Report: 38th IVRN PBMC cryopreservation QA round, May 2023

Executive Summary

The 38th IVRN QA exercise took place on 2nd May 2023, and laboratory assessment of returned PBMC specimens was completed in May/June 2023. The primary outcomes of this QA round are:

- A substantial improvement in performance after the previous QA round.
- New laboratory (Lab X) joined the QAP.
- 11 of 12 labs demonstrated efficient PBMC fractionation recovery;
- 10 of 12 labs provided at least one PBMC sample with post-thaw recovery >75%; the recovery standard was met in 21 of 36 PBMC samples;
- Good quality PBMC: very high viability and function results;
- 10 of 12 participating laboratories passed this QA round, and 11 labs are currently certified by the IVRN for PBMC cryopreservation.

PBMC fractionation recovery

The total PBMC content in blood samples provided by IVRN (approx. 14.5ml per 15ml tube) 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	67.6	68.7	Coulter DXH500
lab B/R	69.9	69.9	Sysmex XN550
lab J	53.7	67.3	Coulter DxH500
lab O	63.8	66.7	CellDyn Emerald 22
lab P	52.5	80.6	Coulter DxH500
lab U	62.1	64.7	Abbott Alinity
lab X	63.8	66.7	CellDyn Emerald
mean	60.6	67.9	

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 fractionation recovery standard is 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 56%, which is at the upper level of recovery expected from careful Ficoll centrifugation (40-60%). Errors in cell counting were demonstrated in Table 2, showing an association between high apparent fractionation recoveries associated with an overestimation of PBMC counts, resulting in low post-thaw recoveries. One laboratory failed this QAP round because a calculation error halved the apparent fractionation recovery and resulted in thawed recovery results of 200%.

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 this QAP round was repeated on two days. PBMC were 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. In the first test thawing, stained samples are viewed but not counted unless some stained dead cells were present; the IVRN samples from Lab X had high neutrophil counts in both fresh and thawed PBMC, which resulted in thawed viability of 83% and 85%. During the repeat thawing and assessment, more dead cells were noted and Trypan blue viability counts were performed on all samples, reported in Table 2.

Post-thaw PBMC viability and recovery

The quality of thawed PBMC in the first round of thawing was excellent, with very few non-viable cells observed manually via haemocytometer (Figure 1, Table 2). Reduced viability was observed where contaminating neutrophils in freshly isolated PBMC were 30% in some thawed specimens. In the second thawing exercise, viability counts were performed soon after thawing, instead of the end of the assessment day after all ELISPOT cultures were setup, as per usual practice. This may have contributed to more dead neutrophils remaining countable, instead of disintegration into general debris buy the end of the day and therefore not counted.

Thawed PBMC recovery in this QA round improved on the previous round, returning to average levels observed during the previous 10 QA rounds (Figure 1, Table 2).

The analysis of recoveries (Figure 2) demonstrates an association between high apparent fractionation recovery and low thawed recovery, or very low fractionation recovery and excessively high thawed recovery, which is the result of cell counting errors (*see following discussion on counting errors*). Most 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 30-60% 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. The response to the CEFX 32-peptide pool was high in both IVRN donors, whereas local donor covered a wide response range, confirming immunogenicity of this peptide pool. One PBMC sample 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 because the sample taken for counting represents are large proportion of available PBMC, 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.

Most labs used cell counters capable of differentiating lymphocytes and monocytes from neutrophils (Table 4). One lab used the white cell count (WCC) despite availability of differential counts (Table 3). Non-differential counters by default provide only WCC, while haemocytometer counting requires experience and a good microscope to visually distinguish PMNs from PBMC.

Calculation errors were noted on the worksheets provided by two labs: Lab B gave over generous rounding up of cell counts which introduced an immediate 10-13% reduction in cells relative to what was claimed per vial; Lab J did not multiply the cell count by the volume (2ml), swapped HIPO with HINE counts/details on the worksheet, and recording process date instead of collection date for the IVRN blood samples.

Certification status of participating laboratories after the 38th QA round

Ten of the 12 labs that participated in the 38th QA round provided at least one PBMC specimen that passed all quality standards, and therefore passed this QA round (Table 5). 11 labs are currently 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.

38th IVRN QAP report was produced by Dr Wayne Dyer, on behalf of the IVRN Executive.

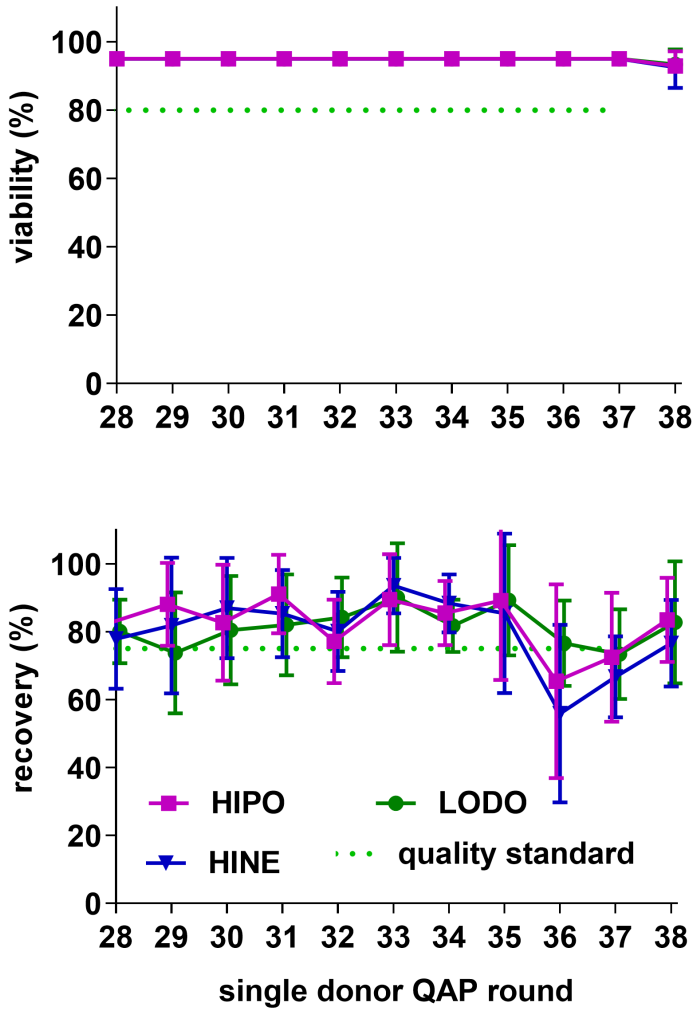

Figure 1. Viability and post thaw recovery compared with the 10 previous QA rounds. Mean and standard deviation; maximum post-thaw recovery was defined as 100%.

Table 2. 38th 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	1/05/2023	29	60.6	10	3	30.0	49.5	NA	6.50	65.0%	32.2	95.6%	0	481	>5000	yes	no	yes	
	HIV neg	1/05/2023	29	67.9	10	3	30.0	44.2	NA	4.96	49.6%	21.9	80.0%	1	642	>5000	yes	no	yes	fail
	local donor	2/05/2023	18	35.6	10	3	30.0	84.3	NA	5.58	55.8%	47.0	97.8%	3	1765	>5000	yes	no	yes	
E	HIV-pos	1/05/2023	29	60.6	9.4	4	37.6	62.0	NA	6.64	70.7%	43.9	88.3%	2	1310	>5000	yes	no	yes	
	HIV neg	1/05/2023	29	67.9	9.9	4	39.6	58.3	NA	7.52	76.0%	44.3	96.5%	0	2297	>5000	yes	yes	yes	pass
	local donor	2/05/2023	27	56.7	10.2	4	40.8	72.0	NA	5.90	57.8%	41.6	93.7%	1	886	>5000	yes	no	yes	
F	HIV-pos	1/05/2023	29	60.6	10	3	30.0	49.5	NA	10.16	101.6%	50.3	89.5%	6	1340	>5000	yes	yes	yes	
	HIV neg	1/05/2023	29	67.9	10	4	40.0	58.9	NA	9.10	91.0%	53.6	97.5%	3	2217	>5000	yes	yes	yes	pass
	local donor	2/05/2023	27	75.6	9.6	3	28.8	38.1	NA	10.50	109.3%	41.7	83.9%	4	0	>5000	yes	yes	yes	
J	HIV-pos	2/05/2023	29	60.6	5	3.3	16.5	27.2	14.0	8.62	172.3%	46.9	96.5%	3	1783	>5000	no	no	yes	
	HIV neg	2/05/2023	29	67.9	5	2.2	11.0	16.2	10.1	10.62	212.4%	34.4	95.0%	3	577	>5000	no	no	yes	fail
	local donor	2/05/2023	26	40	5	2.2	11.0	27.5	0.8	11.26	225.1%	61.9	95.0%	1	400	1350	no	no	no	
K	HIV-pos	1/05/2023	29	60.6	5.52	5	27.6	45.5	7.4	5.22	94.5%	43.1	93.0%	3	1020	>5000	yes	yes	yes	
	HIV neg	1/05/2023	29	67.9	6.8	7	47.6	70.1	4.8	4.64	68.3%	47.9	88.5%	1	2248	>5000	yes	no	yes	pass
	local donor	2/05/2023	27	82.9	5.75	4	23.0	27.7	4.6	5.52	96.0%	26.6	87.0%	3	0	>5000	no	yes	yes	
O	HIV-pos	1/05/2023	29	60.6	8.6	5	43.0	71.0	1.0	6.13	71.3%	50.6	100.0%	1	1367	>5000	yes	no	yes	
	HIV neg	1/05/2023	29	67.9	9.6	5	48.0	70.7	0.7	7.50	78.1%	55.2	97.4%	1	2653	>5000	yes	yes	yes	pass
	local donor	2/05/2023	27	92.4	9.25	8	74.0	80.1	0.2	5.02	54.2%	43.4	96.2%	1	349	>5000	yes	no	yes	
P	HIV-pos	1/05/2023	29	60.6	7.7	3	23.1	38.1	3.1	5.66	73.6%	28.0	96.6%	3	1011	>5000	yes	no	yes	
	HIV neg	1/05/2023	29	67.9	7.46	5	37.3	54.9	1.2	5.66	75.9%	41.7	98.5%	0	2345	>5000	yes	yes	yes	pass
	local donor	2/05/2023	25	70.3	8.11	5	40.6	57.7	6.9	5.83	71.8%	41.4	94.5%	3	622	>5000	yes	no	yes	
R	HIV-pos	1/05/2023	29	60.6	8.19	5	41.0	67.6	10.4	6.61	80.8%	54.6	95.0%	3	890	>5000	yes	yes	yes	
	HIV neg	1/05/2023	29	67.9	8.18	6	49.1	72.3	4.7	6.71	82.0%	59.2	95.3%	2	2040	>5000	yes	yes	yes	pass
	local donor	2/05/2023	17	33.7	7.99	3	24.0	71.1	0.8	7.19	90.0%	64.0	96.5%	1	1740	>5000	yes	yes	yes	
T	HIV-pos	1/05/2023	29	60.6	8.08	4	32.3	53.3	NA	6.13	75.8%	40.5	89.1%	0	1287	>5000	yes	yes	yes	
	HIV neg	1/05/2023	29	67.9	8.51	4	34.0	50.1	NA	5.91	69.4%	34.8	93.5%	2	2747	>5000	yes	no	yes	pass
	local donor	2/05/2023	30	NA	8.83	3	26.5	low	NA	8.72	98.7%	NA	96.8%	7	<50	>5000	no	yes	yes	
U	HIV-pos	1/05/2023	29	60.6	8.58	3	25.7	42.5	2.5	7.40	86.3%	36.6	94.6%	3	940	>5000	yes	yes	yes	
	HIV neg	1/05/2023	29	67.9	8.25	4	33.0	48.6	2.5	7.00	84.8%	41.2	94.6%	1	2007	>5000	yes	yes	yes	pass
	local donor	2/05/2023	25	81.25	6.01	9	54.1	66.6	0.5	5.01	83.3%	55.5	90.5%	3	71	>5000	yes	yes	yes	
W	HIV-pos	1/05/2023	29	60.6	9.86	3	29.6	48.8	NA	8.53	86.5%	42.2	92.4%	3	463	>5000	yes	yes	yes	
	HIV neg	1/05/2023	29	67.9	8.23	2	16.5	24.2	NA	5.49	66.7%	16.2	90.7%	3	940	>5000	no	no	yes	pass
	local donor	2/05/2023	9	NA	5.1	2	10.2	OK	NA	4.76	93.4%	NA	97.4%	0	569	>5000	yes	yes	yes	
X	HIV-pos	1/05/2023	29	60.6	8.5	6	51.0	84.2	32.3	8.30	97.6%	82.2	84.7%	4	1117	>5000	yes	yes	yes	
	HIV neg	1/05/2023	29	67.9	9	6	54.0	79.5	29.5	6.92	76.9%	61.1	82.5%	2	2197	>5000	yes	yes	yes	pass
	local donor	2/05/2023	25	67.5	9	6	54.0	80.0	1.1	8.25	91.7%	73.4	92.2%	3	700	>5000	yes	yes	yes	
R	LD SepMate	2/05/2023	17	33.7	8.86	3	26.6	78.9	1.4	6.95	78.4%	61.8	90.4%							
W	LD SepMate	2/05/2023	4.5	NA	5.15	1	5.2	OK	NA	4.98	96.6%	NA	95.0%							

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 (HIV+ & neg, respectively): PMA/Ionomycin: >5000/10^6 PBMC; CEF (mean - 2SD) ≥ 333 & 403 SFC/10^6 PBMC; control spots (mean +2SD) ≤ 6 & 4 spots/well.

(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.

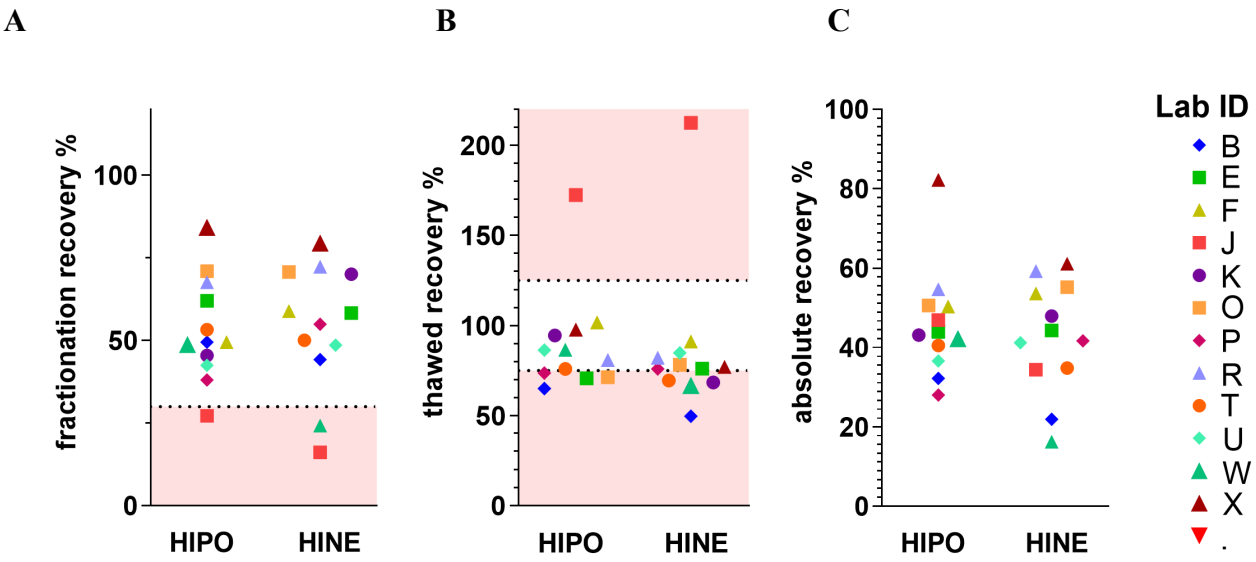

Figure 2. Comparison of relative vs. absolute recovery of PBMC: (A) lab reported fractionation recovery; (B) thawed PBMC recovery relative to vial contents, and (C) absolute recovery (thawed PBMC x total number of vials)/(whole blood PBMC count). Shaded areas in panels A and B define data outside the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

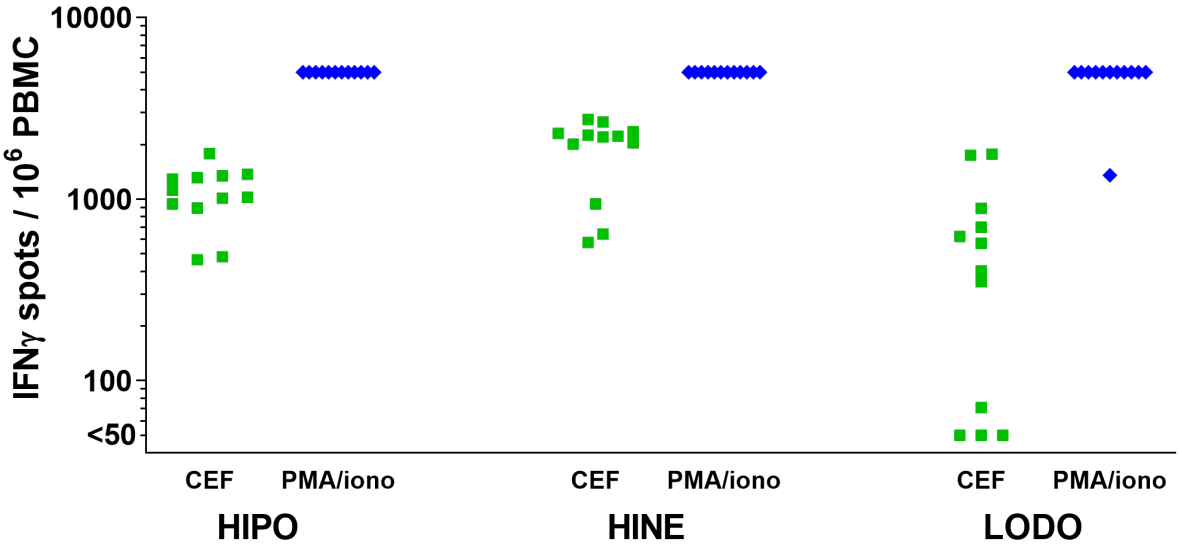

Figure 3. PBMC function results determined by IFN-γ ELISPOT. Antigen-specific responses were determined by stimulation and overnight culture with the CEFX peptide pool, and maximal cytokine release with PMA + ionomycin.

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

Protocol issue	Lab ID
PBMC reconstitution volume too small (<5ml):	B, W (1ml), J (2ml).
Use of non-differential cell counter, or incorrect use of differential count results:	B, (FBC available, but used WCC); T, W (non-differential counter); E (haemocytometer)
Calculation and/or data entry errors on worksheets:	B, J

Table 4. Cell counting method used by each lab.

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
X	CellDyn Emerald	yes

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	38th round	(passed 2 of 3 QAP rounds)
B	fail	fail	fail	fail	**Unsatisfactory**
E	pass	pass	fail	pass	**Certified**
F	pass	pass	pass	pass	**Certified**
J	pass	NA	pass	fail	**Certified**
K	pass	fail	pass	pass	**Certified**
O	pass	fail	pass	pass	**Certified**
P	pass	pass	pass	pass	**Certified**
R	pass	fail	fail	pass	**Certified**
T	pass	pass	fail	pass	**Certified**
U	pass	pass	pass	pass	**Certified**
W	pass	pass	fail	pass	**Certified**
X				pass	**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. A certificate of satisfactory performance will be issued after each QA round.

Remedial action if a laboratory fails to maintain certification:

- After two consecutive failed attempts at satisfactory performance, the laboratory will be classified as "Certified - Under Review", which recommends that the laboratory continue participation in current clinical trials and cohort studies, but recommendation for 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 three 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. "Certified Under Review" status will be reinstated upon attainment of a satisfactory result in the subsequent QA round, then "Certified" status after two consecutive pass results. If negotiation and remedial actions are unsuccessful, termination of Tier One laboratory status will be recommended to the IVRN Steering Committee.