Study Title: Long Term Immune Memory Responses to Human Papillomavirus (HPV) Vaccination Following 2 Versus 3 Doses of Quadrivalent HPV Vaccine

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List of Abbreviations

AEFI	Adverse event following immunization
CIN	Cervical intraepithelial neoplasia
CRF	Case report form
eCRF	Electronic case report form
GCP	Good clinical practices
GMT	Geometric mean titers
HPV	Human papillomavirus
hr-HPV	High risk HPV
ICC	Invasive cancer of the cervix
ICF	Informed consent form
IM	Intramuscular
PID	Participant identification number
Q-HPV	Quadrivalent human papillomavirus – Gardasil®
REB	Research ethics board
SAE	Serious Adverse Event
SID	Secondary identifier
SOP	Standard of practice

PROTOCOL

Long Term Immune Memory Responses to Human Papillomavirus (HPV) Vaccination Following 2 Versus 3 Doses of Quadrivalent HPV Vaccine

1.0 INTRODUCTION/BACKGROUND

Cervical cancer is the second most commonly diagnosed cancer among females worldwide and affects over half a million women globally each year. (1) In Canada, cervical cancer is the second most common cancer in women aged 20-49 (2). Human papillomavirus (HPV) is known to be the etiological agent of cervical cancer and causes cancer by targeting the basal cell of squamous epithelium located in the transformation zone of the cervix (3). HPV types 16, 18, 31, 33, 35, 45, 51, 52, and 56 have been consistently associated with severe grades of cervical intraepithelial neoplasia (CIN) and invasive carcinoma and are thus classified as high risk HPV (hr-HPV) (4). Sexual activity and age of first sexual intercourse are among the primary determinants of HPV infection and therefore interventions aimed at preventing acquisition of oncogenic HPV infection and cervical cancer should begin before the onset of first sexual activity (5-7).

Persistent HPV Infection: HPV is a highly prevalent infection, and it is estimated that over 75% of women have a cervical HPV infection at some time in their life. (8). Natural history studies show that most cervical infections with hr-HPV clear spontaneously (9), but in 10-20% of women, these infections persist and it is these persistent infections that can lead to grade 2/3 cervical intraepithelial neoplasm (CIN) (10) and eventually invasive cancer of the cervix (ICC) (11). Cross sectional prevalence studies show that the burden of HPV infection is concentrated in younger women (<30), but these infections in younger women are often transient and most will clear spontaneously (12). Meta-analyses indicate that women who have hr-HPV types 16 and 18 persisting for 6-12 months or more have significantly increased risk of developing incident high grade CIN (13).

HPV vaccine and cervical cancer prevention: Until recently, screening and treatment of cervical abnormalities, through public health programs, were the primary methods of controlling cervical cancer. However, the availability of the HPV vaccine has changed the focus of cervical cancer prevention, and primary prevention of cervical cancer is now a critical opportunity (14-15). Gardasil® (Q-HPV) is approved for a 3 dose schedule at 0, 2 and 6 months or a 2 dose schedule at 0 and 6 months, and is highly efficacious and immunogenic (16-17). Phase III trials have shown that a three-dose regimen of Q-HPV is 98 - 100% effective in preventing HPV 16, and 18 related CIN2/3 out to 5 years (16-18). Cervarix® (GlaxoSmithKline), a bivalent HPV vaccine approved in Canada (19), is also highly immunogenic and studies have shown 100% effective out to 60 months in preventing HPV 16 and 18 related CIN2/3 with geometric mean titers (GMTs) substantially higher than natural infection titers.

Current HPV Vaccination Programs in Canada: All Canadian provinces have school based HPV vaccine program for girls. Although all programs aim to provide HPV vaccine prior to girls' sexual debut, each jurisdiction incorporated the HPV vaccine into existing school based programs and thus, nationally, girls are given HPV vaccine during different grades. In addition, some provinces have catch up programs. In 2008, Quebec launched its program using a 2 dose program (0 and 6 months; plus 1 booster at 60 months) for girls in Grade 4 (20). In 2010, based on findings from the clinical trial BCGOV01(21-23), British Columbia

started using 2 doses in its Grade 6 program with a booster possible at Grade 9 or 11. Most other jurisdictions now use 2 dose programs following the approved HPV vaccine schedule.

Immunogenicity: HPV vaccine is highly immunogenic (1). Peak antibody concentrations at Month 7 range from 1 to 4 times greater than those found with natural HPV infections. The antibodies to HPV 6, 11, 16 & 18 (the four types contained in the vaccine) typically increase after each vaccine dose, and a peak response is achieved after the final dose. Concentrations wane in the following 12-18 months and then stabilize. Challenges with HPV type specific antigens at 60 months after dose 1 show an anamnestic response for all four subtypes, with antibody levels rising within 3-5 days to levels greater than those achieved at the peak in the initial immunization schedule (24-265).

The long-term goal is to understand the memory response to HPV vaccination. Although memory responses can be detected shortly after immunization, the best approach to measure the long-lasting anamnestic response is to challenge with a booster dose years (> 5) after the original exposure.

2.0 OBJECTIVES

Primary objective:

- To compare the B memory and plasmablast populations between girls and young women that received either a primary 2 or 3 dose series, who are then challenged with a subsequent dose after 120 months.

Secondary objectives:

- To compare the extent of somatic hypermutation and the variable gene usage between girls that received either a primary 2 or 3 dose series.
- To compare the serum antibody responses to HPV 6, 11, 16 & 18 at month 120 in females that received either a primary 2 or 3 dose series, who are then challenged with a subsequent dose.

3.0 STUDY DESIGN AND PARTICIPANT OVERVIEW

This is a single center, interventional study to evaluate long term memory response to HPV 6, 11, 16 & 18. Memory response will be assessed by measuring seroprotection 8-10 years post Q-HPV vaccination and to challenge with a booster dose of HPV9 vaccine (Gardasil® 9, Merck Canada, Inc.) years after the original exposure to measure the long-lasting anamnestic response. This is a 3 arm study with 6 girls per group:

Group 1: girls who received 2 doses of Q-HPV vaccine at 0, 6 month schedule 8-10 years ago when they were between 9-13 years of age at the time of the first dose.

Group 2: girls who received 3 doses of Q-HPV vaccine at 0, 2, 6 month schedule 8-10 years ago when they were between 9-13 years of age at the time of the first dose.

Group 3: young women who received 3 doses of Q-HPV vaccine at 0, 2, 6 month schedule 8-10 years ago when they were between 16-26 years of age at the time of the first dose.

3.1 Study plan

At the first visit, enrolled participants will have a health assessment, provide a blood sample (45ml), and a booster dose of licensed HPV9 vaccine will be given.

At the second visit, 7 days later, and again at the third visit at 30 days, a 45ml blood test to assess the response to the booster vaccine will be drawn.

Participants will be offered two additional doses of HVP9 vaccine at 2 and 6 months as a courtesy to complete the vaccine series (27, 28). The visits are optional and no additional serologic testing will be done.

Duration of study for each participant

The study will include three regular visits and two optional visits. At the first visit, about 60 minutes will be required to complete informed consent, record demographic and health information, draw a blood sample, vaccinate, and to observe vaccinees for 15 minutes afterward to detect and treat any rare allergic reaction. The subsequent visits for a follow-up blood test will require only about 30 minutes, to confirm ongoing consent and eligibility, and to draw a post vaccination blood sample. The two optional vaccination visits to complete will require 20 minutes including a 15 minute post vaccination observation period.

The time commitment to complete the study will be 2 hours. Study duration for each participant will be 1 month.

For participants who choose to return for additional HPV9 vaccination at 2 and 6 months to complete the vaccine series, the time to complete will be 2.6 hours over 6 months.

3.2 Study population

Participants will be females who were immunized with 2 or 3 doses of Q-HPV vaccine at ages 9-13 and 16-26 years.

3.3 Schedule of Events

Visit Activities Visit Window	Telephone	Visit 1 Day 0	Visit 2 Day 7 +/- 1d	Visit 3 Day 30 +/- 3d	Optional visit 4 2 months	Optional visit 5 6 months
Recruitment/Screening	X					
Eligibility Inclusion/Exclusion		X	ongoing	ongoing		
Informed Consent		X	ongoing	ongoing		
Blood Sample		X	X	X		
Pregnancy Test		X		X		
HPV9 Vaccine		X			X	X
Adverse Events			X	X		

4.0 CONDUCT OF STUDY

4.1 Good Clinical Practice

4.1.1 Institutional Review Board

This study is being conducted according to the Good Clinical Practice Guidelines of the International Committee on Harmonization (ICH Guidance Document *E6: Good Clinical Practice (GCP): Consolidated Guideline)*. The investigators will utilize the Research Ethics Board at BC Children's and Women's Hospitals, which conforms to section 3 regarding Responsibilities; Composition, Functions and Operations; Procedures; and Records.

4.1.2 Enrollment

Volunteers will be screened for eligibility before the first visit is scheduled. Participants will provide written informed consent for themselves. The informed consent process must be completed, with the informed consent form (ICF) signed and dated before any procedures specified in this protocol are performed.

Prior to beginning the study, the investigator will have REB written approval of the ICF and any other written information to be provided to participants. Both the informed consent discussion and the ICF will include the necessary content to meet the Good Clinical Practice Guidelines.

4.1.3 Regulatory considerations

Only vaccine supplied for use in this trial will be administered.

4.1.4 Monitoring

To ensure that the trial is conducted and documented appropriately, the following activities will be carried out:

- The site will have up to date **standards of practice** regarding REB approval processes; essential document storage; vaccine handling and storage; determination of eligibility; study staff delegation, qualification, training processes; and the process for Informed Consent.

- **Refrigerator temperature stability** will be demonstrated prior to receiving study vaccine and documented daily thereafter to ensure that the study refrigerator maintains a temperature within 2-8 °C.

- The site will have an **Essential Documents** box specific to this study, to contain copies of the REB approvals and amendments, refrigerator assessments, source documents, AEFI reports, waivers, and vaccine supply inventory.

- The site will devise **source documents** appropriate to this study.

- **Protocol compliance checks** will be made, including: consent date and time, visit dates; immunization details (product, date, time, administration method, cold chain compliance); blood sample details (sample volume, date and time, storage temperature); completion of data.

- **Training** of participating staff and investigators will be conducted prior to study start.

4.2 Recruitment and volunteer identification

4.2.1 Recruitment

This is a closed recruitment cohort from the BCGov01 study and, if needed, girls and young women who were immunized in the BC provincial program who have given permission to be contacted about future studies with the VEC.

4.3 Eligibility criteria

4.3.1 Inclusion criteria

The participant must meet all of the following inclusion criteria to participate in this study. Inclusion criteria include:

- Written informed consent provided by the participant.
- Participant whom the investigator believes can and will comply with the requirements of the protocol.
- General good health.
- Immunized with Q-HPV vaccine between the ages of 9-13 or 16 to 26 years on the BCGov01 study or the BC provincial program.
- Participant who is of child bearing potential must be willing to ensure that they or their partner use effective contraception during the study. Examples of effective methods of birth control include:

  * Abstinence (no sexual activity)
  * Hormonal contraceptives including oral, injectable, implants & skin patches
  * Intrauterine device (IUD)
  * Male partner sterilization
  * Male condom combined with a vaginal spermicide (foam, gel, film, cream or suppository)
  * Male condom combined with a female diaphragm, whether with or without a vaginal spermicide (foam, gel, cream, or suppository)
  * Adequate contraception does not apply to participants with same sex partners, when this is their preferred and usual lifestyle

4.3.2 Exclusion criteria

The participant may not enter the study if any of the following exclusion criteria apply:

- Received more than 3 doses of Q-HPV vaccine
- Received any doses of HPV9 vaccine

- Systemic hypersensitivity to Q-HPV or HPV9 vaccines or severe reaction to any previous dose of Q-HPV vaccine
- Receipt of blood or blood product within 3 months prior to Visit 1
- Receipt of a live vaccine within 28 days or an inactive vaccine within 14 days of Visit 1
- Immune compromise resulting from disease or immunosuppressive systemic medication use within 3 months prior to Visit 1
- Inadequate participant fluency in English to provide fully informed consent
- Participant who is currently pregnant or planning a pregnancy during the course of the trial

4.4 Health assessments

A health history will be reviewed at Visit 1 to identify any current health conditions. The health assessment will be sufficiently detailed to confirm eligibility to participate. Height, weight and oral temperature will be obtained. If oral temperature is greater than or equal to 38.0 C then immunization will be delayed until the participant has been afebrile for at least 24 hours. A urine sample for pregnancy testing will be performed at Visit 1 and Visit 3, and concomitant medications and contraindications to immunization will also be sought prior to immunization.

At visit 2 and 3, an interval health history will be obtained to confirm ongoing eligibility.

4.5 Vaccine and immunization

The study vaccine will be Gardasil®9 (Merck Canada, Inc), a recombinant, 9-valent vaccine that protects against HPV. A single manufacturing lot of vaccine will be obtained for this study, if possible.

4.5.1 Storage

Vaccine should be stored refrigerated at 2°-8°C, avoiding freezing. Vaccine storage temperature will be monitored and recorded daily.

4.5.2 Immunization

Study vaccine must be injected intramuscularly, with the deltoid muscle of the non-dominant arm being the preferred site. Immunizers will use 25 gauge needles, 1 inch in length, with built-in safety caps. Sharps will be disposed of in appropriate containers.

4.6 Serologic Testing (PBMC details in appendix 1)

4.6.1 Sample collection and handling of specimens

Blood samples (35 ml) for B cell testing will be collected by venipuncture in 4 yellow caps (ACD BD Vacutainer tubes). Each tube of blood will be clearly labeled with PID and visit number, secondary identifier (SID) and date and time of collection. Samples will be collected from all participants at visits 1 to 3. The

volume of blood to be collected at each visit is up to 45 ml. The minimum sample volume is 20 ml. Each tube should be gently inverted 8 times to mix blood and anticoagulant. The blood sample should be held at room temperature until processed, which should take place within 4 hours of the blood draw. Processing should occur between 30 minutes and 4 hours post draw. The samples will be centrifuged to separate the plasma from the cells. Plasma should be stored in numerical order (by PID) and in sequence per participant in a vertical position at -20 °C to -80 °C until shipped to the designated laboratory. An accurate list of serum samples will be maintained.

Blood samples (10 ml) for serology testing will be collected by venipuncture in clotted SST Vacutainer tubes. Clotted specimens must be transferred to cold storage (2 ºC - 8 ºC) after clotting has fully completed (30-60 minutes). Cold storage must be maintained after 60 minutes until serum separation. Clotted specimens will be separated using manufacturer specifications within 24 hours of collection. Sera must be aseptically transferred to standard, labeled cryovial tubes and stored at -20 °C to -80° C.

4.6.2 Processing, storage and transport of specimens

PBMC should be labeled by PID and in sequence per participant. An accurate list of PBMC samples will be maintained. PBMC will be processed by Ficoll Paqe density gradient centrifugation, stored and shipped as per the lab manual of Appendix 1. Both PBMC and plasma samples will be stored at -20 °C to -80° C and shipped to the Galloway lab in Seattle, WA on dry ice. Serum samples will be stored at -20 °C to -80° C and shipped to Merck Canada Inc. research facility for antibody response testing.

4.6.3. Immune response testing

PBMC will be enriched for B cells through negative selection with beads. The HPV specific Bmem express a cell surface BCR that binds to Alexafluor labeled HPV pseudovirus. That together with phenotypic markers allows us to isolate HPV-specific Bmem by FACS. Variable regions of the heavy and light chains will be cloned from singly sorted cells to allow us to assign V gene usage and to determine the extent of somatic hypermutation. The sequences from the leader region to the constant region are cloned into antibody vectors, expressed in 293 cells and antibodies are purified and tested for their ability to bind to HPV L1 proteins and to neutralize pseudoviruses. In addition to sequencing antibody genes from single cells next generation sequencing (NGS) will be used to sequence bulk populations of Bmem and plasmablasts to more extensively cover the repertoire. Because plasmablasts do not express a BCR, HPV specific PBs cannot be isolated but it has been estimated that approximately 70% of PBs are antigen specific within a week following vaccination.

Merck will perform serology testing for antibodies to HPV 6, 11, 16 and 18.

4.7 Vaccine safety

4.7.1 Immediate post-vaccination observation for anaphylaxis

Participants will be observed for at least 15 minutes after vaccination for any signs or symptoms of anaphylaxis or other distress. Study staff will be equipped to assess such changes and to administer appropriate initial medication.

4.7.2 Reporting of adverse events following immunization

Surveillance for provincially reportable adverse events following immunization (AEFI) will conform to recommended practices in British Columbia. At Visits 2 and 3 participants will be asked about any AEFI of concern to them, especially if medical consultation was required. Adverse events that qualify for reporting will be reported to British Columbia Centre for Disease Control using the provincial AEFI reporting form. Participants will be advised when reporting is done on their behalf by study staff.

4.7.3 Reporting of serious adverse events (SAEs)

Participants will be asked to advise the investigator as soon as possible of any Serious Adverse Event that occurs while enrolled in the study. Details of the SAE are to be explored in sufficient detail to enable the investigator to assess the likelihood that it was caused by the vaccination. SAEs will be identified on the adverse event section of the e-CRF and reported on the paper SAE form. All SAEs should be reported, including those that are considered unrelated to study vaccination. The study site will report all SAEs to the REB as is applicable per REB guidance notes.

An SAE is any untoward medical occurrence that:

- results in death
- is life threatening
- requires inpatient hospitalization
- results in significant incapacity or disability (significant disruption in conducting normal life functions)
- is a congenital anomaly/birth defect in the offspring of a study subject

4.8 Ongoing Eligibility

4.8.1 Contraindications to subsequent blood draws

A blood sample will not be drawn if in the interim:

- Blood or blood-derived product administration was required
- Additional dose of any HPV vaccine was administered
- Immune compromise developed as a result of illness or medication
- New bleeding disorder developed that contraindicates blood collection

4.8.2 Deviations

Participants may be retained in the study in the following circumstances but will be flagged as having deviated from the protocol:

- Any visits outside of window
- If the volume of blood collected is insufficient to obtain a lab result
- No blood obtained at Visit 2 (Day 7)

4.8.3 Withdrawals

Study staff will attempt to contact participants who do not return for a scheduled visit, making at least 4 attempts by various means of communication. Each attempt should be documented in the source document/study file. Due diligence must be made to ascertain the well-being of participants who were immunized.

Withdrawals will be documented, including date of withdrawal and reason if known. All data obtained before withdrawal may be included in the study analysis.

Participants should be withdrawn from the study in the following circumstances

- Withdrawal of consent to participate
- Unresolved condition beginning after enrollment that renders participant ineligible and is unlikely to resolve (e.g. immune compromise, bleeding disorder)
- Missing blood at Visit 3 (Day 30)

4.9 Non-study vaccines

For the purposes of post vaccine adverse event collection, other routine vaccinations should not be administered concurrently with the HPV9 vaccination. In addition, leading up to study enrollment, participants should not have received a live vaccine within 28 days or an inactive vaccine within 14 days of Visit 1.

5.0 DATA COLLECTION

Data will be collected on source documents (paper) and transferred as applicable to an electronic case report form (e-CRF). Data entry will be completed by trained center personnel shortly after each scheduled encounter with participants. The following data elements will be collected:

Demographics: birth month and year.

Baseline Health History: height, weight, and any health conditions requiring ongoing medical follow-up or care, any currently prescribed medications taken over the study.

HPV immunization history: date (month and year) of each dose is required

Protocol compliance: consent date and time, visit dates; immunization details (protocol adherence regarding administration including participant temperature prior to immunization, cold chain maintenance, date, time); blood sample details (protocol adherence regarding cold chain, volume obtained, dates and times); occurrence of reportable or serious adverse events, any deviations; any withdrawals, with specific reason.

5.1 Data entry

Data entry will be performed by trained staff of the VEC. To minimize transcription errors, the study database will contain numerous pre-programmed edit checks and prompts to enter missing data.

5.2 Data security measures

Data will be entered into the database platform, Dacima Clinical Suite. Dacima Clinical Suite is an encrypted data management platform with strict user access settings and is hosted in a secure data facility in Montreal. The facility has 24 hour security, internal and external back-up measures and disaster recovery plans in place.

5.3 Confidentiality protection

Source documents containing names, addresses and other contact information of participants will be stored in individual folders, identified by the assigned PID number. These documents are stored in lockable filing cabinets in a secure research facility, with controlled access, limited to authorized staff and investigators. No personal identifiers will appear in the CRF or on serum and PBMC tubes, laboratory requisitions or reports. No personal identifiers will appear in any summary or report of the study data.

In keeping with legal obligations, investigators will report any reportable AEFIs to the local Health Department using the prescribed provincial report form. This form requires inclusion of personal identifiers to enable public health to add this information to the participant's central immunization record and to follow-up the event if warranted by its nature or severity.

All VEC staff and investigators participating in this study will have completed PHSA's Confidentiality training module and have a completion certificate on file.

6.0 DATA ANALYSIS PLAN

6.1 Demographics and participation analysis

Frequency analysis will be conducted on demographic data, baseline health history and immunization history. De-identified data will be provided to the Seattle laboratory.

6.2 Immune response analyses

HPV type specific Bmem will be obtained as described above; their number and frequency will be analyzed at the third visit. The difference between the visit 1 and visit 3 Bmem will be obtained for all subjects. The extent of somatic hypermutation will be determined by comparing the nucleotide sequences of the heavy and light chain variable region sequences of each antibody cloned in this study to germ line sequences of these genes in public databases. These data bases will allow us to assign V, D and J gene usage.

Plasmablast numbers and frequencies will be obtained by FACS analysis of the week one samples.

Human monoclonal antibodies will be cloned and expressed from the Bmem at the first and third visit samples. The purified antibodies will be tested in binding assays to the L1 proteins of numerous HPV types to determine their type-specificity. The antibodies will be tested in pseudovirus-based neutralization assays to any type to which they bind to determine the $IC_{50.}$

Mean antibody levels and seropositivity prior to and after the booster dose of vaccine for HPV types 16, 18, 6, and 11 and the geometric mean titres of antibody levels against HPV 16, 18, 6, and 11 will

be compared between cohorts using two-sample t-test. To allow GMT calculation, samples with undetectable anti-HPV will be assigned half of the lowest detectable value.

All measurements will be compared between the two dose vs. three dose girls as well as the 3 dose women.

7.0 OTHER

7.1 Protocol amendment procedure

No modification of this protocol will be made without formal approval of the local REB. Documentation of amendments and REB approvals will be maintained with the Essential Documents collection.

7.2 Records retention

Data and study documents will be stored securely for 25 years beyond completion of the study. Destruction will occur in keeping with privacy and confidentiality requirements of the local REB and Standards of Practice at the investigational site at the time. The storage location of study files will be clearly documented.

Appendix 1. Isolation and Freezing of PBMC

Notes: Ficoll 1.077 (Ficoll-Paque Premium GE Healthcare17-5442-02; 17-5442-03) needs to be at room temperature (RT) and inverted several times before use; Freezing media (50% FBS (heat inactivated):40% RPMI:10% DMSO) once made should be kept on ice; Mr. Frosty (Sigma Aldrich) should be stored at 4C until use; isopropanol in Mr. Frosty should be changed every 5 uses; have at least 300ml PBS (sterile/endotoxin free)/subject sample (i.e., 4 ACD tubes) on hand. Always use sterile materials, endo-toxin free where possible, as endo-toxin can cause cell lysis.

Materials needed:
2 x 50ml Ficoll-Paque Premium (1.077 g/ml)
A clean, sterile (autoclaved) 5-inch underlay needle
Autoclave sleeves for underlay needle
1 x 30ml syringe
1 x 10ml syringe
50ml conical tubes
Mr. Frosty
Nunc cryotubes
One ice bucket with ice
20 ul, 200 ul and 1 ml pipettes
PBS (sterile/endotoxin free)
RPMI
FBS (heat inactivated in 56°C water bath for 1 hour)
Trypan blue
Eppendorfs for cell counting
Hemocytometer
70% ethanol
100% ethanol for cleaning underlay needle
Pen for notes

- Remove 2 x 50 ml Ficoll-Paque tubes from refrigerator and place in 37C bead bath, (typically *before* you go pick up sample from reception) or use unopened Ficoll-Paque at room temperature
- Spray down hood and equipment (i.e., pipettes, filter pipette tip boxes, media bottles) with 70% ethanol and wipe clean with paper towels.
- Set up 6 x 50 ml conical tubes in hood
- Make sure 4 yellow caps (ACD BD Vacutainer tubes) have been mixed (i.e., there is not separation at the top indicating that tubes have not been inverted 8 times, as recommended by BD).
- Remove yellow caps and set aside (do not discard)
- Pipette 10 ml blood into each of 4 x 50 ml conical tubes using a 10 ml stripette
- Consolidate any remaining blood in each glass tube to one tube.
- If there is sufficient blood remaining (i.e., after setting aside 2 ml for plasma), divide extra blood equally into four 50 ml conical vials (typically 4 ml extra blood remains after setting aside 2 ml for plasma, so an additional 1 ml can be added to each 50 ml conical vial).
- Re-cap tube with 2 ml blood for plasma and an additional empty glass tube.
- Centrifuge these two tubes 900xg, 10 min, 25C (with brake)
- Add 2 parts PBS to 1 part blood in each 50 ml conical vial (e.g., if there is now 16 ml blood in each vial, add 32 ml PBS to each tube)
- Invert 50 ml conical tubes and then distribute 1 part PBS/blood mixture from each 50 ml conical equally to two clean 50 ml conical vials (i.e., 16 ml each from two conical vials into a clean conical vial and then repeat).
- Each of the 6 x 50 ml conical vials should now have 32 ml PBS/blood mixture.
- Remove 2 x 50 ml Ficoll-Paque tubes from 37C bead bath, spray down with 70% ethanol and wipe clean with paper towels.

- Invert Ficoll-Paque tubes multiple times, then underlay 15 ml Ficoll-Paque using ***underlay*** needle and 30 ml syringe to each of 6 x 50 ml conical vials with PBS/blood mixture – **ADD FICOLL-PAQUE VERY SLOWLY AT START** to allow a good gradient to form – you should see a clearly delineated interface between the Ficoll and PBS/blood mixture.
    - Always underlay 15 ml Ficoll-Paque for each 30-35 ml PBS/blood mixture. If there is less blood total, adjust number of conical vials accordingly or add more PBS to PBS/blood mixture to total 30 ml *BEFORE* underlaying Ficoll-Paque. There can be as much as 3 parts PBS to 1 part blood, though 2 parts PBS to 1 part blood is preferred.
    - When underlaying with Ficoll-Paque, the very slow initial rate should be ~2 seconds per ml. After underlaying first 15ml of Ficoll-Paque in conical tube, remove needle and place at the bottom of next conical tube and start again. DO NOT expel last bit of Ficoll-Paque from 30 ml syringe, as bubbles will disturb gradient.
- Once centrifuge has stopped, remove plasma sample and empty glass tube from centrifuge and place 6 x 50 ml conical vials with PBS/blood mixture into centrifuge
- Centrifuge 6 x 50 ml conical vials 400xg for 35 min NO BRAKE at 18°C
- Remove 105 ul plasma from glass tube to a cyrotube and then remaining plasma into a separate cyrotube
- Label 105 ul plasma sample with 1. Subject ID, 2. Date, 3. Sample contents (i.e., 'plasma'). Mark cap with a dot.
- Label additional plasma sample with 1. Subject ID, 2. Date, 3. 'plasma', 4. Study group (e.g., 'Exp' for *HPV-Exposed* group), 5. Time point (e.g., 'M6' for month 6).
- Store 105 ul sample in 'Jody' box at -70C and additional plasma sample in 'Plasma box #N' at -70C.
- Once spin has stopped, remove lymphocytes (white, cloudy, 'buffy coat') at interface to 2 x 50 ml conical tubes with sterile 1 ml filter pipette
- You should have approximately 20-25 ml/conical when no more lymphocytes are visible
- Add PBS to each 50 ml conical vial containing isolated PBMC up to 50 ml and invert 1-2 times
- Centrifuge 300xg for 10 min NO BRAKE at 20°C
- During spin, prepare 2 x eppendorf tubes for counting (100 ul trypan blue, 50 ul PBS each)
- After removing conical vials from centrifuge, spray down with 70% ethanol and wipe clean with paper towels.
- Gently decant off supernatant and dislodge pellets by tapping on hood. (N.b., from this point forward, keep cells on ice when not manipulating.)
- Resuspend each pellet in 1 ml PBS. Then add 19 ml PBS to one conical vial, invert, and transfer over cells/PBS to 2nd vial. Then add a second 19 ml PBS wash to 1st vial, invert, and transfer over wash to 2nd vial. Use a 1 ml pipette to collect any remaining wash from 1st vial and transfer to 2nd vial. Now there should be 40 ml cells/PBS in 1 x 50 ml conical vial. If < 60 ml blood contributed to PBMC isolation, adjust accordingly: **1 ml PBS/ 1.5 ml blood** (e.g., 40 ml PBS/60 ml blood)
- Cool down centrifuge by changing temperature from 20°C to 4°C
- Invert conical of cells before taking out 50 ul for counting to ensure homogeneity
- Add 50 ul cells to 1 eppendorf tube with trypan blue/PBS, vortex briefly, and count 10 ul of this sample. Repeat dilution and count for accuracy (adding cells immediately before counting each tube). Dilution factor is 4.
- Calculate how many million cells total (e.g., 2.5x10^6 cells/ml x 40 ml = 100x10^6 cells). Figure out how many cryotubes to freeze cells with. Freeze 1 tube with 10x10^6 cells/ml freezing media; and ≥ 3 tubes with 10x10^6-40x10^6 cells/ml freezing media/tube.
- When aliquoting cells in freezing media (f.m.) into cryotubes, where there will be:
-
- 1.0x10^7 cells 1.0-4.0x10^7 cells 1.0-4.0x10^7 cells 1.0-4.0x10^7 cells …
- 1 ml f.m.| 1 ml f.m. 1 ml f.m. 1 ml f.m.
- 1 tube 1 tube 1 tube 1 tube
-
- if there are any remaining cells in freezing media in the multi-aliquot conical vial (and there probably will be a couple of hundred microliters), then add to one of the 1.0-4.0x10^7 cell tubes.
- Centrifuge 50 ml conical tubes (one with 10x10^6 cells and the other with remaining cells) at 300xg, 5 min, 4°C (with brake)

- During spin, make freezing media using 5 parts FBS: 4 parts RPMI: 1 part DMSO in excess of total # of cryotubes, assuming 1 ml freezing media per cryotubes (e.g., if there will be 4 cryotubes total, make 5 ml freezing media). Vortex to mix freezing media and then place on ice.
- Prepare cryotubes for cells with the following label: 1. Subject ID, 2. Date, 3. $nx10^7$ PBMC, 4. Study group, 5. Time point
- After removing conical vials from centrifuge, spray down with 70% ethanol and wipe clean with paper towels.
- Gently decant off supernatant and dislodge pellets by tapping on hood. (Keep cells on ice when not manipulating.)
- Bring up cells in appropriate amount of freezing media (1 ml freezing media/cryotube). Add freezing media to multi-aliquot conical ml by ml, washing sides of conical as freezing media is expelled.
- Pipet cells up/down gently before removing each ml to a cryotube (this ensures even cell density). Transfer each cryotube to Mr. Frosty after adding cells.
- Transfer Mr. Frosty to -80°C and leave at least overnight.
- Spray down outside of underlay needle with 70% ethanol and rinse 4 times with 100% ethanol using a 10 ml syringe before placing in autoclave sleeve.
- Autoclave underlay needle before using again – or use disposable, sterile, endotoxin free underlay needles.
- Can remove cells from Mr. Frosty and store in -80°C freezer until shipping to Galloway Lab – BUT DO ***NOT*** STORE IN LIQUID NITROGEN VAPOR UNDER ANY CIRCUMSTANCES!
- Please ship to Galloway Lab on dry ice

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