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Title: Purification of HLA-G⁺ Extravillous Trophoblasts from Human Term Placental Tissues for Phenotyping and Functional Analysis

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Author Questionnaire

- 1. Microscopy:** Does your protocol require the use of a dissecting or stereomicroscope for performing a complex dissection, microinjection technique, or something similar? **No**

- 2. Software:** Does the part of your protocol being filmed include step-by-step descriptions of software usage? **No**

- 3. Filming location:** Will the filming need to take place in multiple locations? **No**

- 4. Testimonials (optional):** Would you be open to filming two short testimonial statements **live during your JoVE shoot**? These will **not appear in your JoVE video** but may be used in JoVE's promotional materials. **Yes**

Current Protocol Length

Number of Steps: 29

Number of Shots: 57

Introduction

Videographer: Obtain headshots for all authors available at the filming location.

Authors: Please note that **the questions will not appear on screen**. Please answer in **stand-alone full sentence with sufficient context**.

INTRODUCTION:

~~**REQUIRED:** Summarize your research focus and the main questions it aims to answer.~~

- 1.1. **Tamara Tilburgs:** We aim to investigate the phenotypic and functional properties of human extravillous trophoblasts from placenta, under physiological and pathological conditions.
 - 1.1.1. INTERVIEW: Named Talent says the statement above in an interview-style shot, looking slightly off-camera.

~~Describe the research gap or limitation in existing methods and how this protocol addresses them.~~

- 1.2. **Tamara Tilburgs:** This protocol enables a robust and reproducible platform for advancing understanding of primary human EVT overcoming their fragility.
 - 1.2.1. INTERVIEW: Named Talent says the statement above in an interview-style shot, looking slightly off-camera.

CONCLUSION:

~~What does this protocol allow researchers to study or measure?~~

- 1.3. **Tamara Tilburgs:** This protocol provides viable EVT that are suitable for immunological functional assays, protein and gene expression analysis.
 - 1.3.1. INTERVIEW: Named Talent says the statement above in an interview-style shot, looking slightly off-camera.

~~Describe the most important consideration or challenge when performing this protocol.~~

- 1.4. **Tamara Tilburgs:** It is crucial to start EVT isolation from the placenta immediately after delivery and to not refrigerate the placenta at any time.
 - 1.4.1. INTERVIEW: Named Talent says the statement above in an interview-style shot, looking slightly off-camera.

~~Are there any additional methods or analyses that can be performed following this procedure?~~

- 1.5. **Tamara Tilburgs:** Primary EVT cultures maintain viability for 96 h, allowing co-culture with immune cells and subsequent flow cytometric analysis.

- 1.5.1. INTERVIEW: Named Talent says the statement above in an interview-style shot, looking slightly off-camera.

~~How can future studies build on this work? Describe the questions or research directions that can be investigated next.~~

- 1.6. **Tamara Tilburgs:** Future studies can utilize the cells for genome editing to provide further mechanistic insights in the workings of maternal-fetal immune tolerance and inflammation.

- 1.6.1. INTERVIEW: Named Talent says the statement above in an interview-style shot, looking slightly off-camera.

Videographer: Obtain headshots for all authors available at the filming location.

Testimonial Questions (OPTIONAL):

Videographer: Please capture all testimonial shots in a wide-angle format with sufficient headspace, as the final videos will be rendered in a 1:1 aspect ratio. Testimonial statements will be presented live by the authors, sharing their spontaneous perspectives.

- Testimonial statements will **not appear in the video** but may be featured in the journal's promotional materials.
- **Provide the full name and position** (e.g., Director of [Institute Name], Senior Researcher [University Name], etc.) of the author delivering the testimonial.
- Please **answer the testimonial question live during the shoot**, speaking naturally and in your own words in **complete sentences**.

What problem in your field of research does your article address?

1.6. **Tamara Tilburgs, [Associate Professor]**: (authors will present their testimonial statements live)

1.6.1. INTERVIEW: Named Talent says the statement above in an interview-style shot, looking slightly off-camera.

What value do you expect this video article to have for other researchers working in your field?

1.7. **Tamara Tilburgs, [Associate Professor]**: (authors will present their testimonial statements live)

1.7.1. INTERVIEW: Named Talent says the statement above in an interview-style shot, looking slightly off-camera.

AUTHORS: Please provide your position in the institute

Ethics Title Card

This research has been approved by the Institutional Review Board (IRB) at the Cincinnati Children's Hospital

Protocol

2. Isolation and Separation of Chorionic Membrane and Decidua Parietalis from Human Placenta

Demonstrators: Tamara Tilburgs, Angel Dasenbrock,

- 2.1. To begin, obtain a human placenta sample [1]. Place the placenta with the fetal side facing upward so the cord insertion site is visible [2].
 - 2.1.1. WIDE: Talent holding a dish with the placenta. NOTE: 2.1.1 & 2.2.1 were filmed together
 - 2.1.2. Talent positioning the placenta with the fetal side upward showing the cord insertion.
- 2.2. Manually peel the amnion from the lateral membrane toward the umbilical cord insertion site and remove it [1]. Then use scissors to cut and discard the amnion [2].
 - 2.2.1. Talent manually peeling the amnion toward the cord insertion site.
 - 2.2.2. Talent cutting and discarding the removed amnion.
- 2.3. Cut the choriodecidual membrane into 2-inch strips starting from the base of the placenta upward while keeping each strip attached to the placenta [1].
 - 2.3.1. Talent cutting membrane strips while keeping attachment to the placenta.
- 2.4. Place the choriodecidual membrane strip across two fingers with the decidual side facing upward [1]. With a pair of sterile curved tip forceps, gently scrape the maternal decidua parietalis from the chorion [2].
 - 2.4.1. Talent positioning the membrane strip on fingers with the decidual side up. NOTE: 2.4.1 & 2.4.2 were filmed together
 - 2.4.2. Talent scraping decidua parietalis from the chorion with curved forceps.
- 2.5. Collect the chorionic membrane into a 50-milliliter conical tube containing 20 milliliters of Wash Medium A [1]. Then, collect the decidua parietalis into a 50-milliliter conical tube containing 20 milliliters of PBS for lymphocyte isolation [2].
 - 2.5.1. Talent placing chorionic membrane pieces into a labeled 50 milliliter tube containing Wash Medium A. NOTE: 2.5 was taped together/Take 3
 - 2.5.2. Talent transferring decidua parietalis into a tube with phosphate buffered saline.
- 2.6. Using scissors, cut the chorionic membrane inside the 50-milliliter tube into pieces

smaller than 2 square millimeters [1].

2.6.1. Talent cutting membrane pieces into very small fragments inside the tube.

NOTE: 2.6.1 - 3.3 were filmed together

2.7. Discard Wash Medium A from the tube [1]. Wash the tissue repeatedly with PBS until the supernatant becomes clear [2].

2.7.1. Talent removing Wash Medium A from the tube.

2.7.2. Talent adding PBS to the tissue and removing it.

AUTHORS: Ideally, show only the last wash or a representative wash

3. Dissection and Preparation of Decidua Basalis from Human Placenta

3.1. For placental dissection, place the placenta with the maternal side facing upward and remove blood clots [1]. Using sterile scissors, cut pieces of placenta basal plate while avoiding necrotic, calcified, or ischemic areas [2].

3.1.1. Talent positioning placenta maternal side upward and clearing clots.

3.1.2. Talent cutting basal plate pieces of specified size.

3.2. Place the decidua basalis piece on the fingers with villi facing upwards [1]. Then use scissors to carefully remove the villi [2] to obtain 2 to 3-millimeter membrane with only a thin villous layer remaining [3].

3.2.1. Talent positioning decidua basalis on fingers with villi upward.

3.2.2. Talent trimming villi.

3.2.3. Shot of the membrane with a thin villous layer.

3.3. Collect the decidua basalis tissue into a 50-milliliter conical tube containing 20 milliliters PBS [1]. Chop the tissue into pieces smaller than 2 square millimeters [2].

3.3.1. Talent transferring decidua basalis to tube with phosphate buffered saline.

3.3.2. Talent chopping tissue into very small fragments.

3.4. Now discard the PBS from the tube [1]. Wash the decidua basalis repeatedly with PBS until the supernatant is clear without visible blood [2]. Then split 15 milliliters of tissue suspension into two 50-milliliter conical tubes [3].

3.4.1. Talent removing phosphate buffered saline. **NOTE: 3.4 was filmed together**

3.4.2. Talent adding PBS to the tissue until no visible blood remains.

3.4.3. Talent dividing tissue suspension equally into two tubes.

4. Enzymatic Digestion of the Chorionic Membrane and Decidua Basalis

- 4.1. Fill all tubes with PBS [1]. Centrifuge the tubes at 200 *g* for 1 minute [2] and pour off the supernatant [3].
 - 4.1.1. Talent filling tubes with phosphate buffered saline.
 - 4.1.2. Talent placing tubes in centrifuge.
 - 4.1.3. Shot of the supernatant being poured out. **NOTE: 4.1.3 - 4.3.2 were filmed together**
- 4.2. Add 150 millilitres of warm tissue digestion enzyme cocktail into a 300-milliliter glass bottle [1]. Add 30 millilitres of chorionic membrane to the bottle [2].
 - 4.2.1. Talent pouring enzyme cocktail into glass bottle.
AND
LAB MEDIA: Table 1
Video Editor: Please play both shots side by side in a split screen
 - 4.2.2. Talent adding chorionic membrane tissue to the bottle.
- 4.3. Add 20 millilitres warm DMEM/F20 (*D-M-E-M-F-Twenty*) and 80 millilitres warm tissue digestion enzyme cocktail into two separate 300-milliliter glass bottles [1]. Then pipette 7.5 millilitres decidua basalis into each bottle [2].
 - 4.3.1. Talent adding 20 mL DMEM/F20 and 80 mL enzyme cocktail into 2 different 300 mL bottles.
 - 4.3.2. Talent adding 7.5 mL decidua basalis tissue to each bottle.
- 4.4. Incubate the bottles in a water bath [1-TXT]. Shake all bottles every 3 to 4 minutes during the 15-minute incubation [2].
 - 4.4.1. Talent placing bottles into designated water baths. **TXT: Incubation: Chorionic membrane (39 °C), Decidua basalis(37 °C)** **NOTE: 4.4 was filmed together**
 - 4.4.2. Talent intermittently shaking bottles during incubation.
- 4.5. After digestion, filter the tissue through metal sieves with a mesh size of 40 [1]. Then add Wash Medium B to rinse tissues and dissolve gelatinous material [2].
 - 4.5.1. Talent pouring digested tissue through metal sieve. **NOTE: 4.5 was filmed together**
 - 4.5.2. Talent adding Wash Medium B over the sieve.
- 4.6. Transfer the undigested tissue back into glass bottles and repeat digestion [1]. Now filter the cell suspensions through a 100-micrometer filter into labeled 50-milliliter tubes [2]. **NOTE: The VO is edited for the inverted shots**
 - 4.7.1. Talent returning undigested tissue into bottles for second digestion.

- 4.6.1. Talent filtering suspension through 100 micrometer filter into labeled tubes.
NOTE: 2.7.1 is placed before 2.6.1
- 4.7. ~~Transfer the undigested tissue back into glass bottles and repeat digestion [1].~~ Keep cells from the first and second digestions separate [1].
 - ~~4.7.1. Talent returning undigested tissue into bottles for second digestion.~~ **NOTE: This shot is moved before 2.6.1**
 - 4.7.2. Shot of digested tissue in labeled separate tubes.
- 4.8. Then centrifuge the cell suspension at 650 *g* for 8 minutes [1]. Discard the supernatant [2].
 - 4.8.1. Talent placing tubes into centrifuge. **NOTE: 4.8.1 can be reused for centrifugation shots**
 - 4.8.2. Shot of the supernatant being removed from the centrifuged tubes. **NOTE: 4.8.2-4.9.2 are filmed together**
- 4.9. Combine pellets of the same tissue type and digestion number into one tube [1]. Then make up the volume with Wash Medium B up to 25 milliliters [2].
 - 4.9.1. Talent combining pellets into a single tube.
 - 4.9.2. Talent adding Wash Medium B to 25 mL.

5. Ficoll Separation, Antibody Staining, and Sorting of Extravillous Trophoblast Cells

- 5.1. Add 12 milliliters Ficoll to four 50-milliliter conical tubes labeled with tissue type and digestion number [1]. **NOTE: The VO is edited for the deleted shot**
 - ~~5.1.1. Talent labeling tubes.~~ **NOTE: Not filmed**
 - 5.1.2. Talent adding 12 mL Ficoll into each tube. **NOTE: 5.1.2 and 5.2.1 are filmed together**
- 5.2. Carefully layer the cell suspension onto the Ficoll [1]. Then centrifuge at 800 *g* for 20 minutes at room temperature without brake [2].
 - 5.2.1. Talent layering suspension over Ficoll.
 - 5.2.2. Talent placing tubes into centrifuge.
- 5.3. Pipette out the cell layer at the Ficoll-medium interface into clean labeled tubes [1].
 - 5.3.1. Talent aspirating interface layer into new tubes. **NOTE: 5.3.1 and 5.4.1 are filmed together**
- 5.4. Then fill tubes with Wash Medium B [1] and centrifuge at 650 *g* for 8 minutes [2].

- 5.4.1. Talent adding Wash Medium B.
- 5.4.2. Shot of the tubes in the centrifuge.
- 5.5. After discarding the supernatant, resuspend the pellets in the remaining medium [1]. ~~Keep approximately 10 percent of the suspension for phenotyping and use 90 percent for sorting if needed [2-TXT].~~ NOTE: The VO is edited for the deleted shot
- 5.5.1. Talent resuspending pellets in the remaining medium.
- 5.5.2. ~~Talent separating small aliquot for phenotyping. TXT: Count cells for cell number and viability~~ NOTE: Not filmed
- 5.6. Place the resuspended pellets into sterile fluorescence-activated cell sorting tubes labeled with tissue type and digestion number [1]. Add anti-CD45-BV785 (*Anti-C-D-Forty-Five-B-V-Seven-Eighty-Five*), anti-HLA-G-APC (*Anti-H-L-A-G-A-P-C*), and anti-EGFR1-BV711(*Anti-E-G-F-R-One-B-V-Seven-Eleven*) antibodies [2]. Incubate for 20 minutes at room temperature in the dark [3].
- 5.6.1. Talent transferring cells into fluorescence activated cell sorting tubes.
- 5.6.2. Talent adding antibodies to tubes. NOTE: 5.6.2 and 5.6.3 are filmed together
- 5.6.3. Talent placing tubes protected from light for incubation.
- ~~5.7. After incubation, pipette 1 milliliter Wash Medium B into the tubes [1]. Then centrifuge at 650 g for 8 minutes [1].~~ NOTE: 5.7. and 5.8. are not filmed. The VO for the deleted steps is added concisely in the next step
- ~~5.7.1. Talent adding 1 mL Wash Medium B into the tubes.~~
- ~~5.7.2. Shot of the tubes in the centrifuge.~~
- 5.8. ~~Discard the supernatant [1] and resuspend the pellets with Wash Medium B [2-TXT].~~
- ~~5.8.1. Shot of supernatant being pipetted out~~
- ~~5.8.2. Talent resuspending cells to the required concentration. TXT: Final cell concentration: 1×10^6 cells/mL cell suspension~~
- 5.9. After washing and resuspending the cells in wash medium B, immediately pass them through a 40-micrometer cell strainer [1]. Sort CD45-negative HLA G-positive EGFR-positive extravillous trophoblast into tubes containing 1 milliliter EVT Culture Medium A [2].
- 5.9.1. Talent filtering cells through a 40 micrometer strainer.
- 5.9.2. Shot of labeled tubes before sorting.

Results

6. Results

- 6.1. Flow cytometry analysis of lineage markers showed viable primary extravillous trophoblast culture until day 4 [1].
 - 6.1.1. LAB MEDIA: Figure 1B–D. *Video editor: Show panels B, C, and D together. Highlight the downward lines in C and D*
- 6.2. Representative light microscopy images of primary chorionic and decidua basalis HLA-G positive extravillous trophoblast were captured after 24 hours [1], 48 hours [2], and 72 hours of culture [3].
 - 6.2.1. LAB MEDIA: Figure 2A–F. *Video editor: Highlight the images labeled “24hr” for chorionic EVT and d.basalis EVT.*
 - 6.2.2. LAB MEDIA: Figure 2A–F. *Video editor: Highlight the images labeled “48hr” for chorionic EVT and d.basalis EVT.*
 - 6.2.3. LAB MEDIA: Figure 2A–F. *Video editor: Highlight the images labeled “72hr” for chorionic EVT and d.basalis EVT.*
- 6.3. Extravillous trophoblast expressed polymorphic HLA-C and immunosuppressive co-inhibitory molecules [1].
 - 6.3.1. LAB MEDIA: Figure 3A,B. *Video editor: highlight the green plots for Chorionic EVT in A and the corresponding green plots in B*
- 6.4. Extravillous trophoblast increased the expression of HLA-C, PD-L1, PD-L2 and HLA-E [1], but did not increase the expression of PVR and B7H3 when co-cultured with activated lymphocytes for 72 hours [2].
 - 6.4.1. LAB MEDIA: Figure 4A *Video editor: Highlight the pink HLA-C, PD-L1, PD-L2 and HLA-E plots in the bottom row (Chorionic EVT)*
 - 6.4.2. LAB MEDIA: Figure 4A *Video editor: Highlight the pink PVR and B7H3 plots in the bottom row (Chorionic EVT)*
- 6.5. No increase in extravillous trophoblast death was observed following co-culture with activated lymphocytes [1].
 - 6.5.1. LAB MEDIA: Figure 4D.

1. Placenta
Pronunciation link: <https://www.merriam-webster.com/dictionary/placenta>
IPA: /plə'sɛn.tə/
Phonetic Spelling: pluh·sen·tuh
2. Amnion
Pronunciation link: <https://www.merriam-webster.com/dictionary/amnion>
IPA: /'æm.ni.ɑ:n, 'æm.ni.ən/
Phonetic Spelling: am·nee·on
3. Chorion
Pronunciation link: <https://www.merriam-webster.com/dictionary/chorion>
IPA: /'kɔ:r.i.ɑ:n, 'kɒr.i.ɑ:n/
Phonetic Spelling: kor·ee·on; kohr·ee·on
4. Decidua
Pronunciation link: <https://www.merriam-webster.com/dictionary/decidua>
IPA: /dɪ'sɪdʒ.u.ə/
Phonetic Spelling: dih·sij·oo·uh
5. Centrifuge
Pronunciation link: <https://www.merriam-webster.com/dictionary/centrifuges>
IPA: /'sɛn.trəˌfjuːdʒ/
Phonetic Spelling: sen·truh·fyooj
6. Lymphocyte
Pronunciation link: <https://www.merriam-webster.com/dictionary/lymphocytes>
IPA: /'lɪm.fəˌsaɪt/
Phonetic Spelling: lim·fuh·syte
7. Trophoblast
Pronunciation link: <https://www.merriam-webster.com/dictionary/trophoblast>
IPA: /'troʊ.fəˌblæst/
Phonetic Spelling: troh·fuh·blast
8. Phenotyping
Pronunciation link: <https://www.merriam-webster.com/dictionary/phenotyping>
IPA: /'fiː.nəˌtaɪ.pɪŋ/
Phonetic Spelling: fee·nuh·ty·ping
9. Polymorphic
Pronunciation link: <https://www.merriam-webster.com/medical/polymorphic>
IPA: /ˌpɑː.li'mɔːr.fɪk/
Phonetic Spelling: pah·lee·mor·fik
10. Immunosuppressive
Pronunciation link: <https://www.merriam-webster.com/dictionary/immunosuppressive>
IPA: /ɪˌmjuː.noʊ.sə'pres.ɪv/
Phonetic Spelling: ih·myoo·noh·suh·pres·iv
11. Extravillous
Pronunciation link: <https://www.howtopronounce.com/extravillous>

IPA: /ˌek.strəˈvɪl.əs/

Phonetic Spelling: ek·struh·vil·uhs

12. Parietalis

Pronunciation link: <https://www.howtopronounce.com/parietalis>

IPA: /pəˈraɪ.əˈtæɪ.lɪs/

Phonetic Spelling: puh·rye·uh·tal·is

13. Basalis

Pronunciation link: <https://www.howtopronounce.com/basalis>

IPA: /bəˈseɪ.lɪs/

Phonetic Spelling: buh·say·lis

14. Ficoll

Pronunciation link: <https://www.howtopronounce.com/ficoll>

IPA: /ˈfaɪ.kɔːl/

Phonetic Spelling: fy·kawl