Report: 37th IVRN PBMC cryopreservation QA round, Nov 2022

Executive Summary

The 37th IVRN QA exercise took place on 1st Nov 2022, and laboratory assessment of returned PBMC specimens was completed in Jan 2023. The primary outcomes of this QA round are:

- 10 of 11 labs demonstrated efficient PBMC fractionation recovery;
- 8 of 11 labs provided at least one PBMC sample with post-thaw recovery >75%, but the recovery standard was met in only 10 of 33 PBMC samples tested;
- Good quality PBMC: very high viability and function results;
- 6 of 11 participating laboratories passed this QA round, and 10 labs are currently certified by the IVRN for PBMC cryopreservation.

PBMC fractionation recovery

The total PBMC content in the blood samples provided by IVRN was calculated from FBCs performed on fresh blood and by participating labs the following day:

PBMC = (lymphocytes + monocytes) x 10^6/ml x 29ml (Table 1).

Table 1. Average PBMC (lymphs+monos) in 29ml IVRN blood samples: FBC performed fresh and on the day of processing by the labs indicated below.

Laboratory	HIPO ($x10^6$)	HINE ($x10^6$)	cell counter
fresh blood	68.7	77.7	Coulter DXH500
lab B	70.5	79.2	Sysmex XN550
lab J	60.6	79.3	Coulter DxH500
lab O	66.7	72.5	CellDyn Emerald 22
lab P	72.5	80.9	Coulter DxH500
mean	67.8	77.9	

Fractionation recovery was based on PBMC counts reported by each lab divided by the mean whole blood PBMC content reported in Table 1. The minimum expected fractionation recovery was 30% of whole blood PBMC, or >1 x 10^6 PBMC per 1ml blood from the local donor specimen was if a FBC was not performed. The mean fractionation recovery from all specimens received was 60%, which is at the upper level of recovery expected from careful Ficoll centrifugation (40-60%). Fractionation recovery was low in one lab and the reason this lab failed the QAP exercise (Table 2). However, 4 specimens received had a reported fractionation recovery >80%, and one was 114%. These apparently high fractionation recoveries represented an overestimated PBMC count, and were always associated with a low post-thaw recovery.

A fresh FBC is performed and shared with all labs the day before the QAP exercise, and should be used to indicate if the fractionated PBMC count obtained is reasonable, or should be repeated.

Assessment procedures

Thawing and assessment for the QAP is performed on a single day to maximise consistency. PBMC are thawed in groups of four specimens, a 250µl aliquot from the first 16 specimens is counted on a Coulter DxH500 analyser, the PBMC concentration is adjusted to 1 x 10^6/ml, and PBMC are added to prepared antigens in ELISPOT plates for an 18-hour incubation before development. After the remaining PBMC specimens are thawed and processed, the residual 250µl PBMC aliquots are subjected to Trypan Blue viability assessment. Stained samples are viewed but not counted, unless

some stained dead cells are present. A test count was performed on one sample with a few dead cells, and viability was 96.7%. All samples were therefore considered >95% viable.

Post-thaw PBMC viability and recovery

The quality of thawed PBMC in this QA round was outstanding, with very few non-viable cells observed manually via haemocytometer. All specimens were rated >95% viable (Figure 1, Table 2). A high level of neutrophil contamination was measured in fractionated PBMC from the HIV-pos donor (Table 2), and contaminating neutrophils were up to 35% in some thawed specimens. However, this did not translate into low viability. Since viability assessment was performed at the end of the assessment, dead neutrophils may have disintegrated into general debris (not counted).

Thawed PBMC recovery in this QA round improved on the previous round, but remained low when compared to the previous 10 QA rounds (Figure 1, Table 2). 70% of assessed specimens had fractionation recovery <75%.

The analysis of recoveries (Figure 2) demonstrates an association between high apparent fractionation recovery and low thawed recovery, which is the result of errors (overestimation) in cell counting (*see following discussion on counting errors*). All specimens with a fractionation recovery >80% had post-thaw recovery <75%. Since the expected efficiency of PBMC recovery from Ficoll purification is up to 60%, reported fractionation recoveries >60% may represent an overestimation of cell counts, with repeat counting recommended. Absolute Recovery (Figure 2B) was in the 30-50% range for most samples, but individual specimens failed if either fractionation recovery or thawed recovery were out-of-range.

Functional analysis

PBMC function in this QA round confirmed that PBMC were of high quality (Figure 3). Background spots were low for all PBMC samples, again demonstrating improvements over previous QA rounds. The response to the CEF 32-peptide pool was low in both IVRN donors, whereas local donor covered a wide response range, confirming immunogenicity of this peptide pool. Two PBMC samples failed to show sufficient stimulation by PMA and ionomycin.

Discussion: protocol deviations and counting errors

Potential reasons for errors in the fractionated PBMC count were identified and are summarised in Table 3. Suspension of freshly isolated PBMC in a small concentrated volume (eg. 1 or 2ml) is not only wasteful if a minimum volume is taken for counting, but could exaggerate the effect of poor mixing immediately before counting. The IVRN protocol recommends resuspending PBMC in 5-10ml before counting. If an aliquot of cells removed for counting is not mixed adequately, the auto analyser aspiration pin could sample more cells if positioned in a partially resuspended cell pellet, resulting in an overestimated cell count, or if positioned higher this may result in underestimation.

The HIV-pos donor appeared to have a large proportion of low density neutrophils, a feature of certain inflammatory conditions, and exacerbated by overnight storage before processing. Most labs used counting devices capable of differentiating lymphocytes and monocytes from neutrophils (Table 4), but five labs did not use their counter correctly; 4 labs used the white cell count (WCC) which includes neutrophils (Table 3). Non-differential counters by default provide only WCC, while haemocytometer counting requires considerable experience and a very good microscope to visually distinguish neutrophils/PMNs from PBMC.

A number of calculation errors were noted on the worksheets provided by two labs, including swapping HIPO with HINE counts.

Finally, although not a counting error, producing a single aliquot of 14 million cells from the HINE sample (Lab B) represents a waste of a limited resource, and is a protocol violation.

Certification status of participating laboratories after the 37th QA round

Six of the 11 labs that participated in the 37th QA round provided at least one PBMC specimen that passed all quality standards, and therefore passed this QA round (Table 5). Nine labs are considered certified by the IVRN for proficiency in PBMC fractionation and cryopreservation.

Thanks for your ongoing participation in the IVRN PBMC processing QAP. 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 members join your group, please allow time for participating scientists to practice and self-assess performance between QA rounds. All are encouraged to discuss any methods or performance issues with the QAP coordinator.

37th IVRN QAP report was produced by Dr Wayne Dyer, on behalf of the IVRN Executive.

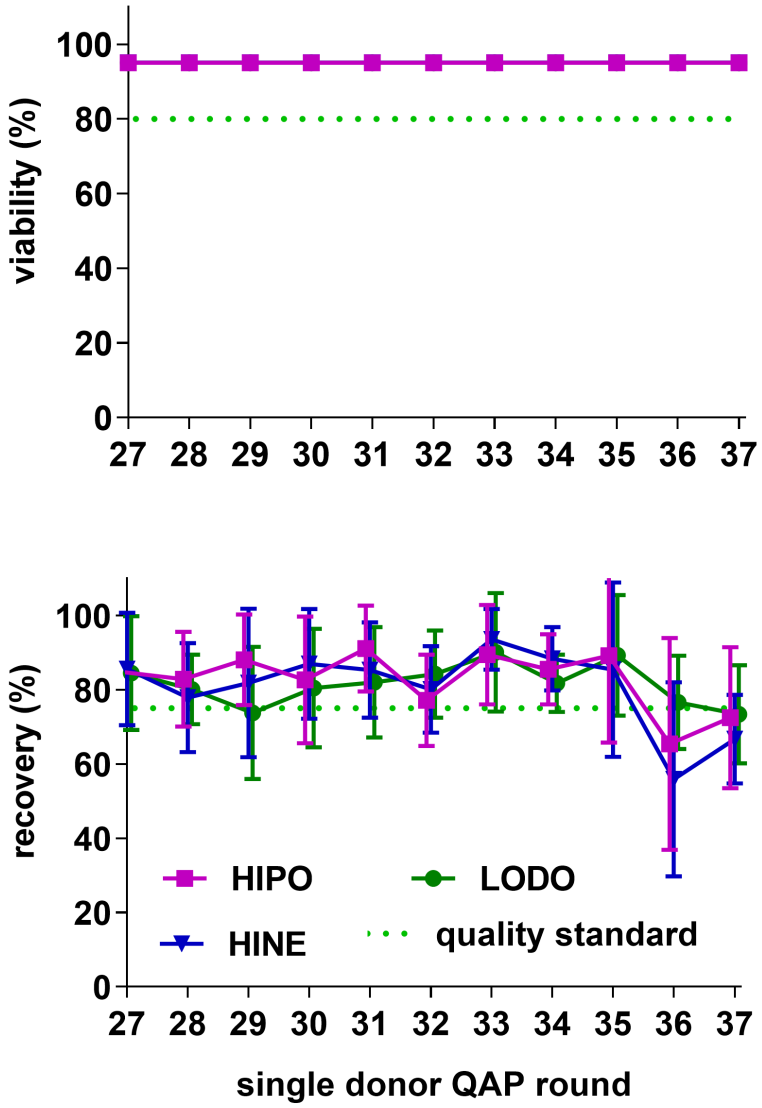

Figure 1. Viability and post thaw recovery compared with the 10 previous QA rounds. Mean and standard deviation; maximum post-thaw recovery was defined as 100% for these mean & SD data.

Table 2. 37th IVRN PBMC Cryopreservation QA Round: PBMC Fractionation Recovery, Viability, Viable Recovery and Function.

									lab data		QAP coordinator data				PBMC function (ELISPOT)					
lab code	donor category	sample date	blood vol	blood PBMC	cells/vial (million)	No. vials	total recovered	[1] fractionation recovery (%)	PMNs in PBMC (%)	thawed PBMCx10^6	[3] post thaw recovery (%)	[6] absolute recovery (%	[2] viability %	control spots/well	net spots/10^6 PBMC CEF	net spots/10^6 PBMC PMA/Iono	[1] Adequate fractionation	Adequate viability/recovery	[4] Adequate function	[5] Overall result
B	HIV-pos	1/11/22	29	67.8	8.8	2	17.6	26.0	11.8	9.85	111.5	29.1	>95	2	<50	3800	no	yes	no	
	HIV neg	1/11/22	29	77.9	14.6	1	14.6	18.7	9.7	14.05	96.2	18.0	>95	0	<50	>5000	no	yes	yes	fail
	local donor	2/11/22	18	22.5	6.6	1	6.6	29.3	0.4	5.95	90.2	26.4	>95	1	350	>5000	no	yes	yes	
E	HIV-pos	1/11/22	29	67.8	9.9	4	39.6	58.4		6.85	69.2	40.4	>95	5	<50	>5000	yes	no	yes	
	HIV neg	1/11/22	29	77.9	9.5	5	47.5	61.0	NA	5.70	60.0	36.6	>95	3	<50	>5000	yes	no	yes	fail
	local donor	2/11/22	27	64.8	10.3	3	30.9	47.7		6.35	61.7	29.4	>95	3	200	>5000	yes	no	yes	
F	HIV-pos	1/11/22	29	67.8	10	4	40.0	59.0	6.6	7.25	72.5	42.8	>95	6	<50	>5000	yes	no	yes	
	HIV neg	1/11/22	29	77.9	10	5	50.0	64.2	5.5	6.65	66.5	42.7	>95	2	<50	>5000	yes	no	yes	pass
	local donor	2/11/22	27	56.7	10	3	30.0	52.9	2.7	9.60	96.0	50.8	>95	8	690	>5000	yes	yes	yes	
J	HIV-pos	1/11/22	29	67.8	10	4	40.0	59.0	46.0	8.35	83.5	49.3	>95	4	<50	>5000	yes	yes	yes	
	HIV neg	1/11/22	29	77.9	10	5	50.0	64.2	18.0	7.10	71.0	45.6	>95	1	120	>5000	yes	no	yes	pass
	local donor	2/11/22	15	15.35	3.7	1	3.7	24.1	2.0	2.35	63.5	15.3	>95	4	790	>5000	no	no	yes	
K	HIV-pos	1/11/22	29	67.8	6.96	5	34.8	51.3	9.6	5.55	79.7	40.9	>95	5	<50	>5000	yes	yes	yes	
	HIV neg	1/11/22	29	77.9	9	6	54.0	69.3	6.3	6.40	71.1	49.3	>95	1	<50	>5000	yes	no	yes	pass
	local donor	2/11/22	27	56.7	7.64	5	38.2	67.4	2.0	4.80	62.8	42.3	>95	4	840	>5000	yes	no	yes	
O	HIV-pos	1/11/22	29	67.8	8.5	6	51.0	75.2	17.8	7.20	84.7	63.7	>95	3	<50	>5000	yes	yes	yes	
	HIV neg	1/11/22	29	77.9	9.17	6	55.0	70.6	9.0	6.20	67.6	47.8	>95	0	110	>5000	yes	no	yes	pass
	local donor	2/11/22	27	54	9.25	4	37.0	68.5	0.1	6.40	69.2	47.4	>95	3	1190	>5000	yes	no	yes	
P	HIV-pos	1/11/22	29	67.8	8.4	5	42.0	61.9	1.8	5.60	66.7	41.3	>95	3	<50	>5000	yes	no	yes	
	HIV neg	1/11/22	29	77.9	8.82	6	52.9	67.9	2.7	5.65	64.1	43.5	>95	1	<50	>5000	yes	no	yes	pass
	local donor	2/11/22	25	67.8	9.03	5	45.2	66.6	4.8	7.05	78.1	52.0	>95	11	680	>5000	yes	yes	yes	
R	HIV-pos	1/11/22	29	67.8	8.6	9	77.4	114.2		4.35	50.6	57.7	>95	2	<50	>5000	high	no	yes	
	HIV neg	1/11/22	29	77.9	7.2	9	64.8	83.2	NA	3.85	53.5	44.5	>95	2	<50	>5000	high	no	yes	fail
	local donor	2/11/22	17	21.3	4.5	2	9.0	42.3		3.25	72.2	30.5	>95	2	360	>5000	yes	no	yes	
T	HIV-pos	1/11/22	29	67.8	8.86	7	62.0	91.5		4.30	48.5	44.4	>95	2	<50	>5000	high	no	yes	
	HIV neg	1/11/22	29	77.9	9.06	6	54.4	69.8	NA	4.70	51.9	36.2	>95	0	110	>5000	yes	no	yes	fail
	local donor	2/11/22	17	NA	5.98	4	23.9	OK		3.45	57.7	NA	>95	1	950	>5000	yes	no	yes	
U	HIV-pos	1/11/22	29	67.8	9.2	7	64.4	95.0	32.9	4.10	44.6	42.3	>95	3	<50	>5000	yes	no	yes	
	HIV neg	1/11/22	29	77.9	8.5	6	51.0	65.5	21.1	5.15	60.6	39.7	>95	2	<50	>5000	yes	no	yes	pass
	local donor	2/11/22	30	62	9.2	3	27.6	44.5	2.5	8.25	89.7	39.9	>95	3	550	>5000	yes	yes	yes	
W	HIV-pos	1/11/22	29	67.8	8.07	4	32.3	47.6		7.90	97.9	46.6	>95	2	<50	3225	yes	yes	yes	
	HIV neg	1/11/22	29	77.9	7.97	4	31.9	40.9	NA	5.65	70.9	29.0	>95	1	<50	>5000	yes	no	yes	fail
	local donor	2/11/22	15	NA	9.65	2	19.3	OK		6.40	66.3	NA	>95	1	1100	>5000	yes	no	yes	
W	LD SepMate	2/11/22	8	NA	6.95	2	13.9	OK		4.60	66.2	NA	>95	1	1060	>5000	yes	no	yes	NA

Notes: **(1) Assessment criteria 1:** fractionation recovery >30% of available PBMC in 30ml whole blood, or >1x10^6 PBMC/ml blood if local donor FBC not available.

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

(3) Assessment criteria 3: Recovery of viable cells: >75% and <125% of stated vial contents.

(4) Assessment criteria 4: ELISPOT IFNg response: PMA/Ionomycin: >5000/10^6 PBMC; CEF (mean - 2SD) >/=0/10^6 PBMC; control spots (mean +2SD) <6.2 & <3.2 spots/well (HIV+ & neg, respectively).

(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 number of vials produced / total PBMC in whole blood sample.

Red Results that failed the assessment criteria. Amber Apparent fractionation recovery >80% is likely erroneous, and associated with low thawed recovery.

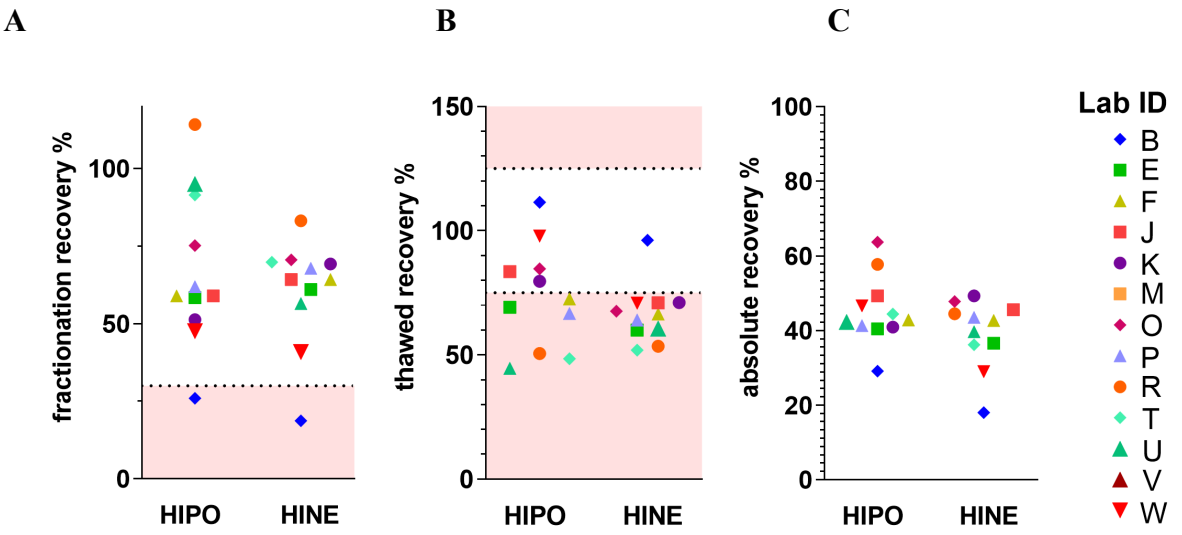

Figure 2. Comparison between relative vs. absolute recovery of PBMC: (A) fractionation recovery; (B) thawed PBMC recovery relative to laboratory cell count, and (C) absolute recovery (thawed PBMC x total number of vials)/(whole blood PBMC count). Shaded areas in panels A and B define data outside the QA specifications.

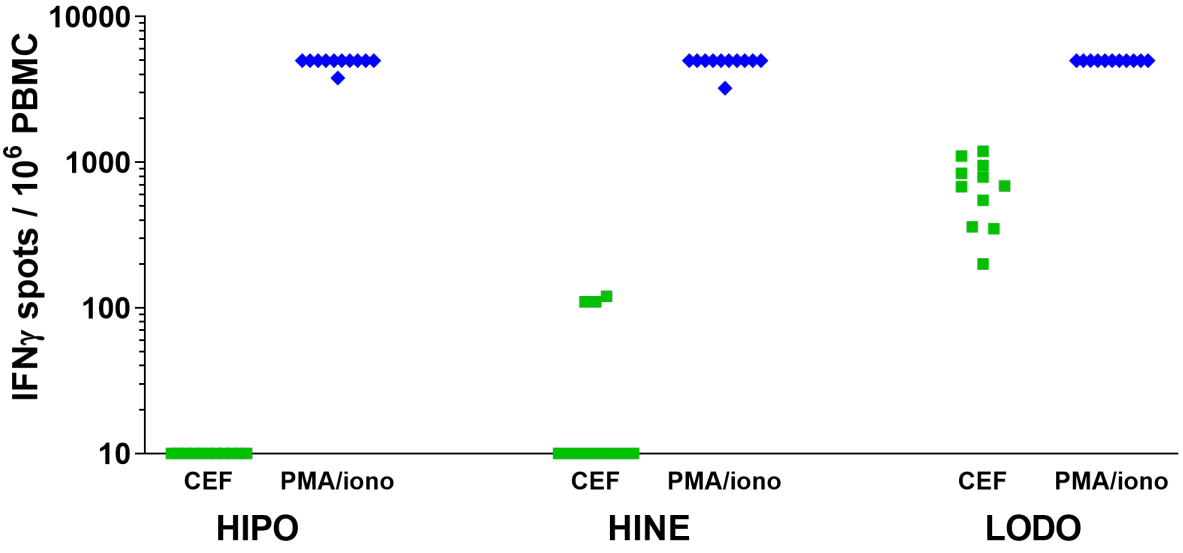

Figure 3. PBMC function results determined by IFN-γ ELISPOT. Antigen-specific responses were determined by stimulation and overnight culture with the CEF peptide pool, and maximal cytokine release with PMA + ionomycin.

Table 3. Summary of protocol issues associated with PBMC counting errors

Protocol issue	Lab ID
PBMC reconstitution volume too small for counting (<5ml)	B, J (2ml); P, R (1ml); E, T W (undefined).
Differential PBMC count not sum of lymphocyte + monocyte, or use of non-differential cell counter	B, F, J, R, T, U (WCC or LØ only); T, W (non-differential counter); E (haemocytometer)
Calculation errors detected on worksheet.	B, J
Other: insufficient aliquots	B (single aliquot of 14 million HINE PBMC)

Table 4. Cell counting method used by each lab in the 37th QA round.

Lab ID	cell counter	Ability to differentiate PBMC (LØ and MØ) from PMNs?
B	Sysmex XN550	yes
E	haemocytometer	visually only
F	Sysmex SN	yes
J	Coulter DxH500	yes
K	Sysmex XN10	yes
O	CellDyn Emerald 22	yes
P	Coulter DxH500	yes
R	Sysmex XN10	yes
T	Logos Luna II	no
U	CellDyn Sapphire	yes
W	Countess II	no

Table 5. Current certification status of Tier 1 labs.

lab code	Adequately performance over the previous QAP rounds? (all 4 quality standards met in at least one PBMC specimen)			current status
	35th round	36th round	37th round	(passed 2 of 3 QAP rounds)
B	fail	fail	fail	**certified under review**
E	pass	pass	fail	**Certified**
F	pass	pass	pass	**Certified**
J	pass	NA	pass	**Certified**
K	pass	fail	pass	**Certified**
O	pass	fail	pass	**Certified**
P	pass	pass	pass	**Certified**
R	pass	fail	fail	**certified under review**
T	pass	pass	fail	**Certified**
U	pass	pass	pass	**Certified**
W	pass	pass	fail	**Certified**

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 until evidence of remedial action to improve performance is provided. 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.