placed on the bucket (Figure 1B).
4. Repeat this operation until the wash solution remains clear (4 L to 8 L needed).

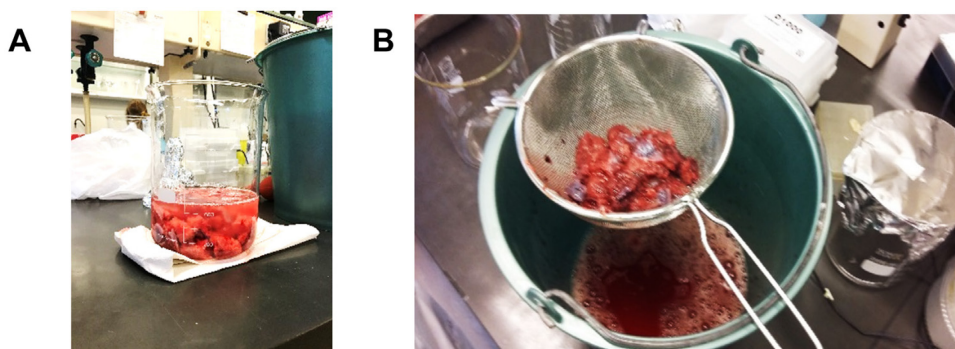


Figure 1. Washing step. A. After cutting the placenta into pieces, put them in the 2 L beaker and add 500 ml of NaCl 0.9%. B. Shake the beaker vigorously and drain the tissue in the sieve. Repeat this operation several times. After 4 to 8 L of NaCl 0.9%, the pieces must turn light red, and the wash solution must remain clear.

5. Place the pieces in the glass container and delicately dry the tissue with paper towels (Figure 2A).
6. With the pair of little scissors and the tweezers, remove the big blood vessels (Figure 2B), the chorionic plate and the remaining amniotic membranes (the white side) (Figure 2C and 2D), the decidua (the pearly opposite side), and the blood clots to facilitate the digestion and to reduce the amount of debris.

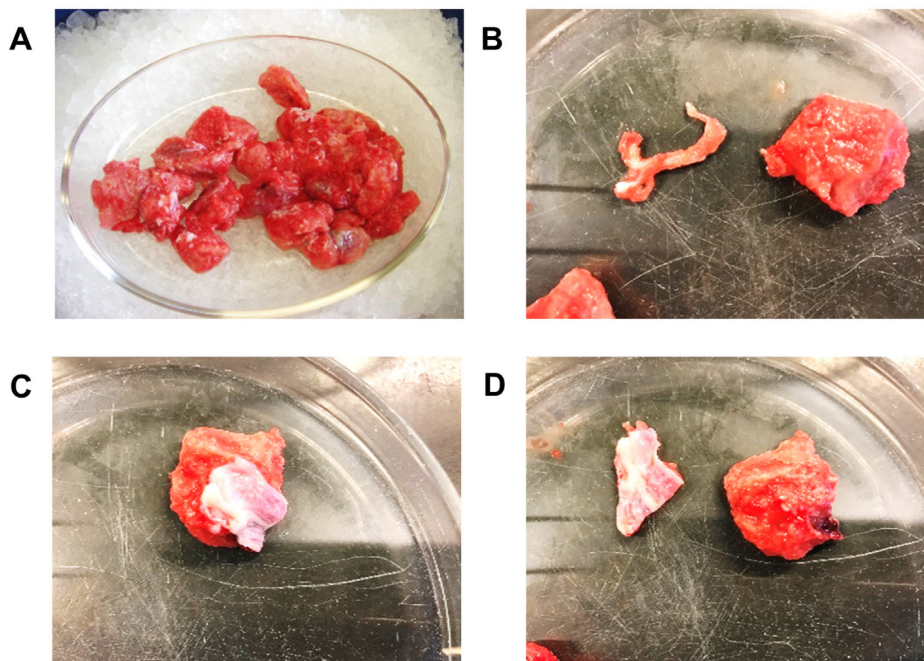


Figure 2. Cleaning step. Prepare the pieces for digestion. A. After drying with paper towels, B. Remove big blood vessels and C-D. the chorionic plate. This step can be time-consuming, but reducing the amount of debris improves digestion and protocol yield.

7. Using the scale, weigh 72 g of tissue and chop the fragments with the pair of big scissors (Figure 3A).

D. Enzymatic Digestion

1. In the water bath, preheat 300 ml of 1× HBSS in the 500 ml baffled flasks with screw closure.
2. Dissolve 720 units of lyophilized dispase I in the preheated 1× HBSS to obtain a solution at 2.4 units/ml.

3. Add the finely minced placental tissue to the dispase solution and incubate 1 h in the water bath under strong shaking (Figure 3B).
4. Add 1.2 ml of DNase I 10 mg/ml to the digested tissue to obtain a final concentration of 100 units/ml.
5. Incubate in the water bath under strong shaking for another 15 min.

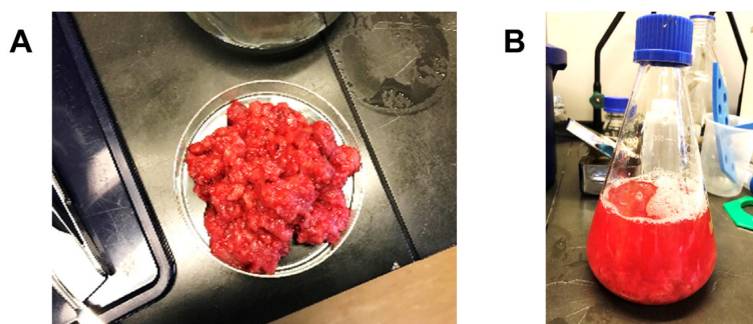


Figure 3. Digestion step. Dissolve 720 units of dispase I in 300 ml of preheated HBSS. A. Chop the pieces with the pair of big scissors and transfer the pulp to the baffled flask. B. Incubate the mixture 1 h in the water bath under strong shaking. Add predissolved DNase I and incubate for another 15 min.

E. Cell Isolation and Purification

1. Prefilter the suspension using in the little sieve placed on the 1 L beaker (Figure 4A).
2. Fix the three test sieves in decreasing order (pore diameter: 200 μm -100 μm -40 μm) on the stand with the clamp.
3. Place the stainless steel funnel and the 500 ml baffled flasks with a straight neck below the filters.
4. Let the suspension pass through the filtration system. If the filtration is difficult, agitate the test sieves to move debris and draw air. Alternatively, a porcelain spoon can also be used to remove blocking dirt (Figure 4B).

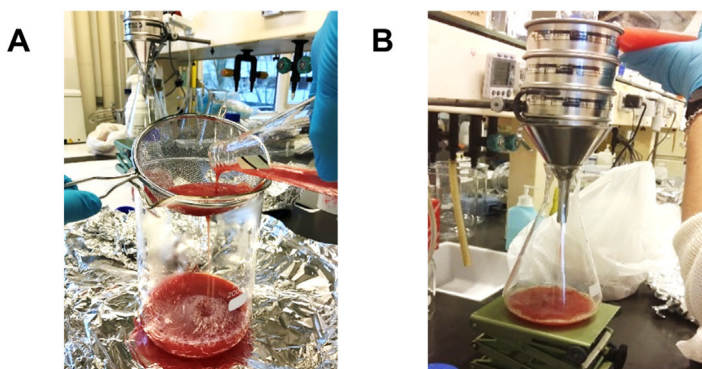


Figure 4. Filtration step. A. After the digestion, the suspension is first prefiltered by using the little sieve. B. The suspension is then passed through the three test sieves, fixed on the stand

by decreasing order. The filtrate is finally centrifuged; the pellet contains red blood cells, lymphocytes, debris, and cytotrophoblasts.

5. Divide the filtrate into two centrifugation bottles and complete with IMDM or DMEM medium supplemented with gentamicin (final concentration 50 µg/ml). This will be referred to as “wash medium” in the rest of the procedure.
6. Centrifuge 20 min at 500 × *g*.
7. Aspirate the supernatant. The pellet contains red blood cells, lymphocytes, debris, and cytotrophoblasts. Be careful not to aspirate the pellet, as it is friable at this step of the procedure.
8. Resuspend the pellet with 10 ml of wash medium and transfer to a new 50 ml tube.
9. Complete to 50 ml with wash medium and centrifuge 5 min at 500 × *g*.
10. Aspirate the supernatant and resuspend the pellet in 6 ml of wash medium.
11. Delicately lay down these 6 ml on the surface of the gradient with the pipet controller (Figure 5A and 5B).

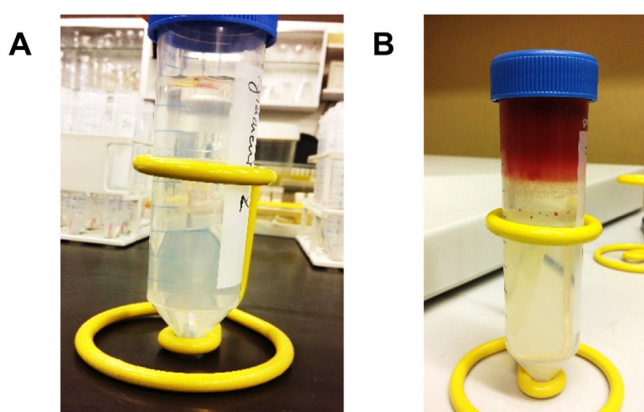


Figure 5. Gradient step. The 6 ml contains a mix of debris, red blood cells, lymphocytes, and cytotrophoblasts. The discontinuous Percoll gradient allows separating the cell type by density. A. The gradient was freshly prepared on the day of isolation and stored at 4°C until use. B. After the filtration step, the cell suspension is meticulously deposited at the surface of the gradient. It is then centrifuged to separate the cells between the different layers.

12. Centrifuge 20 min at 1,200 × *g* without brake.
13. After centrifugation, place the gradient in front of the surgical lamp (cold light) to visualize the cells. The cytotrophoblasts form a thick layer localized between 12.5 ml and 25 ml, which corresponds to a density of 1.048-1.062 g/ml (layers 45%-40%-35%) (Figure 6). This has been previously determined with density marker beads. The debris must be localized in the low-density layers, whereas the lymphocytes can be visualized below the cytotrophoblasts. Some red blood cells may be observed within the cytotrophoblast layer, but the majority of them should be located at the bottom of the tube.

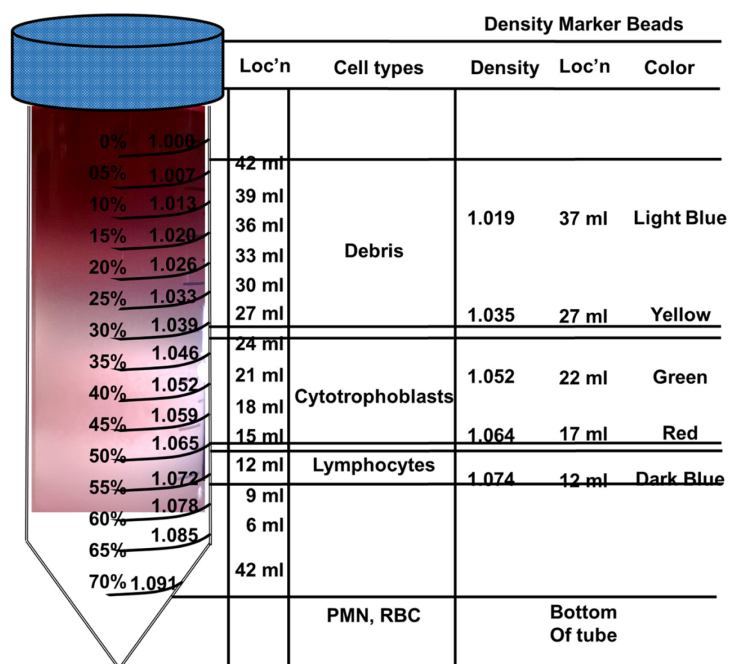


Figure 6. The discontinuous Percoll gradient after centrifugation. The cells form a compact cloud between 12.5 and 20 ml. The debris (red) are above, while the red blood cells form a pellet at the bottom of the tube. Some lymphocytes can be observed around the 12 ml graduation.

14. Remove by aspiration the upper layers corresponding to the debris with a 50 ml syringe and a blunt-end 15 G needle.
15. With a new 20 ml syringe and the other blunt-end 15 G needle, aspirate the cell layer corresponding to the cytotrophoblasts and put them in a new 50 ml tube.
16. Complete the volume to 50 ml with wash medium and centrifuge 5 min at $300 \times g$.
17. The cytotrophoblasts form a white pellet at the bottom of the tube. Some red blood cells can be occasionally observed.
18. Remove the supernatant and thoroughly resuspend the pellet with 6 ml of 0.05% trypsin.
19. Manually agitate the tube 1 min in the water bath and stop the reaction by immediately placing the tube on ice.
20. Add wash medium rapidly to 50 ml and centrifuge 5 min at $300 \times g$.
21. Remove the supernatant and resuspend the cell pellet in 10 ml of wash medium.
22. Filtrate the solution with a 40- μ m disposable cell strainer.
23. Complete the volume to 50 ml with wash medium and proceed to counting.

F. Cell Counting and Culture

1. Dilute 60 μ l of the cell suspension in 260 μ l of wash medium.
2. Add 100 μ l of 0.4% Trypan blue.
3. Count the cells with a hemocytometer (Bürker) and determine the percentage of viability.

4. Centrifuge the cell suspension 5 min at $300 \times g$.
5. Resuspend the pellet in complete culture medium (IMDM-10% FBS, gentamicin 50 $\mu\text{g/ml}$). From now, the cytotrophoblasts must be cultured with IMDM.
6. Plate the cells at a density of 4.0×10^5 cells/cm² to achieve immediate confluence (for example, 1.5×10^6 cells/well in a 12-well plate).
7. Place the cells in an atmosphere-controlled humidified incubator under 21% O₂-5% O₂.
8. The day after, wash the cells with preheated 1× DPBS to remove debris, syncytial knots, and unattached cells.
9. Aspirate the DPBS and add preheated culture medium. Change the medium every 24 h.
10. The cells can be cultured for several days (up to 21 days). Some syncytiotrophoblasts can already be observed after 24 h, and the maximum syncytialization is achieved 72-96 h after isolation.
11. We advise checking the purity of the culture the first few times the protocol is performed ([Supplementary file](#)).

Acknowledgments

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Ethics

The work performed in our lab was conducted with the approval of the Ethical Committee of the Catholic University of Louvain and the Saint-Luc University Hospital (Brussels, Belgium) under the reference 2018/23OCT/397. Informed consent was obtained from all patients.

Competing interests

The authors declare no competing interest.

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