EQAPOL
External Quality Assurance Program Oversight Laboratory
An NIH, NIAID, DAIDS Program

Duke Human Vaccine Institute

EQAPOL 8-color Intracellular Cytokine Staining (ICS) Flow Cytometry Assay Orientation

Updated 11/27/2018

Presentation Outline

- Overview of External Quality Assurance Program Oversight Laboratory (EQAPOL)

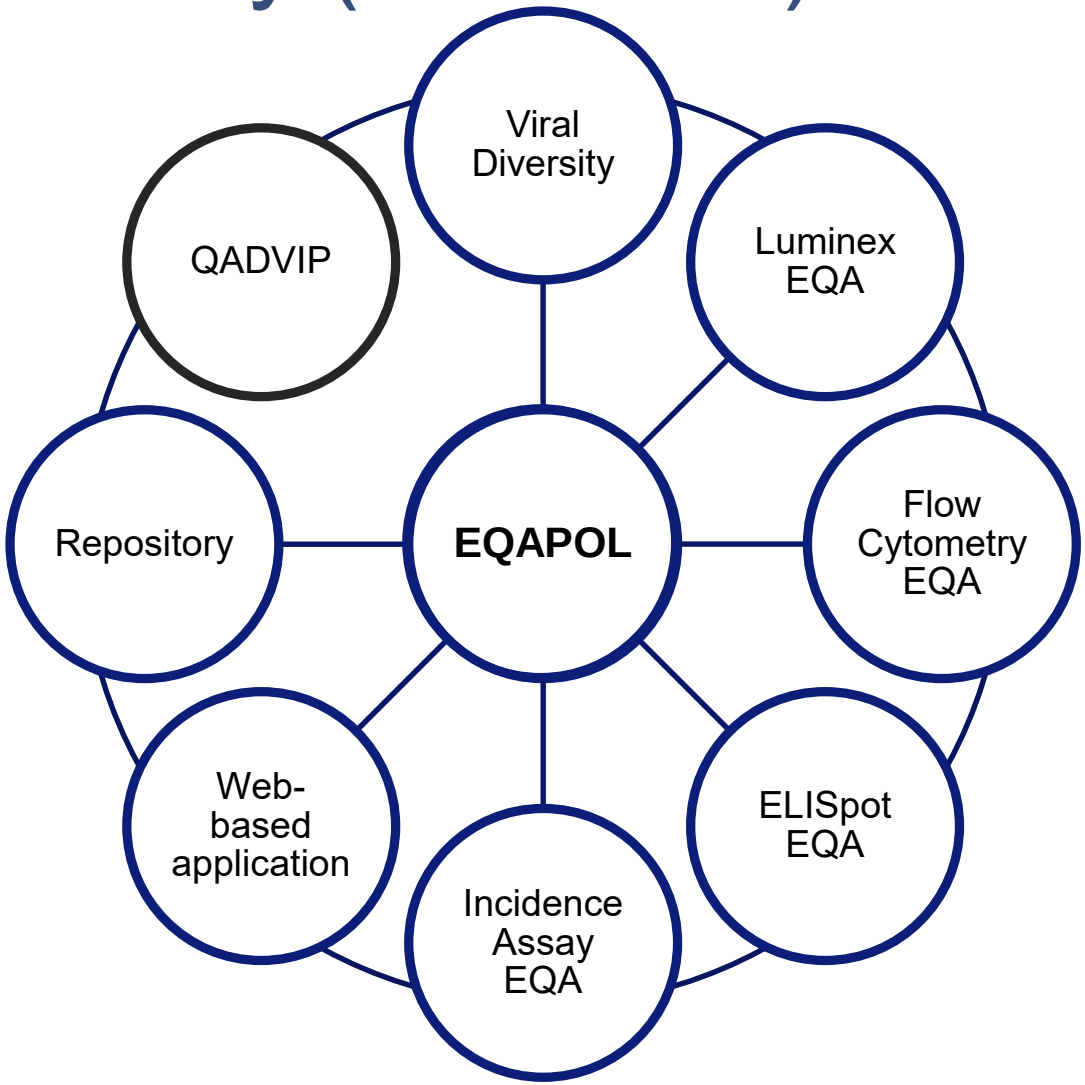
- Overview of EQAPOL Flow Cytometry External Quality Assurance (EQA) Program
  - EQAPOL ICS assay Program Overview
  - EQA Process
  - Data Reporting
  - Assessment
  - Common Problems
  - References
  - FAQs
  - Next steps - DAA

Abbreviations used

- EQAPOL – External Quality Assurance Program Oversight Laboratory
- QAU – Quality Assurance Program
- EQAP – External Quality Assurance Program
- EP – External Proficiency
- EOL – EQAPOL Oversight Laboratory
- ICS – Intra-Cellular Staining
- 8C – 8 Color
- PBMC – Peripheral Blood Mononuclear Cells
- GID – Global ID, sample identifier
- FCS – Flow Cytometry Standard, file format
- EOLm – EOL centralized Manual analysis
- CEF – optimal 8-9mer peptides for **C**MV, **E**BV, and In**f**luenza
- ANR – Assay Not Reliable, used in report template
- AND – Assay Not Done, used in report template
- LN_2 – Liquid Nitrogen
- SA – Site Analysis

External Quality Assurance Program Oversight Laboratory (EQAPOL)

- NIAID/DAIDS-sponsored 7-year contract
- Eight components to support HIV/AIDS research and vaccine trials
- Goal of EQAPOL: help sites improve



EQAPOL EQAP Program Goal

- The goal of a proficiency program, traditionally, is to assess performance of an assay

- The goal of the EQAPOL EQAP is to help sites improve the performance of their own assay

  - Standardized EQAPOL assay - required
    - Graded
    - Site-specific report

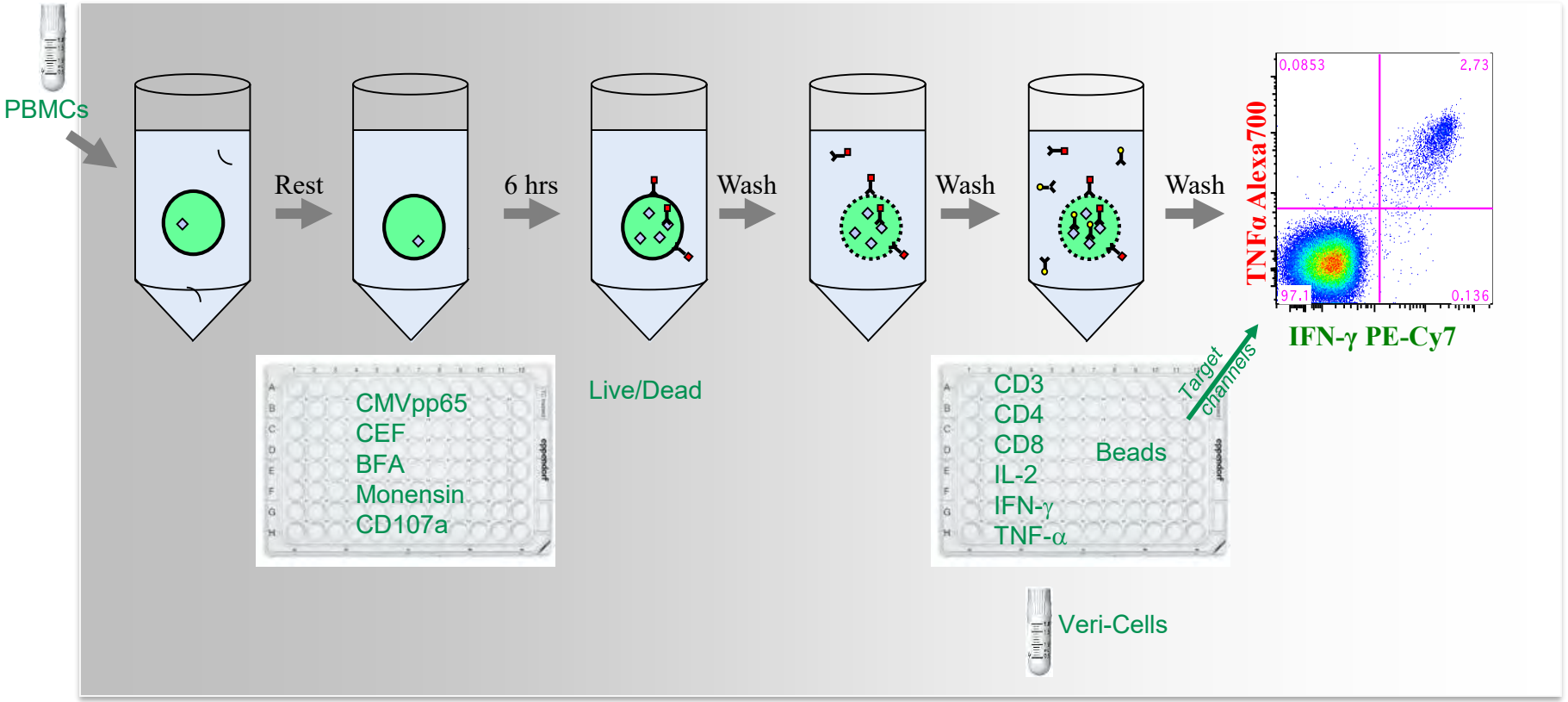
  - Site Choice assay - *optional*
    - Not graded
    - One report

Introduction to 8-color ICS Flow Cytometry Program

- The program promotes improvement through a combination of proficiency testing, *controls, questionnaire, remediation, and training.
  - *Instrument
  - *Staining
- EQAPOL assesses a site's ability to perform and analyze an 8C ICS Flow panel.
- Grading criteria
  - Harmonized across EQAPOL Programs
  - Designed to capture performance in key points of the assay, enabling the identification of technical problems (Site-Specific Comments)

EQAPOL ICS Assay Overview

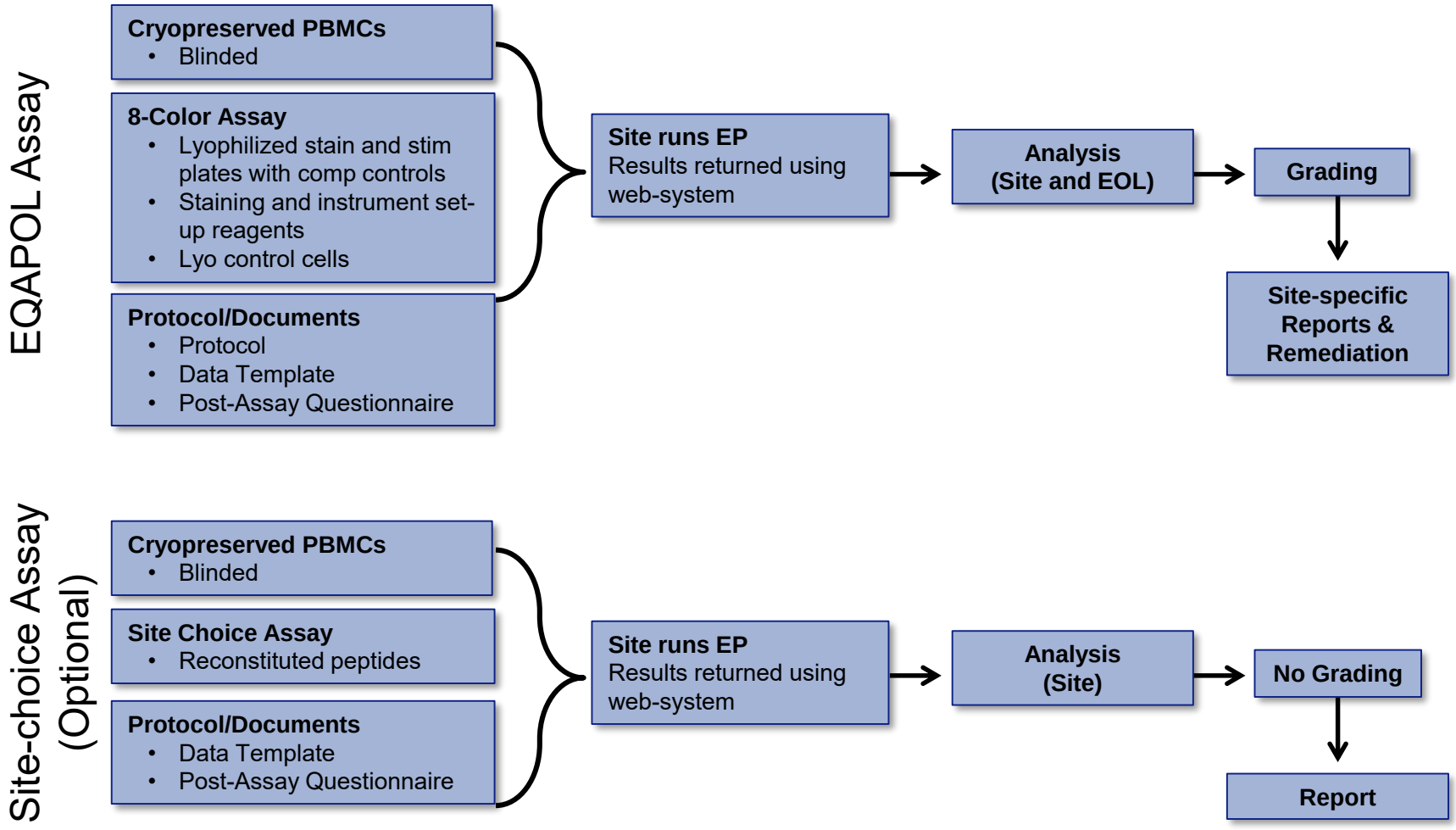
1. Thaw ON rest
2. Count Stimulate
3. Surface Stain
4. Fix/Perm
5. IC Stain Acquisition
6. Analysis



EQAPOL ICS EQAP Grading Categories

- Timeliness
- PBMCs
  - Viability
  - Recovery
- Protocol Adherence
  - Instrument setup
  - Annotation and Gating
- Deviation of EOLm analyzed data from consensus (assay)
- Deviation of EOLm and Site analyzed data (analysis)

EQAPOL Flow Cytometry EQA Approach



Assay Items supplied by EQAPOL

- EQAPOL Assay:
  - Pre-qualified PBMCs
  - Lyophilized Stain and Stimulation plates, including controls
  - Staining and instrument set-up reagents
  - Veri-Cells
    - lyophilized control cells – stim & unstim, stained with zombie dye
  - Protocol, with target channels & gating strategy
  - Data reporting template

- Site Choice Assay:
  - PBMCs
  - Reconstituted Peptide
  - Data reporting template

Stimulation Plate Layout Example

		Stimulation Plate											
		1	2	3	4	5	6	7	8	9	10	11	12
Add **Donor 1** to Row A	A	PTI		PTI + CMV		PTI + CEF							
	B												
Add **Donor 2** to Row C	C	PTI		PTI + CMV		PTI + CEF							
	D												
Add **Donor 3** to Row E	E	PTI		PTI + CMV		PTI + CEF							
	F												
	G	PTI		Unstim Veri-Cells with Zombie	Unstim Veri-Cells No Zombie								
	H	PTI		Stim Veri-Cells with Zombie	Stim Veri-Cells No Zombie								
		PTI		PTI + CMV		PTI + CEF		Veri-Cells					

Note 1: Cells should be added to rows A, C, and E as well as wells G1 and H1 at the time of stimulation according to the protocol and plate layout.

Note 2: LyoCells should be added to wells G3 and H3 on the stimulation plate during the staining portion of the EQAPOL 8cICS assay according to the protocol and plate layout. LyoCells are stimulated and lyophilized and do not need to go through the stimulation part of the EQAPOL 8cICS assay.

Stain Plate Layout Example

	8C mAb Stain Plate											
	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C												
D												
E									TNFa FITC		CD107a PE	
F										CD8 PerCP-Cy5.5		CD4 PE-Cy7
G									IFNg APC		CD3 APC-F750	
H										IL2 BV421		
	mAb cocktail			Unstained PBMC								

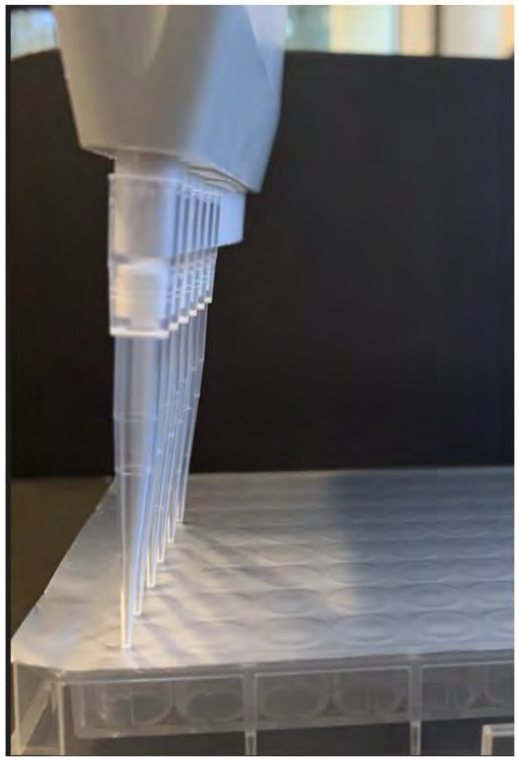
Note 1: The mAb cocktail in wells A1, A3, A5, C1, C3, C5, E1, E3, E5, G3, and H3 on the staining plate should be added to the corresponding wells on the stimulation plate according to the EQAPOL 8cICS protocol.

Note 2: Individual mAb for the compensation beads should be transferred to 12x75 tubes according to the EQAPOL 8cICS protocol.

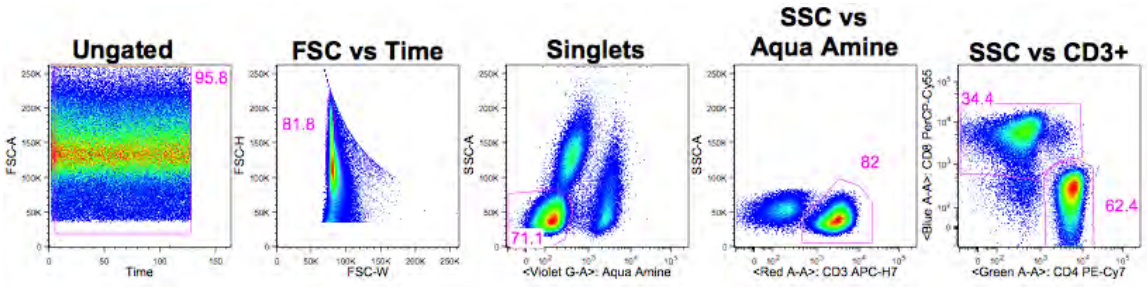
Easy-pierce Seal - reduces loss of lyopellet due to static electricity

Out of package

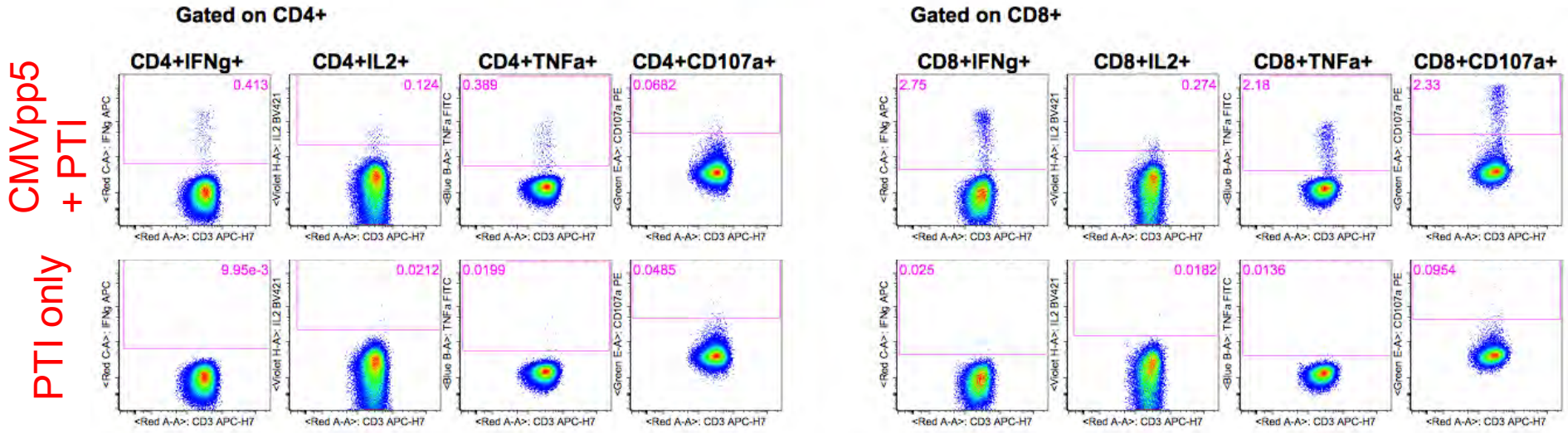
Reconstituted

BAD Piercing Angle	GOOD Piercing Angle
	

EQAPOL 8-color ICS Gating scheme



- Manually check/modify compensation
- Uniform gating
- Exclude acquisition errors
- Include CD3, CD4, CD8 dim+
- Set cytokine gates above "halo" of negative
- Use backgating



Site choice – up to site, harmonize reportables

Reporting Template (.xlsx file)

Enter Exact FCS File Name

Change GID Name

Don't Edit/change

Copy and paste data from FlowJo

File Name	GID	Stimulus	Well ID	Total Event Count	% Time vs FSC	% Singlets	% Aqua-SSClo	% CD3+ SSC	% CD4+CD8-	% CD
	GGGGGGGG_GG	PTI	A01							
	GGGGGGGG_GG	PTI + CMV	A03							
	GGGGGGGG_GG	PTI + CEF	A05							
	GGGGGGGG_GG	PTI	C01							
	GGGGGGGG_GG	PTI + CMV	C03							
	GGGGGGGG_GG	PTI + CEF	C05							
	GGGGGGGG_GG	PTI	E01							
	GGGGGGGG_GG	PTI + CMV	E03							
	GGGGGGGG_GG	PTI + CEF	E05							
	Lyo Cell	Unstim	G03				n/a			
	Lyo Cell	Stim	H03				n/a			

vs	% Singlets	% Aqua-SSClo	% CD3+ SSC	% CD4+CD8-	% CD4+CD8-CD107+	% CD4+CD8-IFNg+	% CD4+CD8-IL2+	% CD4+CD8-TNFa+	% CD4-CD8+	% CD4-CD8+CD107+	% CD4-CD8+IFNg+	% CD4-CD8+IL2+	% CD4-CD8+TNFa+
		n/a			n/a					n/a			
		n/a			n/a					n/a			

Example of Completed Template

File Name	GID	Stimulus	Well ID	Total Event Count	% Time vs FSC	% Singlets	% Aqua-SSClo	% CD3+ SSC	%
008_G6901X4Z_07_A01	G6901X4Z_07	PTI	A01	214,000	100	80.40	94.20	89.00	
008_G6901X4Z_07_A03	G6901X4Z_07	PTI + CMV	A03	221,000	100	78.50	92.60	89.50	
008_G6901X4Z_07_A05	G6901X4Z_07	PTI + CEF	A05	218,000	100	79.40	92.90	88.80	
008_EENB070Z4_07_C01	EENB070Z4_07	PTI	C01	262,000	100	82.00	88.80	76.00	
008_EENB070Z4_07_C03	EENB070Z4_07	PTI + CMV	C03	271,000	100	81.10	88.00	75.40	
008_EENB070Z4_07_C05	EENB070Z4_07	PTI + CEF	C05	259,000	100	82.80	89.30	77.10	
008_J69038D1_07_E01	J69038D1_07	PTI	E01	270,000	100	79.80	79.50	83.80	
008_J69038D1_07_E03	J69038D1_07	PTI + CMV	E03	261,000	100	79.20	84.70	83.00	
008_J69038D1_07_E05	J69038D1_07	PTI + CEF	E05	254,000	100	80.10	85.10	83.40	
008_Lyo cell_G3	Lyo Cell	Unstim	G03	276,000	100	80.90	n/a	78.10	
008_Lyo cell_H3	Lyo Cell	Stim	H03	261,000	100	87.40	n/a	79.00	

% Aqua-SSClo	% CD3+ SSC	% CD4+CD8-	% CD4+CD8-CD107+	% CD4+CD8-IFNg+	% CD4+CD8-IL2+	% CD4+CD8-TNFa+	% CD4-CD8+	% CD4-CD8+CD107+	% CD4-CD8+IFNg+	% CD4-CD8+IL2+	% CD4-CD8+TNFa+
94.20	89.00	61.50	0.05	0.06	0.19	0.06	23.70	0.05	0.11	0.15	0.02
92.60	89.50	61.60	0.04	0.04	0.16	0.08	23.40	0.04	0.04	0.12	0.01
92.90	88.80	61.60	0.04	0.02	0.25	0.07	23.80	1.05	1.11	0.33	1.01
88.80	76.00	49.30	0.10	0.03	0.11	0.10	45.50	0.12	0.05	0.07	0.04
88.00	75.40	49.10	0.12	0.13	0.13	0.17	45.40	3.45	4.77	0.32	3.82
89.30	77.10	49.30	0.09	0.06	0.15	0.06	45.40	3.17	4.51	0.41	3.34
79.50	83.80	40.90	0.13	0.02	0.19	0.06	51.60	0.14	0.01	0.13	0.04
84.70	83.00	41.00	1.37	1.98	0.97	2.03	50.70	0.71	0.45	0.08	0.18
85.10	83.40	41.20	0.13	0.02	0.07	0.05	50.00	0.41	0.33	0.07	0.14
n/a	78.10	61.50	n/a	0.21	2.55	1.67	26.40	n/a	0.01	0.17	0.10
n/a	79.00	60.10	n/a	6.89	8.59	6.99	28.80	n/a	13.40	7.14	9.37

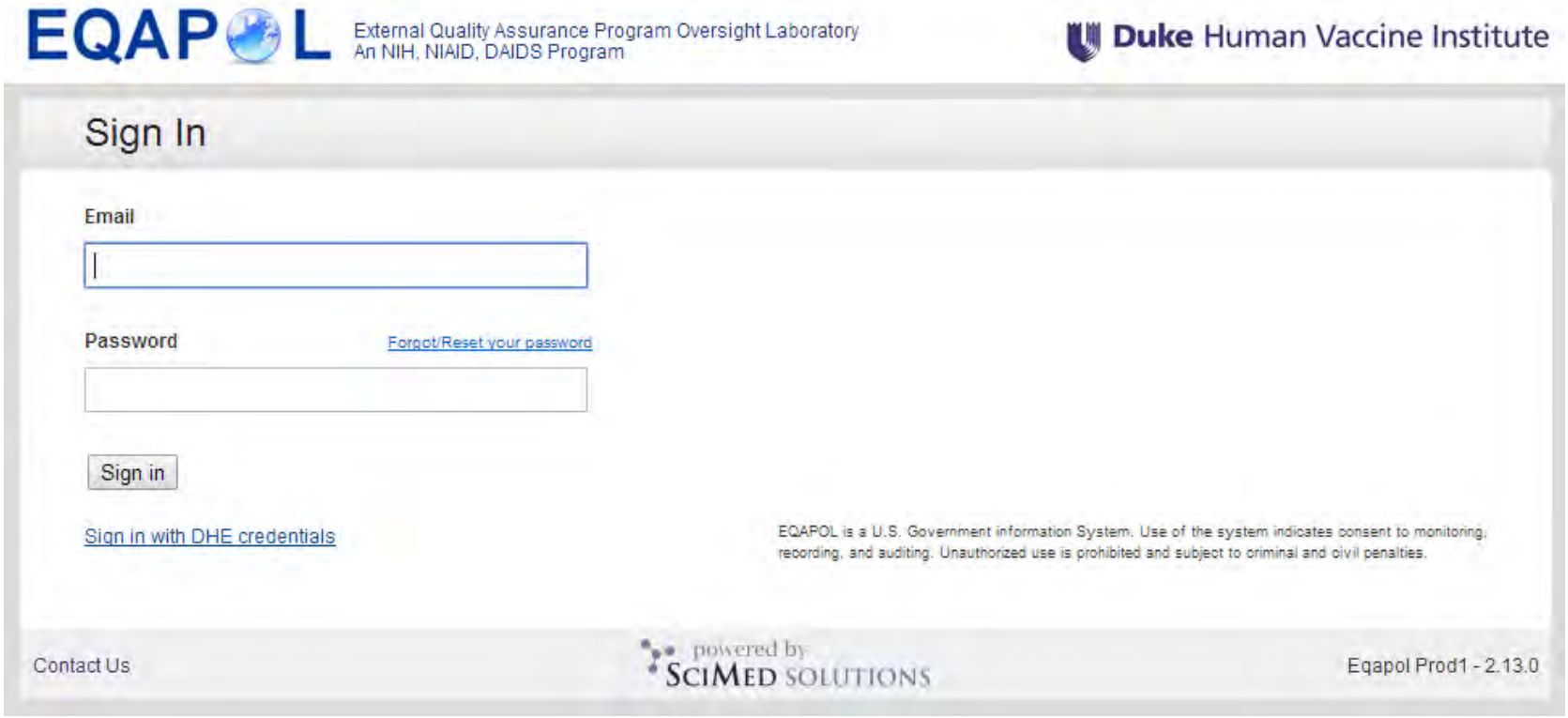
- **Empty cells are not permitted.**
  - Assay Not Reliable (ANR) – assay performed & results are not consistent/reliable for reporting
  - Assay Not Done (AND) – assay not performed & no data to report
  - Not Applicable (n/a) -

Initiating an EP

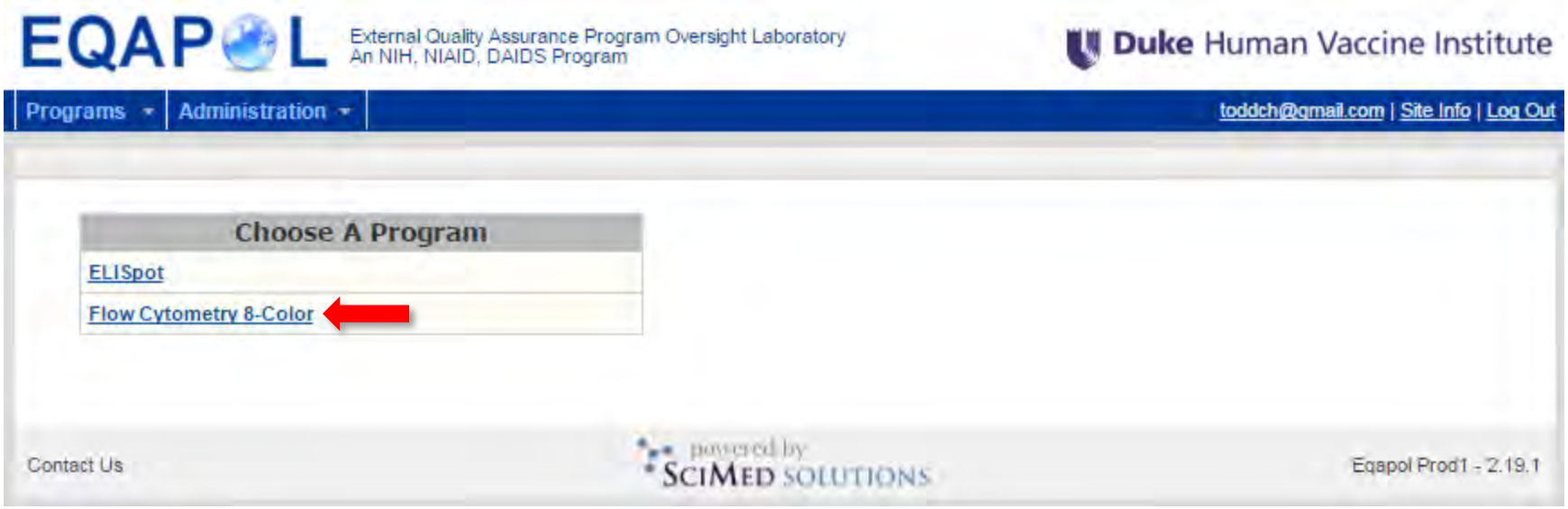
- EPs are scheduled twice per year about six months apart
- EQAPOL will alert sites of a send-out date AT LEAST three weeks in advance
  - Sites may request a delay in shipment due to holidays, etc.
- Sites will be alerted when their EP ships.
- Results are due within 4 weeks of EP receipt.

EQAPOL Web-based System: Log In

https://eqapolapp.dhvi.duke.edu

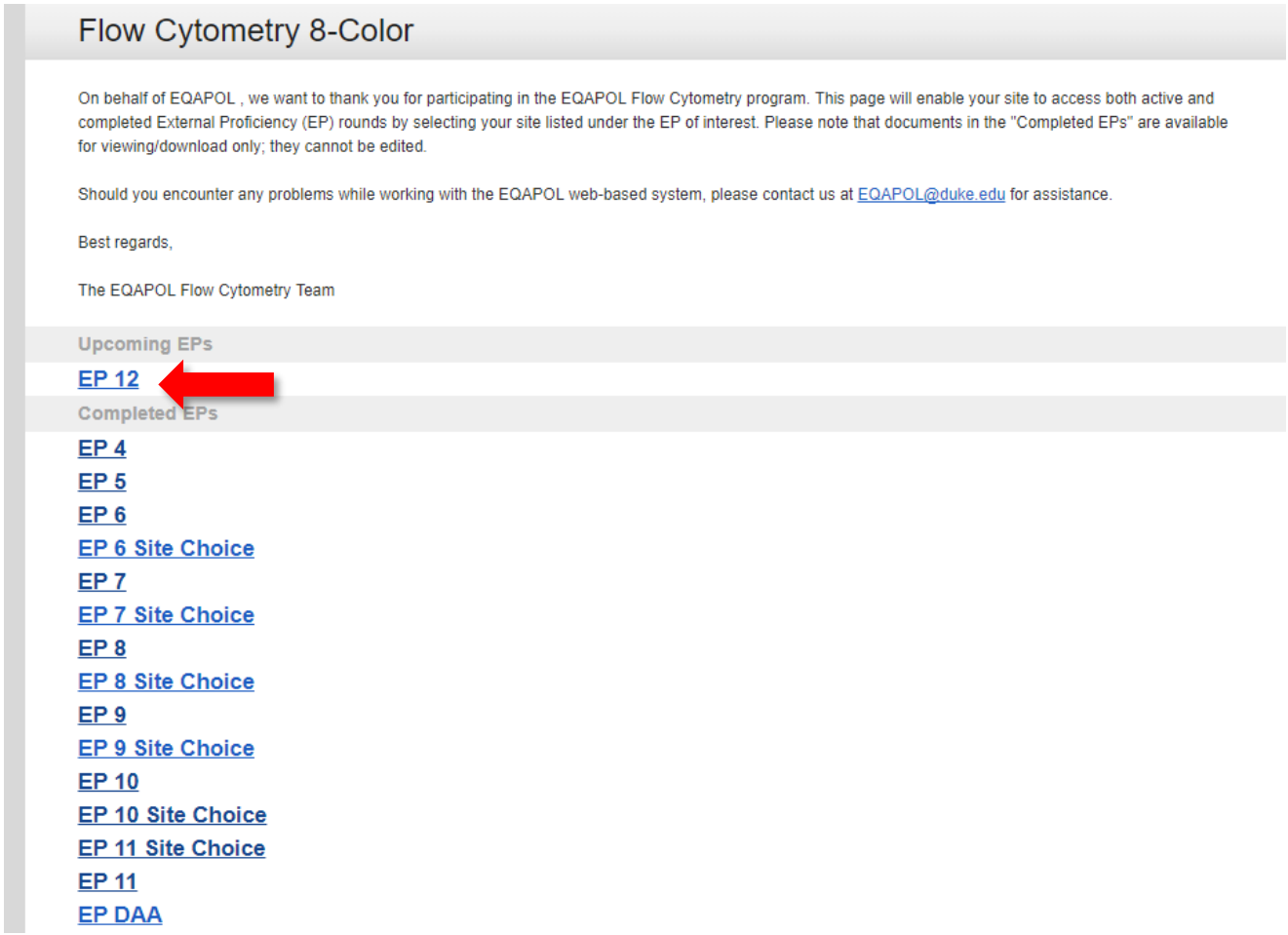


Select “Flow Cytometry” from the Menu



*** Some options will not be available for your site*

Select “EP12” from Flow Cytometry Program Page



Flow Cytometry 8-Color

On behalf of EQAPOL , we want to thank you for participating in the EQAPOL Flow Cytometry program. This page will enable your site to access both active and completed External Proficiency (EP) rounds by selecting your site listed under the EP of interest. Please note that documents in the "Completed EPs" are available for viewing/download only; they cannot be edited.

Should you encounter any problems while working with the EQAPOL web-based system, please contact us at EQAPOL@duke.edu for assistance.

Best regards,

The EQAPOL Flow Cytometry Team

Upcoming EPs

EP 12

Completed EPs

EP 4
EP 5
EP 6
EP 6 Site Choice
EP 7
EP 7 Site Choice
EP 8
EP 8 Site Choice
EP 9
EP 9 Site Choice
EP 10
EP 10 Site Choice
EP 11 Site Choice
EP 11
EP DAA

*** Some options will not be available for your site*

Select your Site under the Current EP

Flow Cytometry 8-Color EP 9 (Active)

We appreciate your participation in EQAPOL Flow Cytometry 8-Color ICS External Proficiency 9 (EP9). Please follow the instructions below to complete EP9:

1. Download the provided protocol,instructions, and Appendices below to perform the 8-Color ICS assay using the EQAPOL-provided kit and samples. Do not deviate from the protocol.
2. Once you have completed the assay, please navigate to the "Results" tab to complete the post-assay questionnaire.
   - Answers will not be final until you select the "Submit" button at the end of the survey.
3. Download the Excel template below and complete the template using the instructions provided in the protocol.
4. Navigate to the "Results" tab and upload your completed Excel template as a "Results Spreadsheet."
5. Navigate to the "Results" tab and upload your final FlowJo analysis file as a "jo File" or "wsp File" according to the protocol.
6. Navigate to the "Results" tab and upload your Cytometer Configuration file as a "csv File" or "jpg File" according to the protocol.
7. Navigate to the "Resutls" tab and upload your CST Baseline Report as a "csv File" according to the protocol.
8. Navigate to the "Resutls" tab and upload your CST Performance Report as a "csv File" according to the protocol.
9. After you have uploaded your results you can either "Save" or "Submit" them. Once the Excel is uploaded, you can save or submit your results. By pressing "Save" you will save a copy of the file to the system, but it will not be recorded as final until you "Submit" the document. Selecting "Submit" will both save your file and submit it as final.
10. Navigate to the "Results" tab and select the link for the Questionnaire and complete the questionnaire.
11. Select the link for Reflow below. Upload your FCS files in the folder titled "EP9."
12. Once your files are submitted you will not be able to add or edit files unless you contact us at EQAPOL@duke.edu.

www.eqapol-reflow.com

https://vimeo.com/167328566

Should you have any questions about EP9 or need assistance with the web-based system, please do not hesitate to contact us.

The EQAPOL Flow Cytometry Team

eqapol_ep9_appendix_a_final.pdf
eqapol_ep9_appendix_b_final.pdf
eqapolep9_8cics_protocol_final.pdf
eqapolep9_reflow_protocol_final.pdf
eqapolep9_site__8cics.xlsx

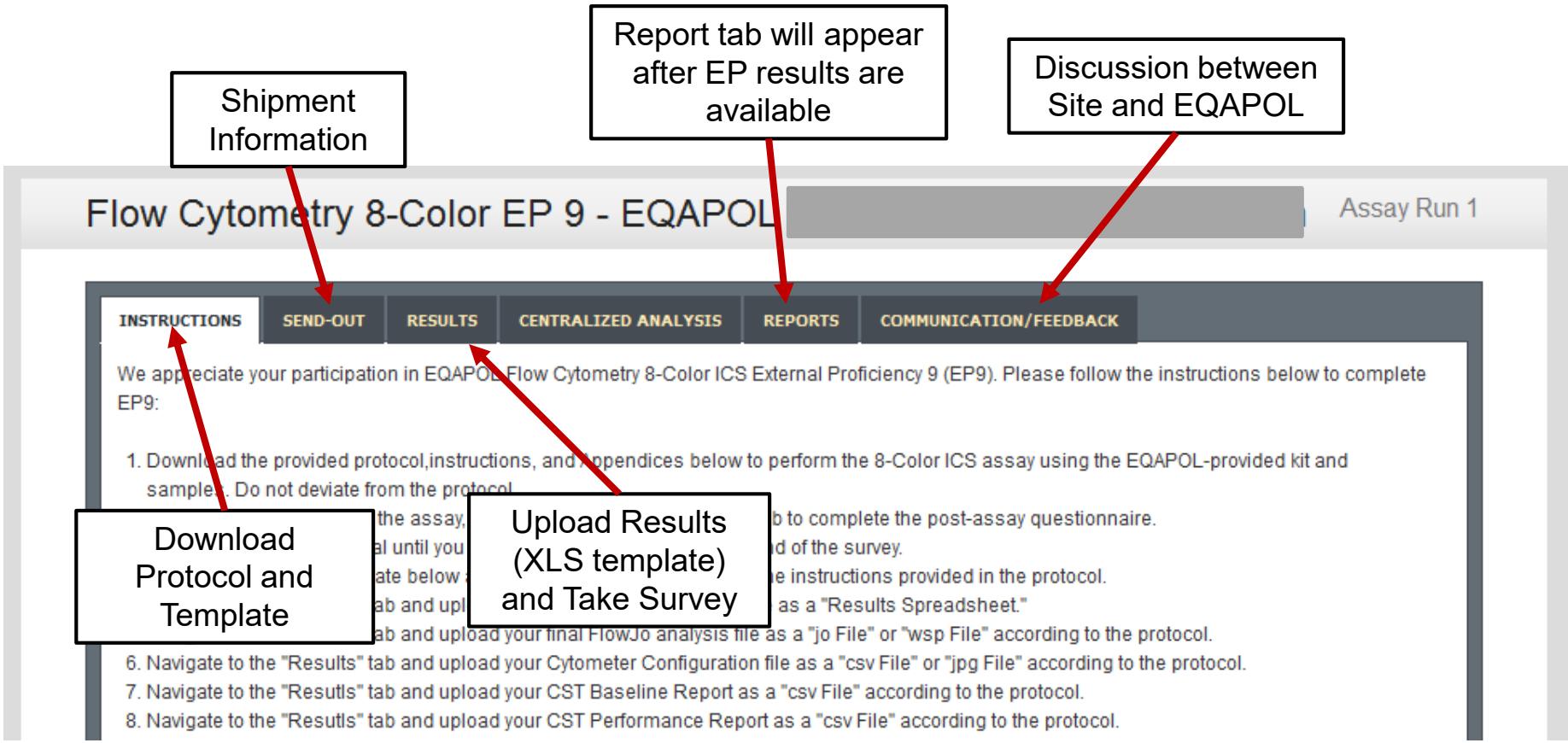
Kit: EP9

Snapshot of EP status

Site	Assay Run	Send-Out	Questionnaire	Results	Reports
EQAPOL [redacted]	1	Received 05/24/2016, Received 05/24/2016, Received 05/24/2016	Submitted 06/20/2016	Submitted 06/20/2016 Imported 06/21/2016	Report Available

Only sites for which you are a part of will display on this page

Navigating Your Site's Home Page



EP Shipment – EQAPOL Assay

- **Three shipments with packing manifests**
  - **Wet ice shipment** containing the reagents for stimulation, staining and instrument set-up.
    - Store materials at 2-8°C
  - **Cryoshipper** containing the PBMCs
    - Store PBMCs in LN_2
  - **Dry ice shipment** containing the Zombie viability dye
    - Store dye at -20°C

- **Data Logger**
  - Upon receipt deactivate the data loggers according to the protocol and return to EQAPOL using the provided shipping envelope and waybill

- **Assay Protocol**
  - Thoroughly familiarize yourself with all aspects of the EP-specific protocol in advance of performing the assay. This is necessary as the assay may vary slightly from your current Flow Cytometry procedures and from previous EPs.
  - Protocol documents include gating instruction appendix, results template and instructions for using ReFlow

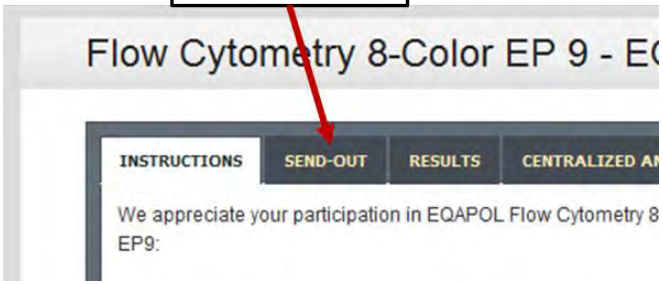
- **Return LN_2 Shipper immediately upon receipt**

EP Shipment – Site Choice Assay

- **Site choice shipment includes:**
  - **Cryoshipper** containing 3 vials of PBMCs and data logger
    - Store PBMCs in LN_2
    - Follow printed instructions for returning data logger
  - **Reconstituted peptides**
    - One 15µL vial of CEF and one 15µL vial of CMVpp65
    - Store at -80 °C

Acknowledge Receipt of Shipments

Shipment Information



Shipment #11740

Shipped: 12/10/2014 05:12 PM
Courier: Fedex
Tracking # 772166719609
Documents eqapol_elispot_ep8_site [redacted] batch_1267.xls

Item	Global Spec ID	Quantity	Volume
Reagent A	09252014	1	200.0 µL
Reagent B	09252014	1	200.0 µL
Reagent C	09252014	1	200.0 µL
Sample 1	J69059VN-36	1	20000000.0 CEL
Sample 1	J69059VN-37	1	20000000.0 CEL
Sample 1	J69059VN-38	1	20000000.0 CEL
Sample 2	E69027ST-20	1	20000000.0 CEL
Sample 2	E69027ST-21	1	20000000.0 CEL
Sample 2	E69027ST-22	1	20000000.0 CEL
Sample 3	F69080LX-11	1	
Sample 3	F69080LX-12	1	
Sample 3	F69080LX-13	1	

Received at:

Shipment issues: None

Please describe the issues with your shipment:

Update Shipment

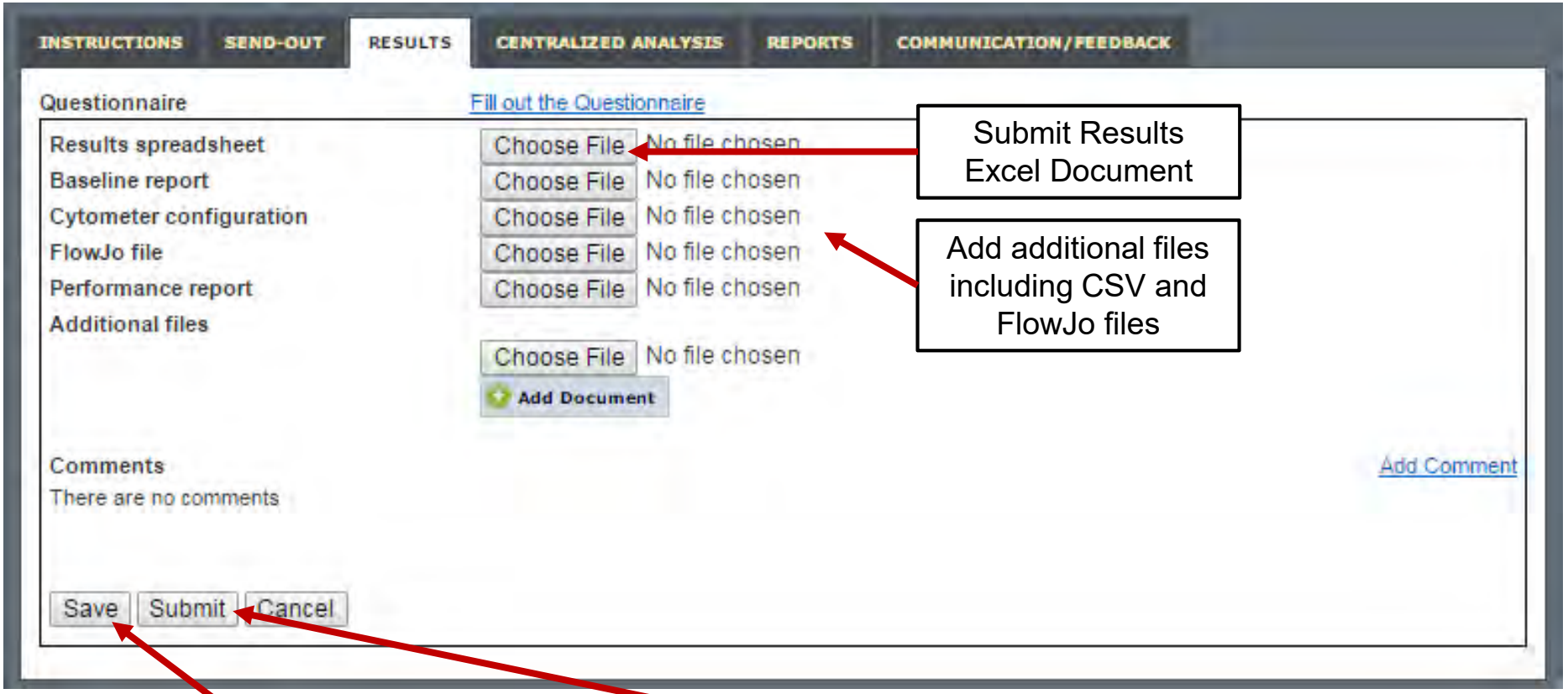
Enter date in “Received at” when your shipment arrives then choose “Update Shipment” below

Change “Shipment Issues” to “Yes” to note any shipping issues and select “Received”

Requesting a New Shipment

- If your shipment had an error, please contact EQAPOL to receive a new kit.
  - The site timeline will be reset based on the new shipment.
- If your site had a technical error that you believe will impact your results, EQAPOL may be able to replace your kit.
  - The site timeline will not be reset based on the new shipment at the discretion of the PI.
- Please contact us if you need to extend your deadline for extenuating circumstances.

Upload Completed Documents: Select "Results"



Document “saved” Example

- “Remove” and “Save” will remove all files selected
- The results sheet will require the proper formatting in order to be successfully imported by EQAPOL

Document “Submitted” Example

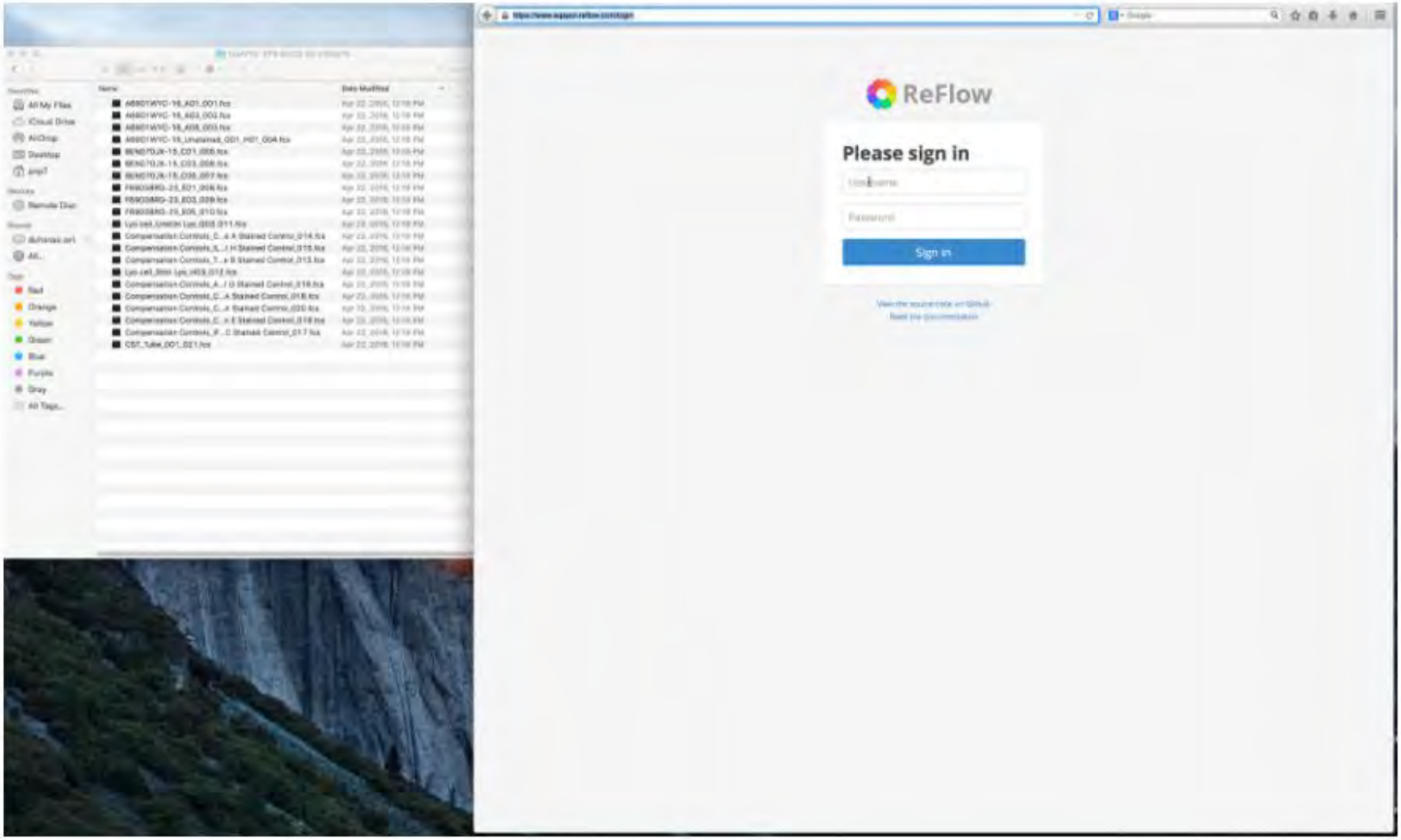
- Submitted documents are final and can only be viewed by sites
- To make any changes contact us (EQAPOL@duke.edu)

ReFlow

- Purpose: standardize data annotation to enable templated centralized analysis
  - Two places one might label parameters in FCS files
    - Instrument configuration
    - Parameter labels
  - Instruments with varying optical platforms create FCS files with inherent differences in instrument configuration metadata
- Web-based application for uploading and downloading FCS files and metadata (www.eqapol-reflow.com)
- Validates annotation against panel specifications
- Exports original and ”cleaned” data files
  - Cleaned files used for EOLm

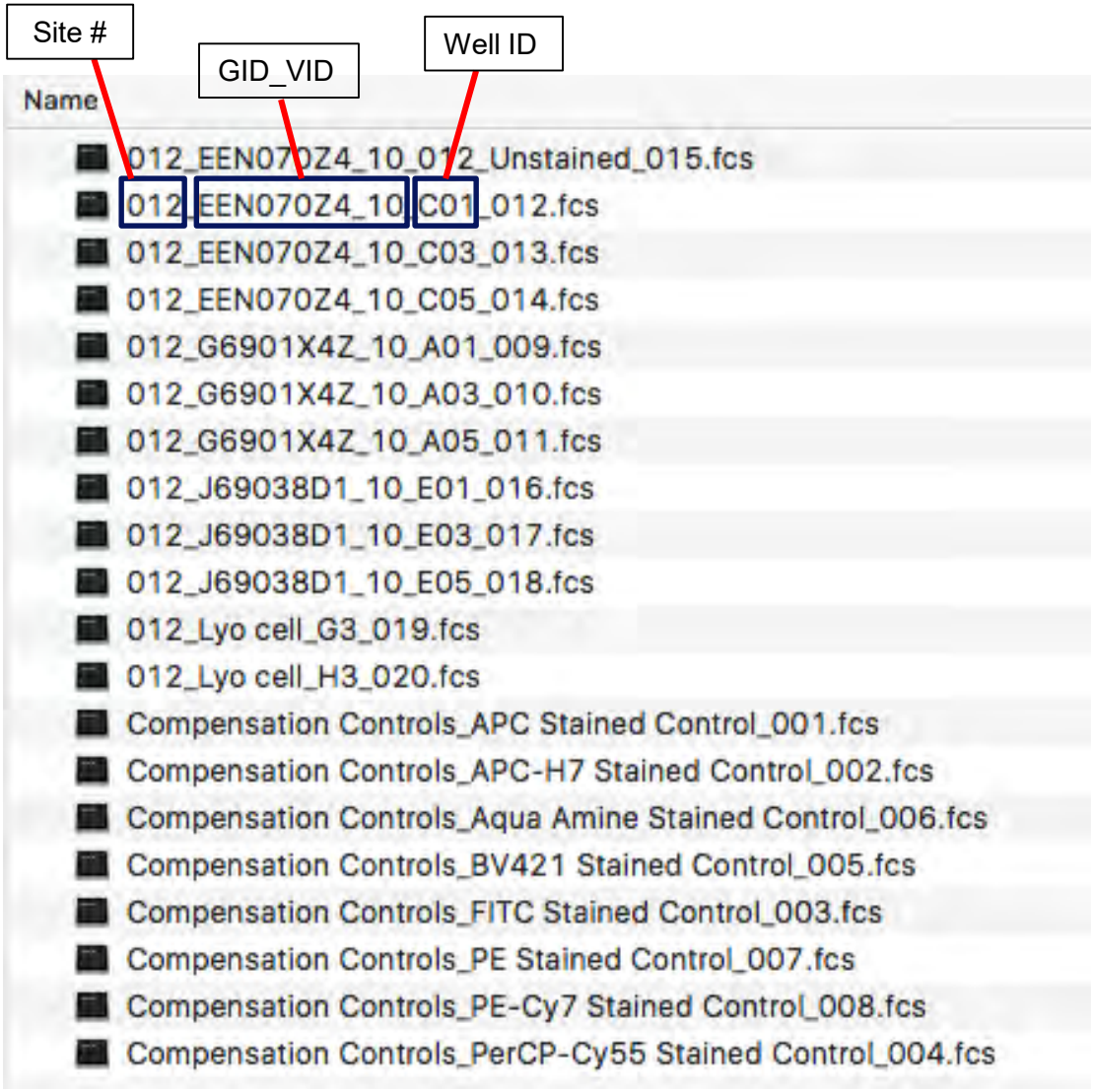
White et al, PMID 26085786

Using ReFlow to Submit FCS files



White et al, PMID 26085786

Example of Properly Annotated FCS Files



- *Site, GID, and Well ID annotation used during EOLm to identify site, sample, and stimulation, respectively.*
- *Improper annotation may lead to out-of-range values for SA & points deducted*

Submitting Data on the Web System

Summary of Documents and files to be uploaded to the EQAPOL Website		
File to be uploaded	**File Name**	**Location to Upload**
Excel template	EQAPOLEP#_Site#_8CICS.xls	EQAPOL Website
FlowJo Analysis document	EQAPOLEP#_Site#_8CICS.jo (or EQAPOEP9_Site#_8CICS.wsp for PC users)	EQAPOL Website
Cytometer Configuration	EQAPOLEP#_Site#_8cICSconfig.csv (or EQAPOLEP#_Site#_8CICSconfig.jpg)	EQAPOL Website
Assay questionnaire	No name, but must be completed	EQAPOL Website
*9 Sample FCS files	Site#_GGGGGGG_GG_WWW	ReFlow
*2 Lyo Cell FCS files	Site#_Veri Cell_WWW	ReFlow
*8 Compensation FCS files	Compensation Controls_Stained Controls (may vary based on FACSDiva version)	ReFlow
*1 Instrument set-up FCS file	Site#_Unstained	ReFlow
1 CST Baseline Report .csv file	EQAPOLEP#_Site#_Baseline report.csv	EQAPOL Website
**1 CST Performance report .csv file	EQAPOLEP#_Site#_Performance report.csv	EQAPOL Website

Assay Assessment

- EQAPOL analyzes data from sites provided via XLS template and questionnaire.
- EQAPOL EOL performs a manual centralized analysis of data files from sites, termed EOLm
- Grading is based on site-reported data and EOLm data, using consensus standards (i.e., not gold standard laboratory)
- EOL reviews each out-of-bounds value, in addition to other relevant materials submitted, to provide detailed “Site-Specific Comments” to improve site performance
- Sites receive a score out of 100 per EP

Grading Flow Cytometry Performance

Acceptability Criteria Used for Grading Flow Cytometry				
Criteria	**Criteria (sub-category)**	**Target**	**Max Points**	**Grading Criteria**
Timeliness		All Valid Files Submitted on or before pre-determined Due Date	10	10 points deducted for late results submission
PBMC Processing	Cell Viability %	≥80%	3	1 point deducted if less than 80%
	Cell Recovery %	Between 70-120%	3	1 point deducted if outside of range
Protocol Adherence	Instrument Set-up	Scatter and Fluorescence Targets are inside range per Protocol	6	0.545 deducted for each channel outside of range
	Data Collection and Analysis	Proper Data Annotation and Gating Strategy Used	5	5 points deducted for deviations
Deviation of EOLm analyzed data from consensus		EOLm value is within 95 CI boundaries of EOLm consensus mean	45	1 point per outlier
Deviation of EOLm and site analyzed data		Deviation of site reported vs EOLm is within 95 CI boundaries of the mean difference	28	1 point per outlier

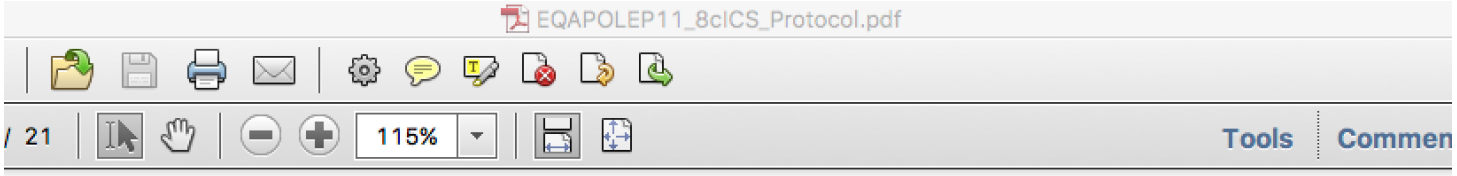
EQAPOL Performance Ranges	
Overall Performance Score	**Performance**
91-100	Excellent
75-90	Good
66-74	Fair
0-65	Poor

Scores triggering remediation

Reports

- Once the EP is closed and the Centralized Analysis is completed, site reports will be made available in the EQAPOL web system. Sites are alerted via email when reports have been uploaded.
- A new tab “Reports” will appear with the report file available for download.
- A Site Choice Report will be available to sites who participated in the site choice component, however the report does not include a numerical score or grade.
- The reports will summarize the results and provide your site with a numerical score and grade category.
- Remediation calls are held with sites that receive a Fair or Poor score to help troubleshoot potential issues.

List of recommendations included in protocol based on common problems



1. **Key Points**
   - 1.1. Verify cytometer performance PRIOR to starting the assay.
   - 1.2. Follow the thawing/resting and cell counting portions of the protocol as instructed.
   - 1.3. Use the instructions provided to properly annotate data (parameter labels and file names).
   - 1.4. Using the samples and target channel instructions provided to calibrate your cytometer. Record target channel values achieved in case you are asked to provide evidence that the channels are within range at the time of sample acquisition.
   - 1.5. Collect 120,000 – 150,000 viable CD3+ lymphocytes.
   - 1.6. Follow the instructions provided for gating your FCS files, ensuring that all viable lymphocytes are included in the viable lymphocyte gate, dead cells are excluded from the viable lymphocyte gate, responding dim cells (CD3+, CD4+, and CD8+) are included in their respective gates, cytokine gates are placed just above the "halo" of the negative or non-responding cells, and that gates are uniform within a single donor
   - 1.7. The EOL strongly recommends that sites check and manually adjust compensation as needed.
   - 1.8. Backgate to maximize positive responses and minimize negative responses.

List of helpful references

Description of EQAPOL program

- Staats JS, Enzor JH, Sanchez AM, Rountree W, Chan C, Jaimes M, Chan RC, Gaur A, Denny TN, Weinhold KJ. Toward development of a comprehensive external quality assurance program for polyfunctional intracellular cytokine staining assays. J Immunol Methods. 2014 Jul;409:44-53. PMC4138230

Tips on gating ICS data

- McNeil LK, Price L, Britten CM, et al. A Harmonized Approach to Intracellular Cytokine Staining Gating: Results from an International Multiconsortia Proficiency Panel Conducted by the Cancer Immunotherapy Consortium (CIC/CRI).Cytometry Part A : the journal of the International Society for Analytical Cytology. 2013;83(8):728-738. PMC4443815

FAQs

- Are reagents tittered?
  - A: Yes, EQAPOL Assay reagents have been optimized and tested across multiple instruments as part of developing the EQAPOL Assay.
  - For the Site Choice Assay, it is highly recommended that each site titer their reagents for use in their own lab.
- Does EQAPOL provide target channels and beads to establish target channels?
  - A: Yes, EQAPOL provides stained compensation beads & Target Channels for use in the EQAPOL Assay. Target channels are provided in the Protocol.
- Do sites need to run CST for each EP?
  - A: Yes, each site needs to run CST before each EP and submit the associated Performance Report as part of the data submission for the EQAPOL Assay.
- Is it possible for more than one operator to participate in an EP?
  - A: Not currently but is being discussed internally
- Is it possible to receive additional kits to run EP samples across multiple instruments?
  - A: Not currently but is being discussed internally
- Is there a specific panel for Site Choice?
  - A: No, as long as your Site Choice panel includes at least 2 of the cytokines (CD107a, IFN-γ, IL-2, or TNF-α), you can use any panel you would like for Site Choice.

FAQs (continued)

- What controls are used?
  - A: An “Unstained” control is prepared as part of the EQAPOL standard ICS assay. The Unstained control is created by the site with the PBMCs provided and is used to set the EQAPOL specific Target Channels for FSC and SSC provided in the EQAPOL ICS assay protocol.
  - An Unstim (PTI only) control well is stained for each donor in the EQAPOL assay. The Unstim control contain cells that are negative for functional markers induced during the 6hr stimulation, but may include residual functional positive cells from any on-going endogenous response that was present at the time of sample collection. The Unstim control should be used to set the cytokine gates for each specific donor by placing the positive region just above the “halo” of the negative cells. When backgating, the Unstim control may be used to minimize the negative response.
  - To keep costs reasonably low, the FMO or Isotype controls are not included as part of the EQAPOL ICS assay; however, they may be useful for sites who wish to observe negative cells in the presence of an endogenous response (or spillover caused by spreading error resulting from sub-optimal panel design) during your on-going research studies.
  - If a site elects to participate in the EQAPOL Site Choice ICS assay, then they should determine the best controls for their individual assay.
- Should sites screen their FBS?
  - A: The EOL highly recommends that each site screen several lots of FBS prior to purchasing to ensure low backgrounds and highest magnitude of responses are observed with each new lot.
- What type of compensation is used?
  - A: Pre-stained lyophilized compensation beads are provided for each fluorescent detector as part of each EQAPOL ICS assay stain plate. If a site elects to participate in the EQAPOL Site Choice ICS assay, then the site should determine the optimal compensation controls to be used. Compensation controls should be used to generate a compensation matrix, either on-line (at the time of acquisition) or off-line (after acquisition). The compensation matrix should be verified manually for accuracy and we highly recommend that any compensation errors corrected prior to analysis.
- What is the timeline for reports being generated and posted?
  - A: DAA and EP reports are generally posted within 6 weeks of completion. For EP specific reports, data from all sites must be submitted prior to analysis. This sometimes results in a significant delay of report generation.

Troubleshooting, Support, Questions

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This project has been funded in whole or in part with Federal funds from the Division of AIDS (DAIDS), National Institute of Allergy and Infectious Diseases, National Institutes of Health, Department of Health and Human Services, under contract No. HHSN272201700061C, entitled External Quality Assurance Program Oversight Laboratory (EQAPOL).