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Report on an International Collaborative Study to Establish the 1st WHO International Reference Panel for HIV-1 Circulating Recombinant Forms for NAT Assays

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Note:
This document has been prepared for the purpose of inviting comments and suggestions on the proposals contained therein, which will then be considered by the Expert Committee on Biological Standardization (ECBS). Comments MUST be received by **4 October 2013** and should be addressed to the World Health Organization, 1211 Geneva 27, Switzerland, attention: Department of Essential Medicines and Health Products (EMP). Comments may also be submitted electronically to the Responsible Officer: Dr Ana Padilla at email: *padillaa@who.int* with a copy to David Wood at email: *woodd@who.int*

Summary

Twenty laboratories were invited to participate in a collaborative study to evaluate the 1st HIV-1 NAT Circulating Recombinant Form (CRF) Reference Panel for use with Nucleic Acid-based Tests (NAT). The Reference Panel consisted of 10 heat inactivated, lyophilised coded samples representing various CRF's with varying degrees of genomic recombination. Each laboratory assayed the blinded panel members which included the 3rd HIV-1 International Standard (IS) on at least two separate occasions and the data were collated and analysed at NIBSC. In total twenty laboratories returned data. Some laboratories returned data using more than one assay type. Nineteen data sets were received from quantitative assays and five from qualitative assays. The results showed that panel members gave a mix of tightly grouped results in the less diverse CRF's such as CRF01 and 02, however there was a greater spread of data in the more genetically diverse viruses such as CRF11 GJ. This highlights that despite the availability of an HIV-1 Subtype Reference panel for the past 10 years, viruses with multiple subtypes within the genome still pose a detection problem for some assay systems. It is therefore considered that this is a valuable panel that continues to provide a means by which to challenge assay development. It is proposed that this panel is established as the 1st International Reference Panel for HIV-1 CRF NAT. No unit is to be assigned to any member of the panel.

Introduction

HIV-1 exhibits substantial genetic diversity and several different subtypes of HIV-1 exist. There is a major group (group M), consisting of subtypes A-K and a more diverse collection of outliers have been referred to as groups N, P and O.

Representatives of the WHO Collaborating Centres involved in the Working Group on Reference Preparations for testing HBsAg, HCV and HIV Diagnostic Kits as well as the WHO International Working Group on Standardisation of Gene Amplification Techniques for the Virological Safety Testing of Blood and Blood Products (SoGAT) agreed at the annual meeting in 1999 that there was a need for a well characterised reference panel of different HIV-1 subtypes and groups. Such a reference panel has been available for 12 years now and has been of great use in regions of the world where non-B subtypes of HIV-1 predominate or in laboratories involved in molecular (NAT) diagnosis and patient monitoring. Kit manufacturers have also benefited greatly from the presence of a panel of known specificity which has enabled them to optimize the assay design, in order to detect as many viral subtypes in circulation as possible.

HIV is however a continually evolving virus and in recent years it has become extremely diverse. Examples of Circulating Recombinant Forms of HIV have been identified with increasing frequency and complexity. Such forms are created when two different subtypes recombine to create a genome that has two or more subtypes present. Genomic regions could be one entire subtype such as the gag region containing all subtype B whilst the Pol region is entirely subtype G; alternatively one region could contain mixed subtypes, such as a gag region containing subtypes A, B, D and F.

As more CRF's are identified so a similar problem was presented to assay manufacturers with regards to ensuring that the assay is optimally designed in the target regions to detect such variants. In conjunction with the Blood Virology SoGAT group, NIBSC agreed to formulate a reference

panel containing a variety of simple and complex CRF's and evaluate it in an international collaborative study. This panel would be intended to complement the 2nd International Reference Panel for HIV-1 Subtypes for NAT assays (NIBSC Code 12/224) established in 2012. This panel, in line with both the HIV-1 and HIV-2 International Standards, would be composed of heat inactivated and lyophilised preparations (Holmes et al, 2011). Heat inactivation has the advantage of (i) being safer to handle during aliquoting and freeze-drying and (ii) simplifies shipping of the material as they are no longer treated as infectious. All viral stocks therefore were heat inactivated at 60^0C for 1 hour and an appropriate dilution of heat inactivated stocks were made into human plasma prior to freeze drying. Approximately 2000 vials of each panel member was prepared each containing, when reconstituted, a volume of 1ml. This randomly coded panel was evaluated in an international collaborative study; the study included a coded sample of the 3rd HIV-1 IS (coded S11).

Materials

Candidate standards

The isolates chosen for the preparation of this CRF panel were, where possible, viral isolates at low passage. All viruses were propagated on human PBMC's and after clarification by centrifugation, a large stock of cell-free culture supernatant was stored under liquid nitrogen vapour as 1ml aliquots.

Selection of virus strains

Table 1 shows the characteristics of viruses used for this panel. The viral strains, subtype group; randomly assigned collaborative study code and NIBSC code are shown. To maintain traceability throughout filling and storage all panel members are referred to by NIBSC code. Where possible, viral isolates with a published full length genomic sequence were chosen.

Source

All viruses were sourced from the Centre for AIDS reagents based at NIBSC. Viruses were chosen where possible where full length sequences were known. The ascension numbers for the full sequence or on cases where only part of the genome is sequenced are shown in table 1.

Heat inactivation of virus

1ml aliquots of virus were placed in a water bath adjusted to 60^oC for a period of 1 hour. The heat-treated virus was pooled, re-aliquoted and stored at $\leq$-80^oC in a laboratory where infectious material was not propagated to avoid any contamination, viruses were then tested using a commercial assay capable of detecting viral reverse transcriptase (RT) activity. During the heat inactivation step the reverse transcriptase enzyme of the virus is denatured rendering it impossible for the virus to replicate. Heat inactivated samples vs the live specimens were tested in parallel. Where no RT activity is seen in the heat treated sample, inactivation is considered successful.

Freeze-drying

This was undertaken at the Centre for Biological Reference Materials (CBRM), NIBSC, Potters Bar, UK, during 2012. The virus subtypes were freeze dried in batches of three, separated by an empty shelf. The freeze dried material consisted of viruses shown in the table 1, diluted in human plasma that had been tested and found negative for HBsAg, HIV antibody and HCV RNA by PCR. For the purpose of traceability during the fill process each candidate was assigned an individual NIBSC code number. Once the panel is established a single, separate, code number will be applied. 1 ml volumes of each subtype member were filled into screw-cap glass freeze-drying vials and the vials freeze-dried.

Post-fill testing

Assessments of residual moisture and oxygen content, as an indicator of vial integrity after sealing, were determined for twelve vials of each of the freeze-dried batches. Residual moisture was determined by non-invasive near-infrared (NIR) spectroscopy (MCT 600P, Process Sensors, Corby, UK). NIR results were then correlated to Karl Fischer (using calibration samples of the same excipient, measured using both NIR and Karl Fischer methods) to give % w/w moisture readings. Oxygen content was measured using a Lighthouse Infra-Red Analyzer (FMS-750, Lighthouse Instruments, Charlottesville, USA). The residual moisture and oxygen measurements can be found in table 2.

Stability studies on the product in the final container

Accelerated degradation studies have been initiated at NIBSC. To date, the results of accelerated degradation studies at 8 months are available, shown in table 3. Samples were incubated at the temperatures and times indicated and were evaluated using the Roche HIV Ampliprep V2 assay. Subsequent testing will take place at a further 12, 24 and 36 months.

Design of collaborative study

Twenty laboratories in seventeen countries were invited to take part in the collaborative study (Appendix 1). Each laboratory received up to 4 heat-inactivated, lyophilised coded panels, each panel member was assigned a number chosen at random between S1-S10, as shown in table 1. The 3rd HIV-1 IS was included as sample 11. Many automated assays now require different minimum input volumes, ranging from 200ul to 1.1ml, therefore each laboratory was sent the study protocol (Appendix 2) in advance and were asked to state how many vials of material they would require to complete the requested testing. Laboratories were requested, where possible, to assay the panel members in duplicate to facilitate the evaluation of intra and inter lab variability. The 3rd HIV-1 IS was also included as a coded panel member in the event that a unitage could be assigned. It was requested that panel members were frozen at -20^{o}C on receipt and participants were requested to return an acknowledgement form sent with the package to report the safe receipt of the samples (Appendix 3). Participants were asked to reconstitute each vial with 1ml of deionised, nuclease-free, molecular biology-grade water and left for a minimum of 20 minutes with occasional agitation before use; a visual check after this time was requested to ensure all vials were fully reconstituted.

The collaborative study was conducted from July to September 2012. All panels were sent with an accompanying Information for Use sheet (IFU). A copy of the IFU can be found in appendix 4.

Statistical Methods

Quantitative Assays: Results from quantitative assays submitted by participants were used directly. Quantitative assays provide estimates in "copies/ml" based on the calibration of the particular assay kit or method. To obtain a single estimate of copies/ml per sample for each laboratory, a single estimate for each assay was obtained by taking the mean of the log10 estimates across replicates and dilutions (after correcting for dilution factor). A mean of the assay means was then calculated to give a single laboratory mean value of log10 copies/ml. The linearity of the assay response across the dilutions range was assessed and in some cases, estimates that fell outside the apparent linear range were excluded from subsequent calculations.

Qualitative Assays: Qualitative assays were analysed by pooling the assay data to give a dilution series of number positive out of number tested. A single estimate of 'detectable units per ml' in the undiluted sample was calculated using the method of maximum likelihood for 'dilution assays' (Collet, 1991). This model assumes that the probability of a positive result at a given dilution follows a Poisson distribution (with mean given by the expected number of "copies" in the sample tested), and that a single "copy" will lead to a positive result. After correction for the equivalent volume of sample amplified, the result is given in "PCR detectable units/ml" (or NAT detectable units) and expressed as a log10 value. As a single value is obtained for each laboratory and sample no assessment of within laboratory, i.e between assay variability can be made.

Results

Data were received from all 20 laboratories invited to participate, laboratories performed either quantitative or qualitative assays. They are referred to by a code number, allocated at random, and not necessarily reflecting the order of listing in the appendix. Some laboratories performed separate assay methods, which have been analysed separately, and are referred to as, for example, laboratory 2A and 2B. In total there were 19 sets of data from quantitative assays, and 5 sets of data from qualitative assays. For the quantitative assays, results were reported as 'copies/ml' for 17 of the data sets, and 'IU/ml' for 2 data sets.

The panel members were scored positive or negative and the results are shown in table 4. The majority of panel members were scored positive in all assays. The quantitative and qualitative assays are grouped together in table 4, as are results from laboratories using the same assay method. Samples determined as positive at neat by the laboratories in both quantitative and qualitative assays are are marked 'P', while samples found negative at neat are marked 'N'. Where a sample had both positive and negative results from repeat assays, or replicates within an assay, they are marked 'P/N'. Some of the samples tested by laboratory 6 (6A and 6B) with qualitative assays were negative at a 1 in 10 dilution, but were not tested neat. These are marked 'N 1/10'.

The laboratory mean estimates of copies/ml or IU/ml (log10) from the quantitative assays are shown in table 5, for all panel members. The results from laboratories using the same assay methods are grouped together, and the overall mean obtained by each method across laboratories is also shown.

The estimates of NAT detectable units/ml (log10) from the qualitative assays are shown in table 6, for all panel members. The laboratory mean estimates (log10 copies/ml) are based on the geometric mean estimates of copies/ml across dilutions and across assays for the quantitative assays, or calculated from the series of number positive out of number tested for the qualitative assays.

The majority of participants returned results as estimates copies/ml. The in-house assay of laboratory 2 was returned as IU/ml. Most laboratories using the Abbott RealTime (ART) returned results as copies/ml, but one laboratory (15) using this kit returned results as IU/ml.

Where laboratories reported data from qualitative assays a $\log_{10}$ PCR detectable units/ml was estimated from the dilution series of positive or negative scores for each sample and laboratory, using the statistical methods described earlier in this report

The estimates from each laboratory and assay method were converted to log10 IU/ml, by direct comparison with the concurrently tested HIV-1 IS, panel member S11, which has an assigned value of 5.27 log10 IU/ml (Morris and Heath). The results are shown in table 7 for quantitative assays, and table 8 for qualitative assays. They are also shown in histogram form in figures 1-10. In the figures, each box represents the mean log10 IU/ml for an individual laboratory and assay method. The boxes are labelled with the laboratory code number and a code indicating the assay method. Results from the qualitative assays are shaded in grey. Negative results are plotted on the extreme left of the scale. The laboratories that obtained negative results at a 1 in 10 dilution, but did not test the samples neat, are plotted next to the negatives, and labelled as Neg.

Estimated copies or IU/ml reported for both quantitative and qualitative assays for the 3rd HIV-1 IS are shown in table 9.

Accelerated degradation studies are underway and the results to date can be seen in table 3. These show that the freeze-dried virus preparation was stable at -20°C, +4°C and +20°C at the 8 month time point. Samples that had been stored at +37°C and +45°C proved difficult to reconstitute, this has been observed in previous studies where samples are based on pooled human plasma. Rather than actual loss of RNA activity, the lower viral copy number seen at elevated temperatures (+37 and +45°C) may reflect an inability to fully reconstitute the freeze-dried pellet. However, in line with other studies, it is expected that this material will show good long term stability (Holmes et al 2001, 2008, 2011). Real time stability studies are also carried out. The panel is included in NAT assays used on site at NIBSC.

Discussion

All panel members were detected by most assays in this study, however large variability in detection efficiencies were observed. Differences exist not only between qualitative and quantitative assays but also across different commercial platforms. Overall, the median estimates across all laboratories and methods range from 3.18 log10 IU/ml (S10) to 4.45 log10 IU/ml (S7).

The laboratory estimates for the panel members S1 to S10 were converted to $\log_{10}$ IU/ml by expressing the results relative to the concurrently tested IS. The laboratory mean estimates (log10 IU/ml) are shown in figures 1-10, in histogram form. Each box represents a laboratory mean

estimate, and the boxes are labelled with the laboratory and assay codes. The scale is log10 IU/ml. From the figures it is clear that the results from the qualitative assays are more variable. However, with limited replication (3-4 assays) of the dilution series – owing to the larger number of samples that were to be assayed in this study it was not reasonable to request further dilutions to be carried out by laboratories, therefore variability is to be expected.

Differences between assay methods are also observed, but these are not consistent across panel members. For example, the ART and CAP assays give identical mean potencies for S5 but differ by over 0.5 log10 for S2. This suggests that the different assay methods may have different efficiencies for quantifying the different CRF/genotypes. The differences in results between methods were significant ($p<0.05$) for all panel members. For S1, S2, S6, and S8 the CAP assay gives significantly higher estimates of IU/ml than the ART or RHP assays, which are not significantly different from each other. For S3, S4, S5, and S10 the RHP assay gives significantly lower estimates of IU/ml than the CAP or ART assays, which are not significantly different from each other. For S07 the CAP assay gives significantly higher estimates of IU/ml than the RHP assay, while the ART assay is in between and not significantly different from either. For S9, all methods are significantly different from each other, with the ART assay giving the highest estimates and the RHP assay the lowest.

Such discordance could be attributed to the amplified target region employed by different assay systems. CRF's with less genetic variation such as CRF 01 AE showed lgreater concordance with the quantitative commercial methods with only some outlying results from in-house qualitative methods. A lower value was observed when the Roche high pure extraction methods was used in conjunction with the Taqman V1 amplification method compared to the same samples extracted and amplified using the Ampliprep/Taqman V2 method. It is known that V1 targets only a region in the gag section of the genome, this region is susceptible to variations and on analysing the genome maps for each CRF (Los Alomos database) variable subtypes are present in these regions. The method V2 employs a dual amplification target, with primers specific to parts of the gag and LTR regions. The LTR area is known to be a highly conserved area of the HIV genome and therefore allowing greater primer/probe efficiency of detection.

It can be seen in figures 1, 2 and 6 that there is in the order of one log difference in detection between the Roche AmpliPrep v 2 assay and the Abbott Real time PCR, the higher or lower reading is not biased towards either assay, with the Roche systems reporting higher in some CRFs and the reverse being seen with others. The Abbott real time system utilises the pol region of the genome for detection

The 3rd HIV-1 NAT IS (NIBSC code 10/152, study code S11) was included in this study and has an assigned value of 5.27 log10 IU/ml (C Morris et al, 2012). The purpose of its inclusion was to allow the determination of potency for each panel member, if sufficient consistence was seen across the assays. The mean of labs using CAP was 5.18 log10 copies/ml, with lab results ranging from 5.05 to 5.23. This would lead to conversion factors of 1 copy = 1.23 IU, or 1 IU = 0.81 copies. The mean of labs using RHP was 4.87 $\log_{10}$ copies/ml, but results were variable between labs, ranging from 4.68 to 5.11 $\log_{10}$ copies/ml. This demonstrates that the conversion of IU to copies depends on the kit being used, and there is close to a 2-fold difference between the main Abbott and Roche kits.

Overall, data for panel members gives a value around 3.5 – 4.0 log10 IU/ml when expressed relative to the concurrently tested IS. However, despite good agreement between some methods, it is still

considered that there is a disparity between some assays and subtypes and therefore it is not beneficial to assign values in IU to panel members. However to allow laboratories to understand the differences observed between methods the report of this study should be made easily available to users. A reference to the report will be made in the IFU of the established panel.

Conclusions

This study has shown that the proposed 1st International Reference Panel for HIV-1 CRF NAT performs well in all assays used within the study. Due to the number of panel members that have been included in the study and the complexity of obtaining clinical patient material of the subtypes assessed in this study it has not been possible to include an assessment of commutability, however should the opportunity arise in the further this will be investigated.

Proposal

We propose that the panel is established as the 1st International Reference panel for HIV-1 CRF NAT. Due to variations in the assays ability to detect different subtypes with equal efficiency we propose that no unit can be assigned to any panel member.

Participant feedback comments

This report was sent to all participants, in total 14 responses were received, the majority if which were to acknowledge that they agreed with the report. Four comments were received concerning minor typographical corrections. Another participant reported they had been missed from the participants list. One comment from a laboratory stated the assay they had used had been incorrectly referenced.

All corrections have been made.

References

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WHO report (to be published by WHO)

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Table 1: Characteristics of the Subtype Reference Panel

Subtype /Group	Strain	Study Code	NIBSC Code	Acession number
Group O	BCF01	1	11/144	AY713425
CRF 11 GJ	MP1307	2	11/148	AF460972
CRF 02 AG	P1261	3	11/140	EU786671
CRF01 AE	CM244	4	11/102	AY494 972
CRF01, A, G, J, U	96CM1849	5	12/146	AJ291720
CRF BG 24	X2456	6	12/144	FJ670526
Subtype J	SE9173	7	11/100	*NA
Subtype C	X1936	8	12/142	EU786673
Subtype G	P962	9	11/138	EU786670
CRF ADG	24203	10	11/146	*NA

* Not Available

Table 2: Fill characteristics

	11/100	11/102	11/138	11/140	11/144
Mean residual oxygen	0.51	0.57	0.13	0.66	0.54
Mean residual moisture	0.230	0.286	0.787	0.520	0.768

	11/146	11/148	12/142	12/144	12/146
Mean residual oxygen	0.71	0.64	0.18	0.24	0.19
Mean residual moisture	0.733	0.722	0.756	1.01	1.02

Table 3: Accelerated Degradation data – 8 months (data shown in copies/ml)

	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10
-20^{o}C	3.69	4.00	3.31	3.67	3.91	4.10	4.47	3.90	3.57	3.22
+4^{o}C	3.68	3.91	3.32	3.73	4.01	4.15	4.44	3.91	3.60	3.20
+20^{o}C	3.63	3.94	3.33	3.57	3.77	4.07	4.30	3.79	3.53	3.12
+37^{o}C	3.15	3.79	2.83	3.31	3.70	3.83	3.90	3.64	3.23	2.83
+45^{o}C	-	-	-	2.52	-	-	3.22	-	2.65	2.01

- No result obtained, sample would not reconstitute successfully.

Table 4: Samples Scored Positive or Negative

Type	Method	Lab	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	S11
Quantitative	ART	10A	P	P	P	P	P	P	P	P	P	P	P
		11A	P	P	P	P	P	P	P	P	P	P	P
		14A	P	P	P	P	P	P	P	P	P	P	P
		15	P	P	P	P	P	P	P	P	P	P	P
	CAP	1	P	P	P	P	P	P	P	P	P	P	P
		3	P	P	P	P	P	P	P	P	P	P	P
		10B	P	P	P	P	P	P	P	P	P	P	P
		11B	P	P	P	P	P	P	P	P	P	P	P
		14B	P	P	P	P	P	P	P	P	P	P	P
		16	P	P	P	P	P	P	P	P	P	P	P
		17	P	P	P	P	P	P	P	P	P	P	P
	RHP	12	P	P	P	P	P	P	P	P	P	P	P
		18	P	P	P	P	P	P	P	P	P	P	P
		20	N	P	P	P	P	P	P	P	P	P	P
	IH	2A	P	P	P	P	P	P	P	P	P	P	P
		4	P	P	P	P	P	P	P	P	P	P	P
		6A	P	P	P	P	P	P	P	P	P	P	P
		9	P	P	P	P	P	P	P	P	P	P	P
		13	P	P	P	P	P	P	P	P	P	P	P
Qualitative	PUP	7	P	P	P	P	P	P	P	P	P	P	P
	PU	19	P	P	P	P	P	P	P	P	P	P	P
	IH	5	P/N	P	P	P	P	P	P	P	P	P	P
		6B	?	P	P	P	P	P	P	P	P	P	P
		8	N	P	N	P	P	P	P	P	P	P	P

P	-	Positive
N	-	Negative or < LLD
P/N	-	Both Positive and Negative replicates or assays
?	-	Not tested Neat but Negative at 1 in 5 and Pos/Neg at 1 in 10

Assay Codes:

ART	Abbott Real Time
CAP	Roche cobas Ampliprep/cobas Taqman
RHP	Roche HighPure System/cobas Taqman
PU	Procleix Ultrio Assay
PUP	Procleix Ultrio Plus Assay
IH	In-House

Table 5: Estimated Copies or IU /ml (log10) from Quantitative Assays

Units	Method	Lab	S01	S02	S03	S04	S05	S06	S07	S08	S09	S10	S11
Copies	ART	10A	3.07	3.11	3.21	3.52	3.56	3.54	4.20	3.37	3.76	2.97	5.06
		11A	2.92	3.06	3.14	3.42	3.43	3.33	4.09	3.29	3.56	2.77	4.96
		14A	3.00	3.06	3.16	3.44	3.50	3.40	4.16	3.33	3.62	2.95	4.98
		Mean	3.00	3.08	3.17	3.46	3.50	3.43	4.15	3.33	3.65	2.89	5.00
	CAP	1	3.60	3.69	3.27	3.66	3.64	3.69	4.29	3.58	3.60	3.04	5.23
		3	3.64	3.79	3.51	3.71	3.67	3.85	4.35	3.70	3.75	3.14	5.22
		10B	3.98	4.04	3.63	3.98	3.91	4.13	4.58	3.82	3.90	3.36	5.32
		11B	3.50	3.66	3.27	3.53	3.57	3.73	4.20	3.65	3.51	3.10	5.05
		14B	3.71	3.77	3.39	3.75	3.68	3.78	4.36	3.72	3.52	3.12	5.14
		16	3.74	3.81	3.37	3.74	3.69	3.86	4.37	3.75	3.46	3.15	5.13
		17	3.76	3.85	3.44	3.60	3.73	3.93	4.45	3.76	3.69	3.26	5.20
		Mean	3.71	3.80	3.41	3.71	3.70	3.85	4.37	3.71	3.63	3.17	5.18
	RHP	12	2.94	3.16	2.86	3.24	3.12	3.38	4.07	3.29	3.09	2.39	4.82
		18	3.18	3.38	3.08	3.36	3.17	3.57	4.10	3.44	3.32	2.52	5.11
		20	Neg	2.71	2.41	2.92	2.52	3.05	3.42	2.87	2.82	2.13	4.68
		Mean	3.06	3.08	2.78	3.17	2.94	3.33	3.86	3.20	3.08	2.35	4.87
	IH	4	3.01	3.14	3.12	3.50	3.60	3.48	4.22	3.48	3.73	3.07	5.10
		6A	2.50	2.61	2.62	3.13	3.09	3.55	3.73	2.62	2.55	2.90	5.30
		9	2.90	2.84	3.04	3.25	3.33	3.23	3.91	3.13	3.46	2.81	5.03
		13	3.07	3.47	2.94	3.54	3.17	3.68	4.33	3.48	3.45	2.91	5.11
IU													
	ART	15	3.33	3.35	3.46	3.71	3.79	3.58	4.38	3.55	3.88	3.14	5.17
	IH	2	2.14	3.29	2.68	2.59	2.97	3.12	3.98	3.12	3.36	2.77	5.03

neg – Negative or < LLD

Table 6: Estimated NAT detectable units (log10) from Qualitative Assays

Method	Lab	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	S11
PUP	7	2.28	2.82	3.37	3.51	3.35	3.51	3.83	3.14	2.83	2.38	5.16
PU	19	2.86	3.14	2.38	2.86	3.55	3.14	3.14	2.86	3.22	1.85	4.48
IH	5	0.53	1.82	2.82	2.32	2.82	2.82	2.82	2.82	2.32	1.82	3.32
	6B	1.25	2.59	2.59	3.36	2.76	3.18	3.59	2.76	2.59	2.36	3.97
	8	Neg	2.63	Neg	1.56	2.98	2.63	3.64	2.98	2.98	1.92	4.70

Neg – Negative at neat

Table 7: Estimated IU/ml (log10) from Quantitative Assays

Method	Lab	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10
ART	10A	3.29	3.30	3.40	3.71	3.75	3.73	4.39	3.56	3.95	3.16
	11A	3.23	3.35	3.43	3.71	3.72	3.62	4.38	3.58	3.85	3.06
	14A	3.29	3.38	3.48	3.76	3.81	3.72	4.47	3.64	3.94	3.26
	15	3.43	3.50	3.61	3.86	3.94	3.73	4.53	3.69	4.03	3.28
	Mean	3.31	3.38	3.48	3.76	3.81	3.70	4.44	3.62	3.95	3.19
CAP	1	3.64	3.86	3.44	3.83	3.81	3.86	4.46	3.75	3.77	3.21
	3	3.69	3.83	3.55	3.74	3.70	3.89	4.39	3.73	3.79	3.18
	10B	3.94	4.03	3.62	3.98	3.90	4.12	4.57	3.82	3.89	3.35
	11B	3.72	3.96	3.56	3.83	3.87	4.02	4.50	3.95	3.81	3.40
	14B	3.84	3.90	3.51	3.88	3.81	3.90	4.49	3.85	3.64	3.25
	16	3.88	3.90	3.45	3.82	3.78	3.94	4.45	3.83	3.55	3.23
	17	3.83	3.93	3.51	3.68	3.80	4.01	4.53	3.83	3.76	3.34
	Mean	3.79	3.91	3.52	3.82	3.81	3.96	4.48	3.82	3.74	3.28
RHP	12	3.39	3.55	3.25	3.62	3.51	3.77	4.46	3.68	3.48	2.78
	18	3.34	3.54	3.24	3.53	3.34	3.74	4.26	3.60	3.48	2.69
	20	-	3.30	2.84	3.35	2.95	3.48	3.85	3.30	3.25	2.56
	Mean	3.37	3.46	3.11	3.50	3.27	3.66	4.19	3.53	3.40	2.67
IH	2	2.38	3.53	2.92	2.83	3.21	3.36	4.22	3.36	3.60	3.01
	4	3.18	3.28	3.26	3.64	3.74	3.62	4.36	3.62	3.87	3.21
	6A	2.48	2.64	2.65	3.16	3.12	3.58	3.76	2.65	2.58	2.93
	9	3.14	3.03	3.23	3.45	3.53	3.43	4.10	3.33	3.65	3.01
	13	3.23	3.65	3.13	3.72	3.35	3.86	4.52	3.66	3.64	3.09
Overall Median		3.37	3.54	3.43	3.71	3.74	3.74	4.45	3.66	3.76	3.18

neg – Negative or < LLD
nt – Not Tested

Table 8: Estimated IU/ml (log10) from Qualitative Assays

Method	Lab	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10
PUP	7	2.39	2.93	3.48	3.62	3.46	3.62	3.94	3.25	2.94	2.49
PU	19	3.65	3.93	3.17	3.65	4.34	3.93	3.93	3.65	4.01	2.64
IH	5	2.48	3.77	4.77	4.27	4.77	4.77	4.77	4.77	4.27	3.77
	6B	2.55	3.89	3.89	4.66	4.06	4.48	4.89	4.06	3.89	3.66
	8	Neg	3.20	Neg	2.13	3.55	3.20	4.21	3.55	3.55	2.49

Neg – Negative at neat

Table 9: Estimated Copies or IU /ml (log10) from Quantitative Assays for 3rd IS 10/152

Units	Method	Lab	Log10 Copies or IU/ml
Copies	ART	10A	5.06
		11A	4.96
		14A	4.98
		Mean	5.00
	CAP	01	5.23
		03	5.22
		10B	5.32
		11B	5.05
		14B	5.14
		16	5.13
		17	5.20
		Mean	5.18
	RHP	12	4.82
		18	5.11
		20	4.68
		Mean	4.87
	IH	04	5.10
		06A	5.30
		09	5.03
		13	5.11
IU	ART	15	5.17
	IH	02	5.03

Assay Codes:

ART	Abbott Real Time
CAP	Roche cobas Ampliprep/cobas Taqman
RHP	Roche HighPure System/cobas Taqman
IH	In-House

Figure 1: Panel Member 1
NIBSC Code 11/144
BCF01 (Group O)

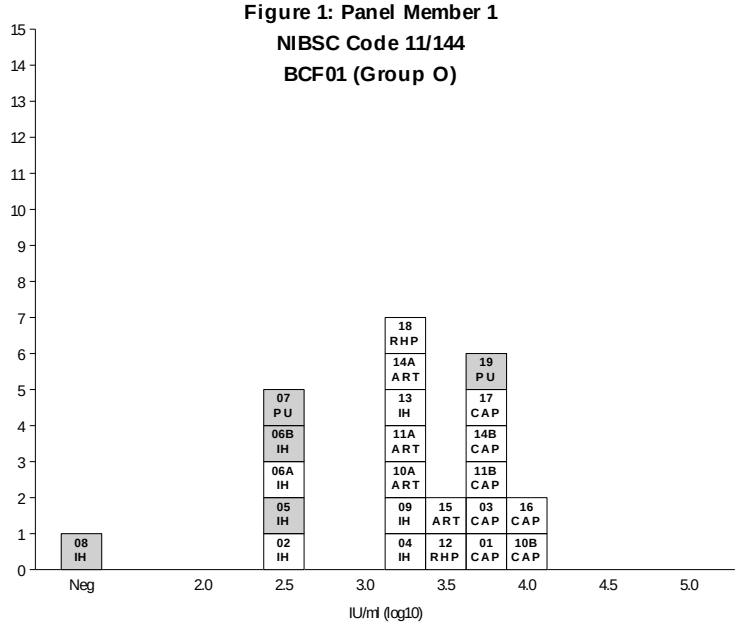


Figure 2: Panel Member 2
NIBSC Code 11/148
MP1307 (CRF 11 GJ)

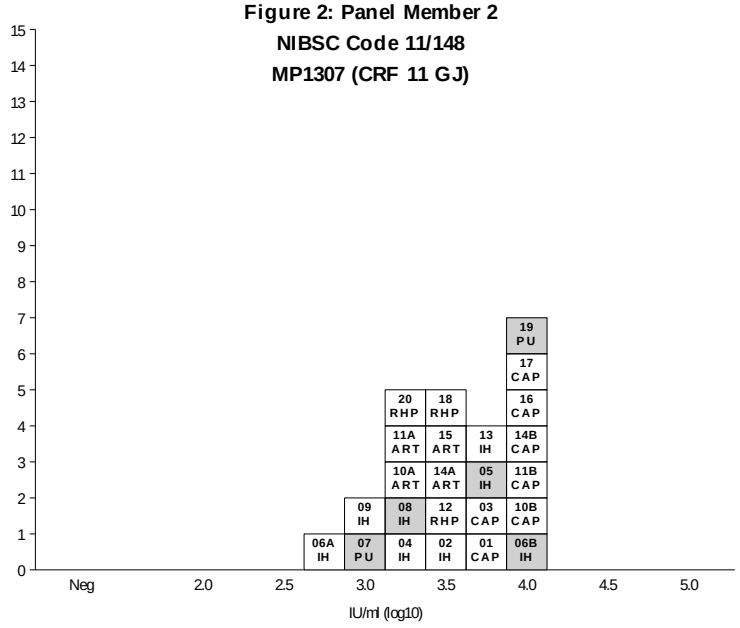


Figure 3: Panel Member 3
NIBSC Code 11/140
P1261 (CRF 02 AG)

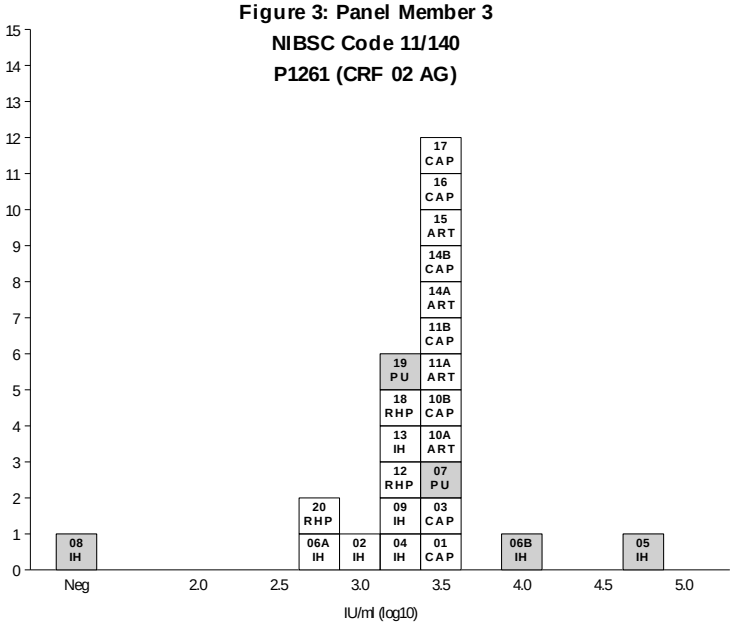


Figure 4: Panel Member 4
NIBSC Code 11/102
CM244 (CRF01 AE)

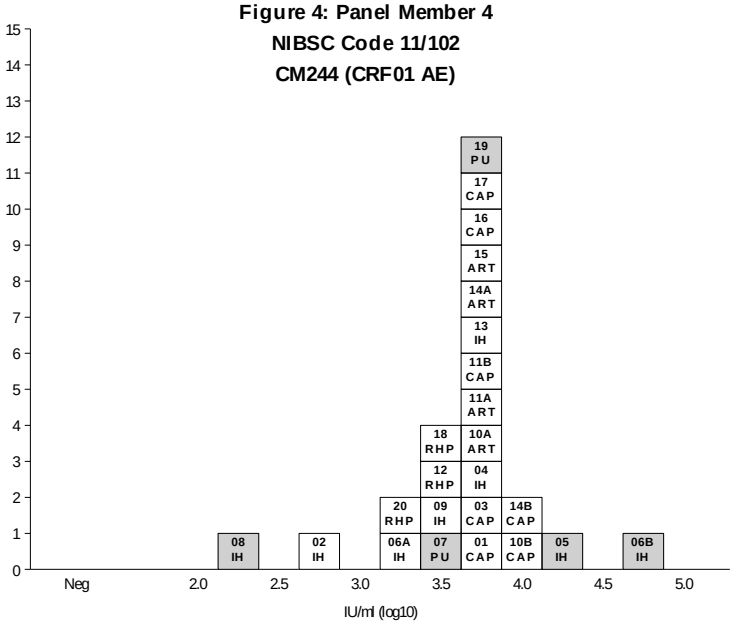


Figure 5: Panel Member 5
NIBSC Code 12/146
96CM1849 (CRF01 A,G,J,U)

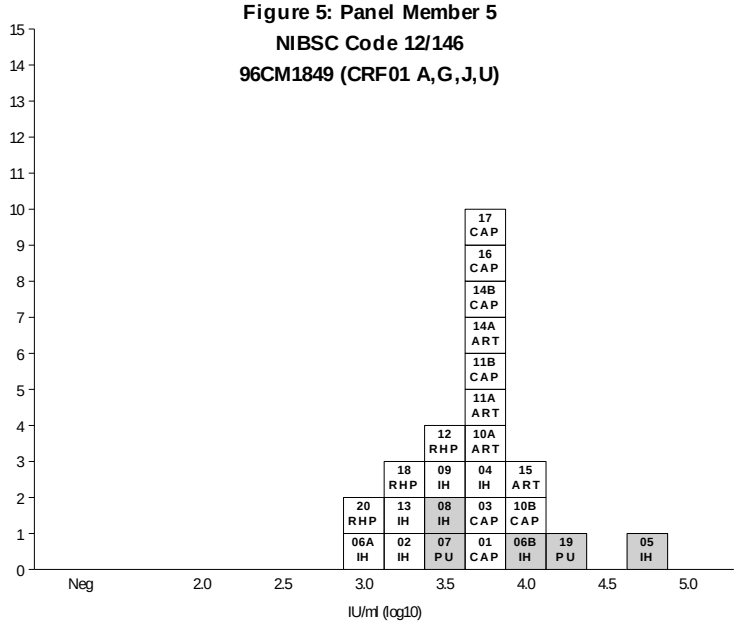


Figure 6: Panel Member 6
NIBSC Code 12/144
X2456 (CRF BG 24)

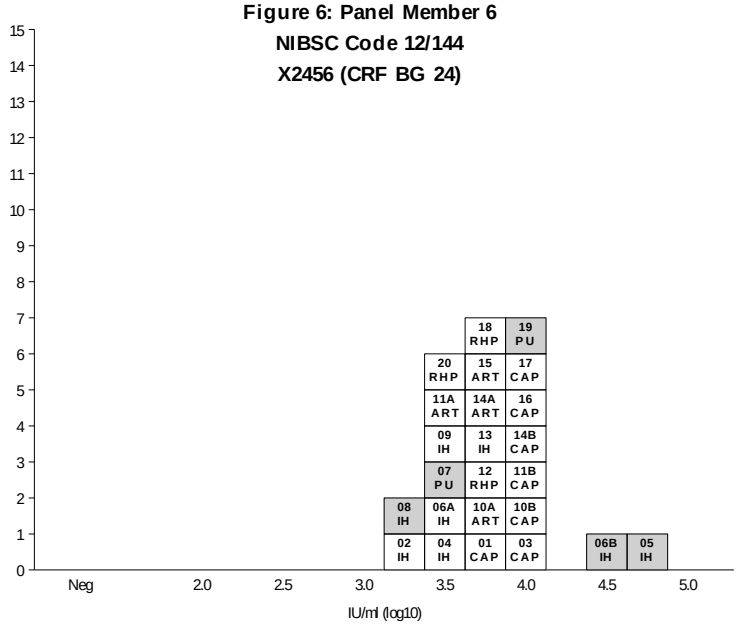


Figure 7: Panel Member 7
NIBSC Code 11/100
SE9173 (Subtype J)

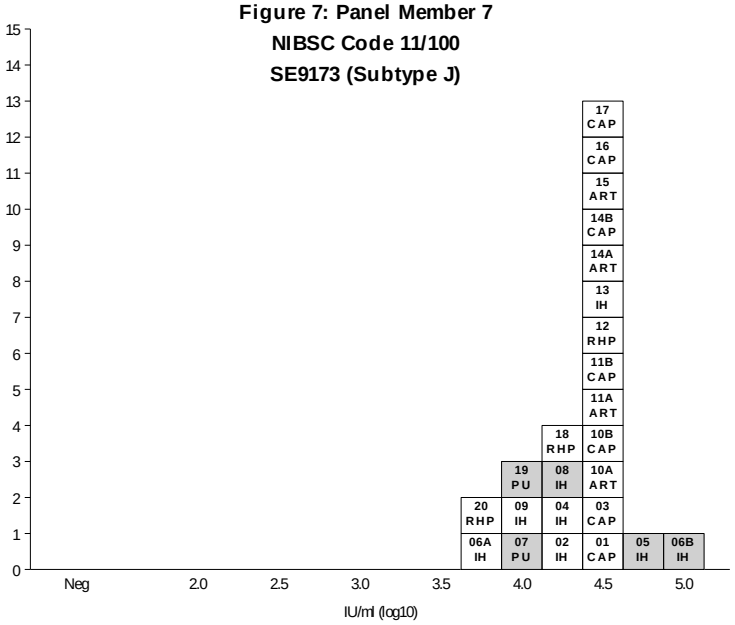


Figure 8: Panel Member 8
NIBSC Code 12/142
X1936 (Subtype C)

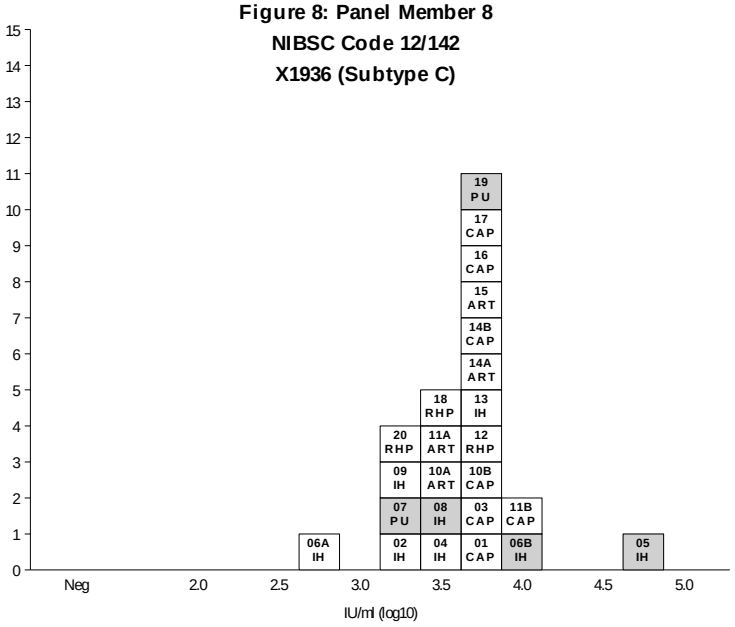


Figure 9: Panel Member 9
NIBSC Code 11/138
P962 (Subtype G)

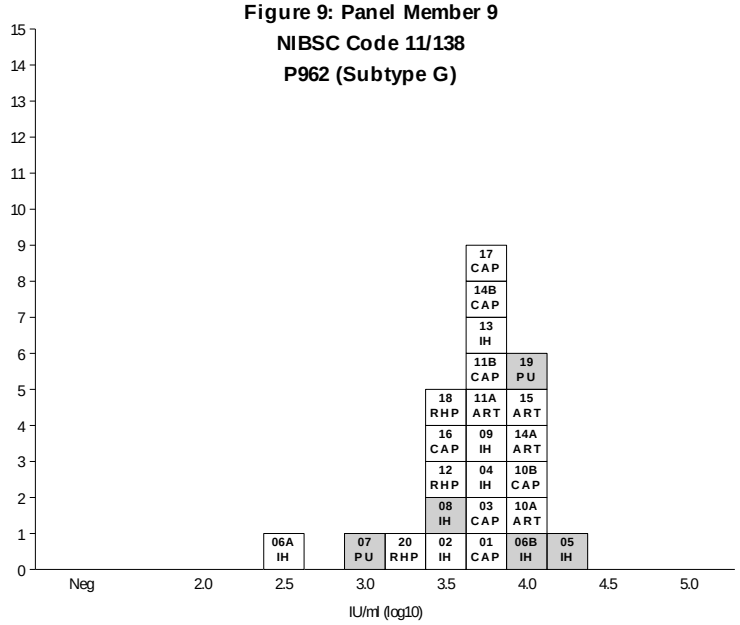
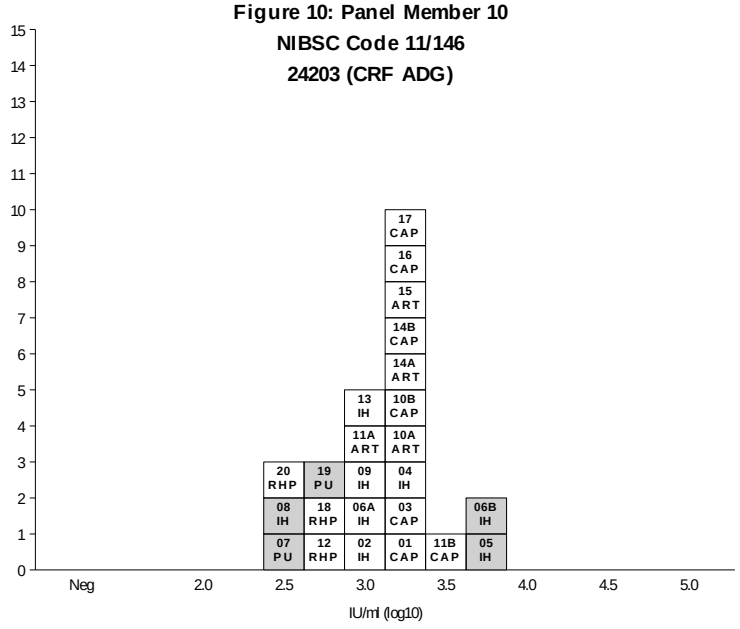


Figure 10: Panel Member 10
NIBSC Code 11/146
24203 (CRF ADG)



Appendix 1: Participants

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Appendix 2 – Study Protocol

Evaluation of the1st HIV-1 NAT CRF panel

Objective.

To assess the ability of various commercial and in-house assays to detect a panel of HIV-1 RNA subtypes.

Background.

It has been known for some time that different subtypes of HIV-1 exist. There is the major group known as M consisting of subtypes A –J and more diverse groups of outliers such as group N, P and O. A few years ago nucleic acid-based tests had a narrow band of specificity targeting the B clade as this was most predominate in the Western World. Improvements have been made in assay design in an attempt to detect a wider range of subtypes.

However it is recognised that recombination events have led to a wider diversity of HIV-1 subtypes and in recent years viruses containing one or more subtype sequence within the genome have become evident, these viruses have become known as Circulating Recombinant Forms (CRF's). It is known from collaborative studies conducted using an HIV-1 NAT panel containing many common subtypes that predominately circulate within in Europe, North America and Asia, that some assays are still poor at detecting such subtypes, thus giving a low or negative result on samples that are known to be positive. In order to allow manufacturers of assays and laboratories running in house assays to validate an assays ability to detect CRF's the WHO endorsed the development of the 1st HIV-1 NAT CRF panel. This panel is being assessed in this collaborative study.

Materials.

The package consists of 10 vials containing representatives from HIV-1 NAT Circulating Recombinant Forms, a member from diverse HIV groups and a standard subtype C virus, the members are as follows – C, G, J, O, CRF 01, 02 11, 13, 24 and CRF ADG, the 3rd International Standard for HIV-1 NAT is also included. All panel members have been coded.

All panel members and the International Standard have been diluted in HBsAg, anti-HIV and anti HCV negative human plasma.

CAUTION.

THESE PREPARATIONS ARE NOT FOR ADMINISTRATION TO HUMANS.

All preparations have been heat inactivated at 60^0C for 1 hour. Successful heat inactivation has been demonstrated by the use of a commercial assay designed to detect Reverse Transcriptase activity in a live virus preparation. In all cases no RT activity was seen indicating successful heat inactivation. However as with all biological preparations the material should be used and discarded according to your own laboratory safety procedures.

Study Design.

You have been sent up to 4* HIV-1 CRF panels (each panel contains 10 members as described above), up to 4* vials of the International Standard for HIV-1 NAT has also been included, all vials have been randomly coded Sample 1 - 11.
*(number of vials is dependent on how many ml's is required in each assay, this information has been derived from the assays that you indicated on your initial study acceptance form)

For each assay, one panel (samples 1-11) should be reconstituted in 1ml of molecular grade water, all panel members should be prepared and tested at the same time in a single assay.

Participants are requested to perform two independent assays and where possible to test each sample in duplicate in each assay.

A fresh vial of each reagent should be used in each assay.

We do not recommend that vials should be used after a freeze thaw cycle.

For Quantitative assays.

Assay samples 1 - 11 undiluted, preferably in duplicate, if sample numbers are restricted by the assay, then test samples singularly.

Where possible two assays should be carried out on separate occasions.

For Qualitative assays.

Assay samples 1- 11 determine the HIV-1 RNA end-point for each preparation. We suggest using neat material and 3 tenfold dilutions.

In the subsequent assays participants are requested to assay a minimum of two half log dilutions either side of the pre-determined end point.

Results.

Results of each assay should be recorded on the appropriate result form included with this information sheet. A separate methods form is provided so that all relevant information can be recorded.

All completed forms should be returned to:

Erin Wigglesworth/Clare Morris
Division of Retrovirology
NIBSC
Blanche Lane
South Mimms
Potters Bar
Herts EN6 3QG
UK

Fax: +44 1707 641060

Appendix 3: Material Receipt form

Recipients name		Lab code Number:
Organisation Address		
Materials sent		
Date sent		
Condition of package		
Condition of contents		
Was the sample still frozen (if applicable)?		
Other comments		
Date received		
Recipients Signature		

Please complete and return this form to:

Erin Wigglesworth/Clare Morris
National Institute for Biological Standards and control
Blanche Lane
South Mimms
Potters Bar
Herts.
EN6 3QG
UK

Fax: +44 (0) 1707 641060
Erin.Wigglesworth@nibsc.hpa.org.uk
Clare.Morris@nibsc.hpa.org.uk

Appendix 4: Panel IFU

International Ref. Preparation
1st HIV-1 NAT CRF Panel
NIBSC code: 13/xxx (TBC after ECBS approval)
Instructions for use
(Version 1.00, Dated)

N/A

1. INTENDED USE

This panel has been assessed in an international collaborative study with 24 laboratories using a range of difference commercial and in house assays. It is intended for use in HIV-1 nucleic acid based procedures and should be used to aid the development of new assays and the optimisation of current assays to detect such a range of HIV-1 RNA Circulating Recombinant Forms (CRF).

The panel consists of different heat inactivated HIV CRF's and subtypes spiking into a matrix of human plasma that have been tested and found negative for for HBsAg, Anti HIV and Anti HCV. The HIV viruses have been grown through tissue culture to high titre prior to spiking in to the plasma.

Panel members contain whole virus and are

2. CAUTION

This preparation is not for administration to humans.

Virus contained within this panel has been heat inactivated at 600C for 1 hour, effective heat inactivation have been demonstrated by the use of a commercial RT activity test. The plasma diluent used has been screened and found negative for HBsAg, Anti HIV and Anti HCV.

As with all materials of biological origin, this preparation should be regarded as potentially hazardous to health. It should be used and discarded according to your own laboratory's safety procedures. Such safety procedures should include the wearing of protective gloves and avoiding the generation of aerosols. Care should be exercised in opening ampoules or vials, to avoid cuts.

3. UNITAGE

No unit has been assigned to any panel member as the differences in detection ability varied too greatly in the data returned from the collaborative study. However each vial is clearly labelled with the subtype and the report outlining this study is available via the WHO website [1].

4. CONTENTS

Country of origin of biological material: United Kingdom.
The panel consist of 10 members individually stored in 5ml screw capped glass vials. Each vial contains 1ml of lyophilised plasma preparation.

5. STORAGE

The panel should be stored at -20^{0}C on receipt.
Please note: because of the inherent stability of lyophilized material, NIBSC may ship these materials at ambient temperature.

6. DIRECTIONS FOR OPENING

Vials have a screw cap; an internal stopper may also be present. The cap should be removed by turning anti-clockwise. Care should be taken to prevent loss of contents. Please note: If a stopper is present on removal of the cap, the stopper should remain in the vial or be removed with the cap.

7. USE OF MATERIAL

No attempt should be made to weigh out any portion of the freeze-dried material prior to reconstitution

Each panel member should be reconstituted with 1ml of deionised, nuclease free molecular grade water and left for a minimum of 20 minutes prior to use. The vials should be gently agitated at least twice during this period. The reconstituted material contains whole virus and is therefore designed to be used in the laboratories extraction system prior to amplification.

8. STABILITY

Reference materials are held at NIBSC with assured, temperature controlled storage facilities and they should be stored on receipt as indicated on the label. It is the policy of WHO not to assign an expiry date to their international reference materials. Accelerated degradation studies have indicated that this material is suitably stable, when stored at -200C or below, for the assigned values to remain valid until the material is withdrawn or replaced. These studies have also shown that the material is suitably stable for shipment at ambient temperature without any effect to the assigned values. Users who have data supporting any deterioration in the characteristics of any reference preparation are encouraged to contact NIBSC. The stability of the material when reconstituted has not been specifically determined. Therefore it is recommended that the standard is for single use only.

9. REFERENCES

1. Morris c, Wigglesworth E, Heath A. Evaluation of the proposed WHO 1st HIV-1 CRF panel for NAT. WHO ECBS report 2013; WHO/BS/2013.xxxx

10. ACKNOWLEDGEMENTS

We gratefully acknowledge the important contributions of the collaborative study participants.

11. FURTHER INFORMATION

Further information can be obtained as follows;
This material: enquiries@nibsc.org
WHO Biological Standards:
http://www.who.int/biologicals/en/
JCTLM Higher order reference materials:
http://www.bipm.org/en/committees/jc/jctlm/
Derivation of International Units:
http://www.nibsc.org/products/biological_reference_materials/frequently_asked_questions/how_are_international_units.aspx
Ordering standards from NIBSC:
http://www.nibsc.org/products/ordering_information/frequently_asked_questions.aspx
NIBSC Terms & Conditions:
http://www.nibsc.org/terms_and_conditions.aspx

12. CUSTOMER FEEDBACK

Customers are encouraged to provide feedback on the suitability or use of the material provided or other aspects of our service. Please send any comments to enquiries@nibsc.org

13. CITATION

In all publications, including data sheets, in which this material is referenced, it is important that the preparation's title, its status, the NIBSC code number, and the name and address of NIBSC are cited and cited correctly.

Medicines and Healthcare Products Regulatory Agency

National Institute for Biological Standards and Control, Potters Bar, Hertfordshire, EN6 3QG
WHO International Laboratory for Biological Standards, UK Official Medicines Control Laboratory

T +44 (0)1707 641000
nibsc.org

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14. MATERIAL SAFETY SHEET

Physical and Chemical properties			
Physical appearance: Lyophilised powder		Corrosive:	No
Stable:	Yes	Oxidising:	No
Hygroscopic:	No	Irritant:	No
Flammable:	No	Handling:	See caution, Section 2
Other (specify):			

Toxicological properties	
Effects of inhalation:	Not established, avoid inhalation
Effects of ingestion:	Not established, avoid ingestion
Effects of skin absorption:	Not established, avoid contact with skin

Suggested First Aid	
Inhalation:	Seek medical advice
Ingestion:	Seek medical advice
Contact with eyes:	Wash with copious amounts of water. Seek medical advice
Contact with skin:	Wash thoroughly with water.

Action on Spillage and Method of Disposal

Spillage of ampoule contents should be taken up with absorbent material wetted with an appropriate disinfectant. Rinse area with an appropriate disinfectant followed by water.
Absorbent materials used to treat spillage should be treated as biological waste.

15. LIABILITY AND LOSS

Information provided by the Institute is given after the exercise of all reasonable care and skill in its compilation, preparation and issue, but it is provided without liability to the Recipient in its application and use.
It is the responsibility of the Recipient to determine the appropriateness of the standards or reference materials supplied by the Institute to the Recipient ("the Goods") for the proposed application and ensure that it has the necessary technical skills to determine that they are appropriate. Results obtained from the Goods are likely to be dependent on conditions of use by the Recipient and the variability of materials beyond the control of the Institute.
All warranties are excluded to the fullest extent permitted by law, including without limitation that the Goods are free from infectious agents or that the supply of Goods will not infringe any rights of any third party.
The Institute shall not be liable to the Recipient for any economic loss whether direct or indirect, which arise in connection with this agreement.
The total liability of the Institute in connection with this agreement, whether for negligence or breach of contract or otherwise, shall in no event exceed 120% of any price paid or payable by the Recipient for the supply of the Goods.
If any of the Goods supplied by the Institute should prove not to meet their specification when stored and used correctly (and provided that the Recipient has returned the Goods to the Institute together with written notification of such alleged defect within seven days of the time when the Recipient discovers or ought to have discovered the defect), the Institute shall either replace the Goods or, at its sole option, refund the handling charge provided that performance of either one of the above options shall constitute an entire discharge of the Institute's liability under this Condition.

16. INFORMATION FOR CUSTOMS USE ONLY

Country of origin for customs purposes*: United Kingdom * Defined as the country where the goods have been produced and/or sufficiently processed to be classed as originating from the country of supply, for example a change of state such as freeze-drying.
Net weight: 1g/vial
Toxicity Statement: Toxicity not assessed
Veterinary certificate or other statement if applicable. **Attached:** No Please add vet cert numbers separated by a space