Report on the 25th IVRN Single Donor PBMC QA round, May 2015

Blood was taken from the IVRN donors on 4th May 2015 for processing the following morning along with a freshly obtained local blood sample at each IVRN Tier 1 processing laboratory. PBMC were shipped back to Sydney for assessment later in May. Since the previous QA round, Lab S formally withdrew from regular participation in the QAP. There are now 10 participating laboratories, with 8 out of 10 passing this QA round. Nine laboratories remain certified for IVRN sponsored PBMC cryopreservation.

PBMC recovery and counting accuracy

Laboratories with access to an automated counter provided full blood counts which were used to calculate the total PBMC (lymphocytes + monocytes) in each 30ml blood sample (Table 1). Based on the mean PBMC content of the IVRN blood specimens, 9 laboratories were capable of achieving at least 30% fractionation efficiency from at least one blood specimen (Table 2). The mean fractionation efficiency of 50% was well above the minimum requirement.

Table 1. Total PBMC in 30ml donor blood samples for 25th QA round.

Laboratory	HIPO ($x10^6$)	HINE ($x10^6$)	cell counter
lab B, R	39.7	74.4	CellDyn Sapphire
lab J	84.1	83.1	Coulter Act Diff
lab K	39.0	69.0	Coulter Max M
lab M	42.3	71.1	Sysmex XE5000
lab P	39.0	78.0	Coulter Act Diff
mean	**40.0**	**75.1**	

The accuracy of all PBMC recovery data depends on the accuracy of post-fractionation cell counting. The QA round was assessed using a Coulter Act Diff automated counter. Several labs used similar counters, and many labs also performed manual counting with a haemocytometer. In cases of high neutrophil or erythrocyte contamination resulting from abnormal or aged blood, an automated counter that can accurately quantify the major leukocyte subsets will be able to give a reasonably accurate PBMC count. Manual counting of the sample described above with a haemocytometer can result in overestimation of PBMC if the operator using a microscope with poor visual clarity and resolution cannot distinguish between the larger neutrophils and monocytes, and between erythrocytes and lymphocytes. Similarly, use of the whole blood count as the PBMC count ignores possible neutrophil contamination (which are all dead upon thawing), thus overestimating the true PBMC count. This is the reason why lab R overestimated the PBMC content of their fractionated sample and therefore failed both this and the 24th QA round.

The other critical factor in accurate cell counting is ensuring that sampling technique and dilution is accurate. It is better to perform a small dilution or count neat from a moderately concentrated cell sample (5 – 10 ml) than to perform a large dilution from a highly concentrated low volume (1-2ml) sample. The sample should be evenly suspended without cell clumps. Cells must be evenly suspended when taking the sample for dilution, and equally important cells must be evenly suspended in the specimen tube in the cell counter. Human error in determining the cell sample volume, in making the dilution, in calculation of the final PBMC count, and in even dispensing into cryovials, should also be scrutinised.

PBMC viability

The viability of all thawed PBMC specimens was >90% (Table 2), as determined by visual inspection in the presence of trypan blue, confirmed by manual counting of selected specimens.

Relative and absolute PBMC recovery

The ability to recover sufficient PBMC during fractionation and for the end user to recover sufficient PBMC after thawing are key components of the QA assessment aimed at ensuring that laboratories entrusted with processing valuable blood samples can provide adequate PBMC samples to the study sponsor. Poor fractionation recovery is a waste of the sample. Low or excessively high post-thaw PBMC recovery is also a waste of sample according to the number of PBMC needed for an assay. Therefore, the IVRN QAP determined that fractionation recovery of >30% of PBMC from whole blood, and post-thaw recovery of PBMC ranging from 75% to 125% is required for a laboratory to be certified as competent for PBMC fractionation and cryopreservation, in addition to measures of PBMC viability and function.

Fractionation recovery and post-thaw recovery data are shown in Table 2. Starting from the 24th QA round report, absolute recovery data was included to illustrate the effect of inaccurate cell counting. The recovery results from both the 24th and 25th QA round are shown in Figure 1, and results from each lab are represented by the same symbol between panels. The PBMC content of whole blood (Table 1) is considered a constant, and the thawing and counting of frozen PBMC is performed so as to minimise variation and hence is considered a virtual constant. As discussed above, and based on differences in results between labs, counting of fractionated cells is the major variable. Therefore, overestimation of the cell count relative to the whole blood PBMC count usually results in a relatively high fractionation recovery result (Figure 1a) but a correspondingly low post-thaw recovery (Figure 1b). However, absolute recovery of total PBMC (based on total vials frozen), relative to the whole blood count (Figure 1c), measures the skills of the scientist in producing PBMC, assuming no significant losses during thawing. In the 24th QA round, both fractionation and absolute recovery results suggested that most scientists could extract and deliver between 40 and 60% of available PBMC from blood. However, in the 25th QA round, slightly lower fractionation and post thaw recoveries resulted in a broad range in absolute recovery results instead of a tight cluster between 40-60% obtained in the 24th QA round. The aim is to return to this uniformly high level of recovery that defined the 24th QA round.

The cumulative trend in viability and recovery over the past 10 QAP rounds is shown in Figure 2. The results from the 25th QA round show a small decrease in post thaw recovery over previous rounds, however the overall proficiency of the IVRN Tier 1 Laboratory Network in processing PBMC from a potentially limited clinical specimen is good.

Reasons for low recovery- shared experiences

A catastrophic accident at lab M resulted in mixing of the HIV-pos and HIV-neg PBMC. Nothing could be done to salvage these samples. Thankfully the local donor specimen was of high quality and resulted in a QA round pass.

The lab P scientist experienced difficulties with fractionation with all 3 specimens shown by very sparse PBMC layers after the main Ficol spin step, and extra PBMC could not be recovered after an attempted salvage. A new local donor sample was obtained and gave normal PBMC recovery (this is allowed). Discussions with the scientist suggested that two possible reasons for low recovery. Poor harvesting of the buffy coat before the Ficol spin step is always an unquantifiable possibility. The other possible reason could have been failure to mix the Ficol before use. The product instructions suggest (at least they used to) to mix the Ficol bottle by inverting before use because

denser Ficol may have settled to the bottom of the bottle, and Ficol taken from the top may not be dense enough to trap a PBMC layer.

To salvage a sample with a very small PBMC layer, first remove most of the buffer above the PBMC layer. Then harvest the PBMC layer and the Ficol layer and the granulocyte buffy on top of the erythrocyte layer. Wash with at least equal volumes of PBS, spinning at 500- 700g to ensure all PBMC are pelleted. Then remove all buffer and mix well in 10 - 15ml fresh PBS, and then underlay with 15ml Ficol, and re-spin.

Low fractionation recovery at Lab B was primarily due to insufficient harvesting of the PBMC layer (the scientist said that some remaining cells were visible in the layer but were not taken).

The lab R scientist repeated the same mistake made in the 24th QA round and used the total White Cell Count for PBMC which resulted in reduced post-thaw recovery (discussed above, and in the 24th QA round report). Why?

Functional analysis

The IFNγ ELISPOT assay was used to determine PBMC function, in response to antigenic stimulation with the CEF peptide pool (representative peptide epitopes from CMV, EBV and Influenza), and maximal stimulation from PMA and ionomycin (Figure 3). In this round, PBMC from the HIV-pos donor gave weak responses while the HIV-neg donor gave uniformly strong responses to the CEF peptide pool, and responses to the CEF peptide pool from the local donors ranged from weak to very high, as expected. PBMC from all of the HIV-neg and control donor PBMC samples showed maximal stimulation in the presence of PMA and ionomycin (in excess of 5000 spots/million PBMC), as expected from functional PBMC. Despite some misgivings about PBMC recovery, the ELISPOT analysis demonstrated that PBMC function and quality was excellent.

Overall conclusions on performance in the 25th QA round

All labs achieved uniformly high viability results, and post thaw recovery results continue to be good for most specimens as seen in recent QA rounds. The ability to fractionate sufficient PBMC of excellent quality is good at most labs. The IVRN's high standard for fractionation efficiency, viability and viable recovery was achieved in at least one sample from 8 of the 10 participating labs. Remedial action is now required to return one lab to full certified status, and this should be a relatively simple process. This positions the IVRN Tier 1 Lab network at the highest of international standards for PBMC fractionation and cryopreservation, with highly capable laboratories around the country available for participation in clinical studies involving PBMC cryopreservation.

Thanks for your ongoing participation in the IVRN PBMC processing QAP, and contributing to the national network of clinical trial support labs. To maintain a high level of proficiency, the IVRN recommends that in the absence of routine PBMC cryopreservation work between QA rounds, or if new staff join your group, time should be set aside for specimen processing scientists to self assess their performance between QA rounds. All are encouraged to discuss any methods or performance issues with the QAP coordinator.

25th IVRN QAP report was produced by Dr Wayne Dyer, on behalf of the IVRN Executive.

Table 1. 25th IVRN Single Donor QA Round: PBMC Fractionation Recovery, Viability, Viable Recovery and Function.

			IVRN Tier 1 lab data					QAP coordinator data				PBMC function (ELISPOT)						
lab code	donor category	sample date	blood vol	cells/vial (million)	No. vials	total recovered	[1] fractionation recovery (%)	thawed cell count (X10^6)	[3] post thaw recovery (%)	[6] absolute recovery (%)	[2] viability %	**control** spots/well	**net spots/10^6 PBMC** CEF	PMA/Iono	[1] Adequate PBMC fractionated	Adequate viability/recovery	[4] Adequate response in function assays	[5] overall result
B	HIV-pos	4/05/15	30	10	1	10	25.0	4.850	48.5	12.1	>90	15	0	>5000	no	no	yes	
	HIV neg	4/05/15	30	10	1.5	15	20.0	8.856	88.6	17.7	>90	14	1030	>5000	no	yes	yes	fail
	local donor	5/05/15	30	10	1.5	15	27.8	4.000	80.0	22.2	>90	8	20	>5000	no	yes	yes	
C	HIV-pos	4/05/15	30	6	3	18	45.0	8.152	135.9	61.1	>90	8	10	>5000	yes	high	yes	
	HIV neg	4/05/15	30	9.7	5	48.5	64.6	9.424	97.2	62.8	>90	0	1840	>5000	yes	yes	yes	pass
	local donor	5/05/15	4	6.6	1	6.6	61.1	6.383	96.7	59.1	>90	3	0	>5000	yes	yes	yes	
E	HIV-pos	4/05/15	30	10.25	2	20.5	51.3	11.224	109.5	56.1	>90	12	0	>5000	yes	yes	yes	
	HIV neg	4/05/15	30	11.3	4	45.2	60.2	11.396	100.9	60.7	>90	3	1720	>5000	yes	yes	yes	pass
	local donor	5/05/15	27	10.75	3	32.25	52.9	10.385	96.6	51.1	>90	4	900	>5000	yes	yes	yes	
F	HIV-pos	4/05/15	30	8	3	24	60.0	5.730	71.6	43.0	>90	109	0	>5000	yes	no	no (high control)	
	HIV neg	4/05/15	30	10	6	60	79.9	7.896	79.0	63.1	>90	23	1660	>5000	yes	yes	yes	pass
	local donor	5/05/15	27	9	5	45	>1E6/ml	5.868	65.2	NA	>90	99	1090	>5000	NA	no	high control	
J	HIV-pos	4/05/15	30	8.9	2	17.8	44.5	4.500	50.6	22.5	>90	14	40	>5000	yes	no	yes	
	HIV neg	4/05/15	30	8.4	5	42	55.9	7.736	92.1	51.5	>90	27	1870	>5000	yes	yes	yes	pass
	local donor	5/05/15	20	8	3	24	61.5	6.860	85.8	52.8	>90	19	510	>5000	yes	yes	yes	
K	HIV-pos	4/05/15	30	5.19	4	20.76	51.9	3.500	67.4	35.0	>90	7	30	>5000	yes	no	yes	
	HIV neg	4/05/15	30	5.9	8	47.2	62.8	3.500	59.3	37.3	>90	1	1630	>5000	yes	no	yes	pass
	local donor	5/05/15	27	5.5	5	27.5	50.9	4.500	81.8	41.7	>90	3	0	>5000	yes	yes	yes	
M	HIV-pos	4/05/15	30															
	HIV neg	4/05/15	30															pass
	local donor	5/05/15	30	6.4	5	32	59.3	5.784	90.4	53.6	>90	4	580	>5000	yes	yes	yes	
O	HIV-pos	4/05/15	30	9.2	2	18.4	46.0	6.143	66.8	30.7	>90	8	10	>5000	yes	no	yes	
	HIV neg	4/05/15	30	8.5	4	34	45.3	5.280	62.1	28.1	>90	1	1180	>5000	yes	no	yes	pass
	local donor	5/05/15	18	7.3	3	21.9	>1E6/ml	5.946	81.5	NA	>90	1	830	>5000	yes	yes	yes	
P	HIV-pos	4/05/15	30	1.2	1	1.2	3.0	1.000	83.3	2.5	>90	5	0	>5000	no	yes	yes	
	HIV neg	4/05/15	30	5.3	1	5.3	7.1	4.950	93.4	6.6	>90	5	2970	>5000	no	yes	yes	pass
	local donor	5/05/15	21	8.1	4	32.4	51.4	7.440	91.9	47.3	>90	3	30	>5000	yes	yes	yes	
R	HIV-pos	4/05/15	30	7.23	3	21.69	54.2	4.770	66.0	35.8	>90	12	0	>5000	yes	no	yes	
	HIV neg	4/05/15	30	9.63	5	48.15	64.1	6.909	71.7	46.0	>90	2	1350	>5000	yes	no	yes	fail
	local donor	5/05/15	15	8.98	3	26.94	88.9	5.898	65.7	58.4	>90	2	10	>5000	yes	no	yes	

Notes:

(1) Assessment criteria 1: The minimum required fractionation recovery was 30% of available PBMC, which averaged 40 million PBMC/30ml blood from HIV-pos and 75.1 million/30ml blood from HIV-neg donor. Local donor fractionation efficiency was based on whole blood counts provided by each lab, or at least 1x10^6 PBMC/ml blood if whole blood counts were not available (denoted as ">1E6/ml").

(2) Assessment criteria 2: Viability >80%, determined by Trypan Blue exclusion, counted in a haemacytometer.

(3) Assessment criteria 3: Required recovery of viable cells: >75% and <125% of stated vial contents. Cell counts performed on a Coulter Act Diff cell counter.

(4) Assessment criteria 4: ELISPOT results: PMA/Ionomycin: >5000/10^6 PBMC (all samples); CEF (mean - SD) 0 & >1223/10^6 PBMC (HIV-pos & -neg); control (mean +2SD) <87.4 &<29.1 spots/well (HIV-pos & -neg).

(5) Adequate results in all 4 criteria from at least one specimen (IVRN or local donor) is required to pass the QAP round.

(6) Absolute recovery = total cells thawed x total nunber of vials produced / total PBMC in whole blood sample.

Red shading indicate results that are outside the performance standards.

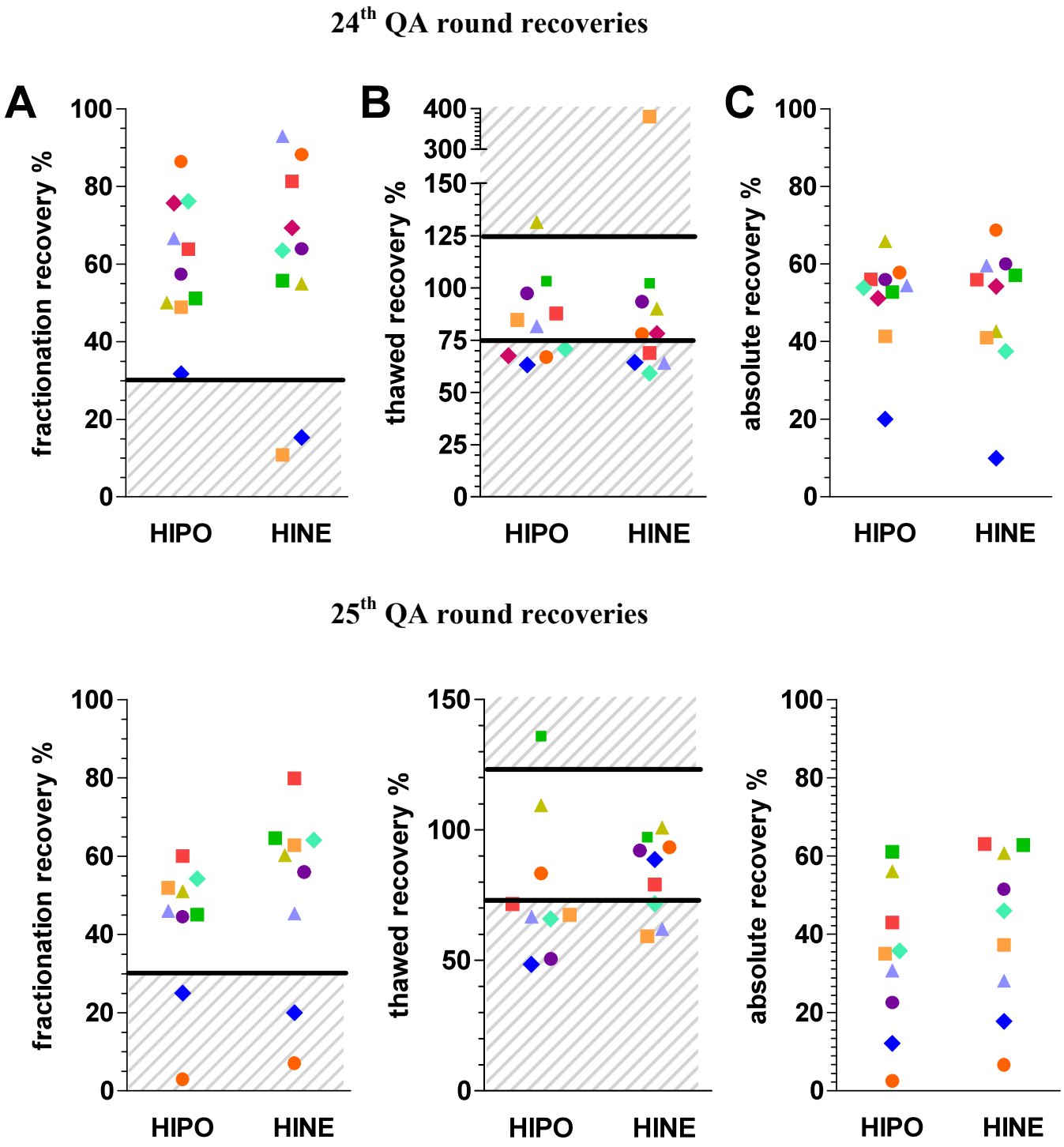

Figure 1. Comparison of relative vs. absolute recovery of PBMC between the 24th and 25th QA rounds, showing (A) post fractionation recovery relative to Tier 1 Lab cell count; (B) thawed PBMC recovery relative to Tier 1 Lab cell count, and (C) absolute recovery of PBMC expressed as the % of the mean whole blood PBMC count. Shaded areas in panels A and B define data outside the QA specifications.

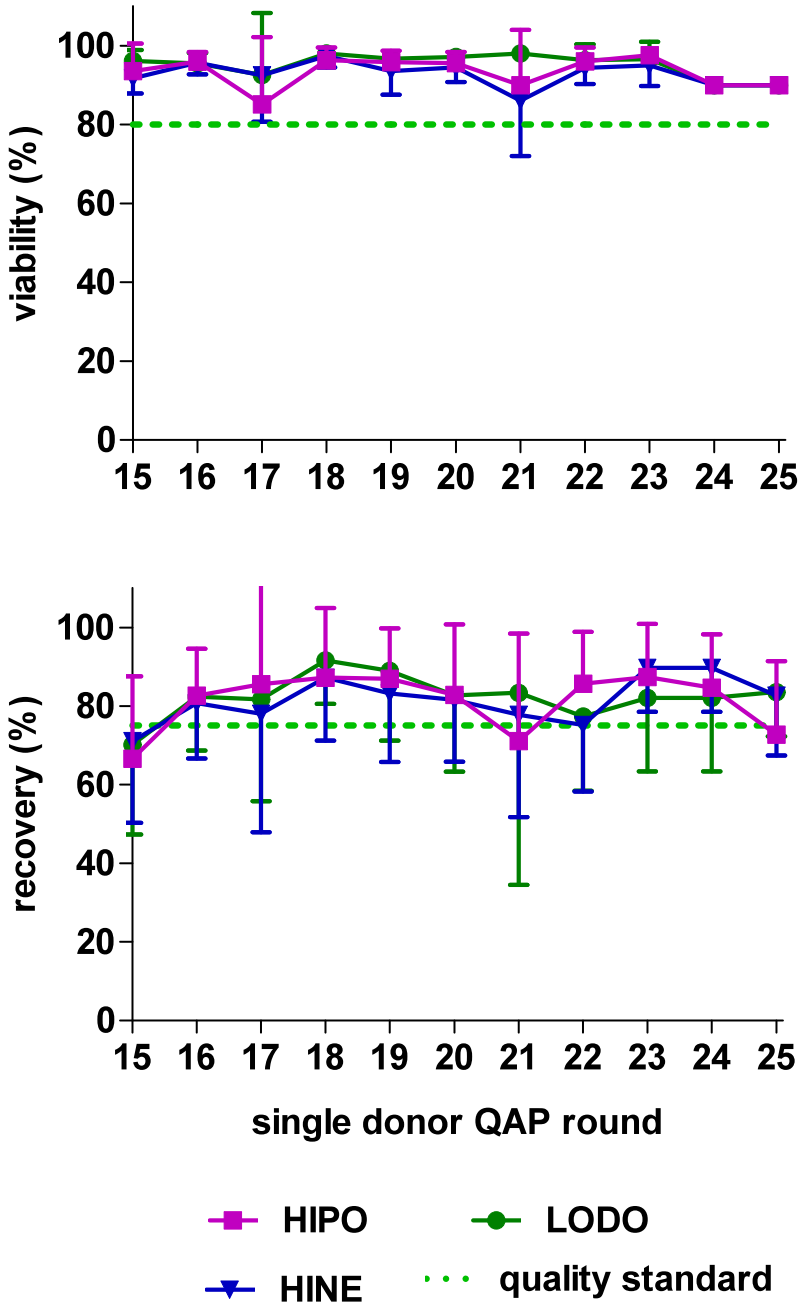

Figure 2. Cumulative trend in viability and post thaw recovery over the previous 10 QA rounds.
Mean and standard deviation; average recovery was calculated using a maximum cell recovery of 100%.

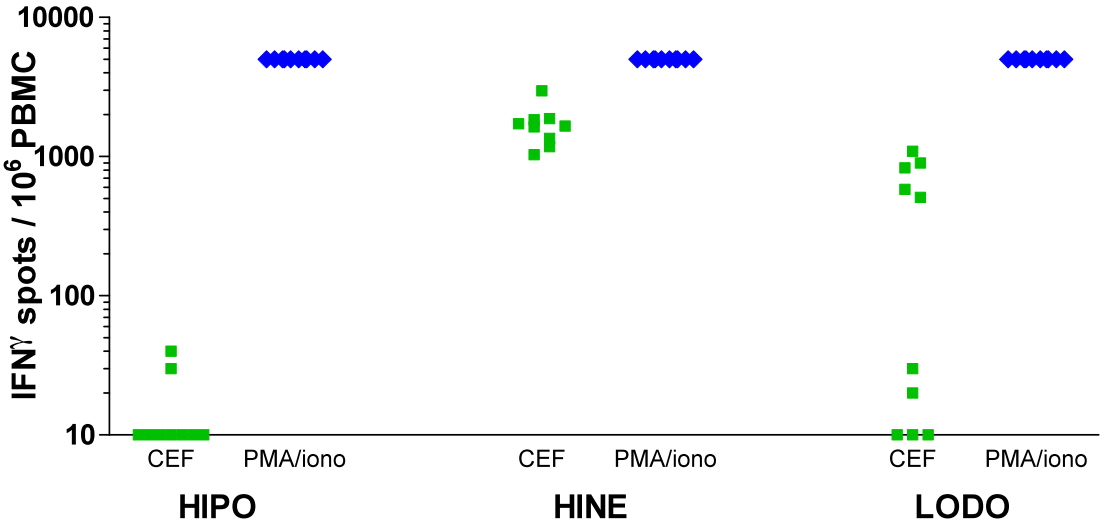

Figure 3. PBMC function results determined by IFN-γ ELISPOT. Antigen-specific responses were determined by stimulation and overnight culture with the CEF peptide pool (green squares), and maximal cytokine release by PMA + ionomycin (blue diamonds).

Table 3. Current certification status of Tier 1 labs.

lab code	Performed adequately over the previous QAP rounds? (all 4 quality standards met in at least one PBMC specimen)			current status
	23rd round	24th round	25th round	(passed 2 of 3 QAP rounds)
B	yes	yes	no	**Certified**
C	yes	yes	yes	**Certified**
E	yes	yes	yes	**Certified**
F	yes	yes	yes	**Certified**
J	yes	yes	yes	**Certified**
K	yes	yes	yes	**Certified**
M	yes	yes	yes	**Certified**
O	no	yes	yes	**Certified**
P	yes	yes	yes	**Certified**
R	yes	no	no	**Certified under review**

Notes (extracted from the IVRN Laboratory Performance Policy):

Performance required for ongoing certification as a Tier 1 Laboratory: The performance standards (above) must be attained from at least one PBMC specimen (IVRN single or local donor), from at least 2 out of the past 3 QA rounds. Non-participation in a QA round is designated as a failed result. A certificate of satisfactory performance will be issued to each successful laboratory after each QA round.

Remedial action if a laboratory fails to maintain accreditation:

- Upon losing fully "Certified" status, a laboratory will be issued with an "Certified - Under Review" report, which recommends that the laboratory continue participation in current clinical trials and cohort studies, but involvement in new studies be deferred. Laboratory staff will be contacted by the QAP coordinator with the aim of identifying potential causes for the below standard performance, and interventions put in place to achieve the quality standard.
- After two consecutive failed attempts at satisfactory performance, the laboratory will be classified as "Unsatisfactory". In due regard for confidentiality of the status of each laboratory, it is the responsibility of the laboratory that is downgraded to "Unsatisfactory" status to notify the relevant clinical trial sponsor of this change of status. The IVRN will not distribute any details of laboratory performance to a third party. The consequence of this change in status is for negotiation between the laboratory and the clinical trial coordinator/sponsor.
- The IVRN Steering Committee will negotiate a remedial plan with the head of a laboratory that becomes "Unsatisfactory" to assist in improving performance. If the response is deemed acceptable, "Certified Under Review" status will be reinstated upon attainment of a satisfactory result in the subsequent QA round. If the negotiation is unsuccessful, termination of Tier One laboratory status will be recommended to the IVRN Steering Committee.