Report on a Collaborative study for proposed NIBSC Working standard for Tumor Necrosis Factor-alpha (TNF-α)

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Summary

Declining stocks of the current 3rd World Health Organization (WHO) International Standard (IS) for human sequence recombinant Tumor Necrosis Factor - alpha (TNF-α) coded 12/154 for assignment of potency to preparations of human TNF-α has necessitated the need for a NIBSC working standard for human TNF-α.

A human sequence recombinant human TNF-α from the same lot as the current TNF-α IS was lyophilized and evaluated in-house prior to a collaborative study. Subsequently, the candidate working standard for human TNF-α (17/232) was assessed relative to the existing International Standard coded 12/154 in bioassays for TNF-α by seven laboratories in seven countries for its suitability as a working standard and for assignment of unitage.

Data from the study showed that the standard is suitable for use in assays which utilise the current TNF-α IS. The mean estimate of the TNF-α bioactivity of the candidate standard, coded 17/232, is 42,607 IU per ampoule. It is proposed that the candidate standard, 17/232 is established as the NIBSC Working Standard for human TNF-α with an assigned bioactivity of 42,607 IU per ampoule (expanded uncertainty 40,720 – 44,582 calculated on a log scale with coverage factor k=2.36 corresponding to a 95% level of confidence).

Responses from study participants

Responses were received from six of the seven participants. Minor comments were received relating to the names and addresses of participants (Table 3) and these have been corrected. All responses received were in agreement with the proposal that the preparation coded 17/232 is suitable as the NIBSC Working Standard for human TNF-α with an assigned bioactivity of 42,607 IU per ampoule (expanded uncertainty 40,720 – 44,582 calculated on a log scale with coverage factor k=2.36 corresponding to a 95% level of confidence).

Introduction

Human Tumor Necrosis Factor - alpha (TNF-α) is a major pro-inflammatory cytokine involved in regulation of infections and in necessary protection of the host. It is clinically approved in Europe as an adjunct to surgery for soft tissue sarcoma of the limbs due to its ability to induce tumour necrosis and cytotoxicity. However, clinical translation has mainly occurred not in its use in cancer therapies but rather in inhibition of its biological effects in autoimmune and/or inflammatory diseases that are associated with abnormal persistent release of TNF-α. Several inhibitors of TNF-α activity have achieved unprecedented clinical success and are approved for treatment of autoimmune and/or inflammatory disorders such as rheumatoid arthritis, psoriasis and Crohn's disease.

The third International Standard (IS) for human TNF-α (12/154) consisting of a rDNA derived TNF preparation was established by the WHO Expert Committee on Biological Standardisation (ECBS) in October 2013 (WHO Technical Report Series 987, 2014). The IS has proved suitable for the potency labelling of the approved TNF-α product (INN tasonermin) and is widely used for calibration of human TNF-α preparations which serve as critical reagents for potency evaluation of anti TNF-α products. The global requirement for such a standard is evidenced by the high sustained demand for the current standard and the continued expansion in the number of anti-TNF-α products approved or in development worldwide.

Stocks of the current 3rd WHO IS, 12/154, are declining and to conserve stocks of this standard, a decision was taken to develop a lyophilized NIBSC working standard for use as a critical reagent in bioassays for anti-TNF-α products. The rationale was that the availability of the working standard would allow unrestricted distribution to some extent of the working standard to current users of the WHO IS and also allow potentially new customers access to the standard. Usage of the WHO IS has increased due to the utility of the IS as a critical reagent for development of biosimilar medicines and for calibration of immunoassays measuring cytokine release for various immunotherapeutics.

It was also agreed that the lyophilized candidate preparation be evaluated for bioactivity in a small study relative to the current 3rd IS for TNF-α and, subject to suitability, be considered to serve as a NIBSC working standard for TNF-α. However, since the protein used in the working standard is the same as the current IS and was previously assessed in TNF-α neutralization assays using a range of antagonists, in particular, etanercept, infliximab and adalimumab, a need for re-evaluation was not considered necessary.

Following endorsement of this strategy by the NIBSC Standards Program Board, the candidate preparation was lyophilized using a stock of the bulk drug substance lot used for the 3rd IS (retained at NIBSC at -80^{0}C) and the formulation and freeze-drying procedures used for the 3rd IS with strict adherence to WHO recommendations (ECBS guidelines - WHO Technical Report Series 932, 2006) for development of the standard were followed. A small multi-centre collaborative study comprising seven expert laboratories was organised to assess the suitability of the proposed standard for TNF-α and to facilitate its value assignment relative to the current 3rd IS for TNF-α as per WHO procedures (WHO Technical Report Series 932, 2006). The results of this study are provided in this report.

Aims of the Study

To characterize a candidate working standard for the bioassay of human TNF-α and assign a unitage for activity, the study sought

1. To assess the suitability of an ampouled preparation of human TNF-α, coded 17/232 to serve as a candidate working standard for the bioassay of human TNF-α by assaying its biological activity in a range of bioassays.

2. To assess the activity of the ampouled preparation, coded 17/232 in different TNF-α bioassays in current use and to calibrate the candidate working standard relative to the 3rd International standard (IS) for TNF-alpha (NIBSC code 12/154).

Materials and Methods

A preparation of recombinant human sequence TNF-α expressed in E coli was kindly donated for development of the candidate working standard; the same lot of the rDNA derived human TNF-α was previously used for the 3rd WHO IS for TNF-α (coded 12/154).

The preparation, coded 17/232 was lyophilized at NIBSC using the same formulation and freeze drying cycle as the 3rd WHO IS for TNF-α according to the procedures used for International Biological Standards (ECBS guidelines - WHO Technical Report Series 932, 2006). For this, buffers, final compositions as shown in Table 1, were prepared using nonpyrogenic water and depyrogenated glassware and solutions filtered using sterile nonpyrogenic filters (0.22μm Stericup filter system, Millipore, USA) where appropriate.

For formulation of the TNF-α preparation, the appropriate volume of TNF-α was added to the buffer to provide a solution of TNF-α at a concentration which, when distributed in 1.0ml aliquots, gives the theoretical protein content per ampoule as 1μg/ml as shown in Table 1. The mass content of the preparation was determined by the manufacturer. As the protein content of the ampoules cannot be verified by direct measurement of absolute mass, the content is assumed to be the theoretical mass, calculated from the dilution of the bulk material of known protein mass content, and the volume of formulated solution delivered to the ampoule. This mass value is given as "predicted μg".

Table 1: Materials used in the study

Ampoule Code	Fill Date	Study Code	No in Stock	TNF-α (predicted Mass-μg)	Type and expression system	Excipients
12/154	14/6/12	Current IS	4600	1.0	157 amino acids, full length; E.Coli expressed	6 salt PBS, 0.6% HSA, 0.1% Trehalose
17/232	1/3/18	A, B	3998	1.0	Same as above	Same as above

For the fill, a percentage of ampoules were weighed. The mean fill weights are shown in Table 2. The filled ampoules were lyophilized and the ampoules sealed under dry nitrogen by heat fusion of the glass and stored at –20°C in the dark. Residual moisture of the preparation, measured by the Karl-Fischer method (Mitsubishi CA100) and headspace oxygen content determined by frequency modulated spectroscopy using the Lighthouse FMS-760 Instrument (Lighthouse Instruments, LLC) are shown in Table 2. Testing for microbial contamination using total viable count method did not show any evidence of microbial contamination.

Following lyophilization, the candidate preparation was compared to the unformulated bulk drug substance and the 3rd WHO IS for TNF-α in a cytotoxicity bioassay (Figure 1) which demonstrated the material had lyophilized successfully with no observable loss in biological activity

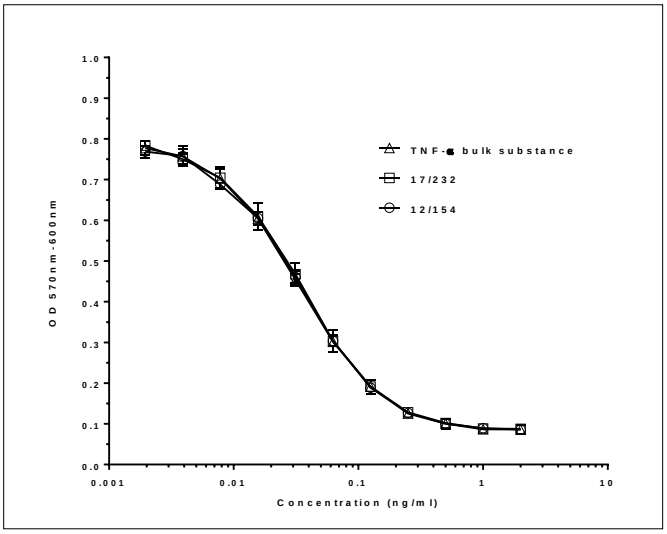

Figure 1. Dose response curves for the TNF-α working standard and the bulk drug substance relative to the 3rd WHO IS for TNF-α in the L929 cytotoxicity assay

Table 2: Mean fill weights and residual moisture content of candidate preparations

Ampoule Code	Study Code	Mean Fill weight (g)	CV Fill weight %	Mean Residual Moisture %	CV Residual Moisture %	Mean Headspace Oxygen %	CV Headspace Oxygen %
12/154	Current IS	1.006 (264)	0.100	1.294 (12)	29.912	0.430 (11)	32.32
17/232	A, B	1.008 (164)	0.194	0.248 (12)	17.02	0.22 (12)	52.21

The numbers in parentheses indicate the number of determinations.

Participants

Samples were coded as shown in Table 1 and despatched in March 2018 to 7 laboratories in 7 countries. The participants comprised 3 control laboratories and 4 manufacturers' laboratories. All participants submitted data and are listed in Table 3.

Assay Methods and Study Design

A summary of the bioassays used in the study is given in Table 4. Cytotoxicity assays using the murine fibroblast cell-line, L929 or the murine fibrosarcoma, WEHI-164 were predominantly used. These assays employed different readouts for assessing the cytotoxic effect of TNF-α. In one laboratory, a reporter gene assay using the HEK-Blue™ CD40L cells, generated by stable transfection of HEK293 cells with the human CD40 gene and an NF-κB inducible secreted embryonic alkaline phosphatase (SEAP) construct (Invivogen, Toulouse, France, https://www.invivogen.com) was used.

Participants were asked to assay the samples including the current IS (12/154) concurrently on a minimum of three separate occasions using their own routine bioassay methods within a specified layout which allocated the samples across 3 plates and allowed testing of replicates as per the study protocol (Appendix). It was requested that participants perform at least eight dilutions of each preparation using freshly reconstituted ampoules for each assay.

Participating laboratories were sent five sets of three study samples coded A and B along with the current IS (12/154) as detailed in Table 1. Samples A and B were coded duplicate samples of the same material (candidate working standard 17/132). Participants were requested to return their raw assay data, using spreadsheet templates provided.

All laboratories are referred to by a code number, allocated at random and not representing the order of listing in Table 3, to retain confidentiality in the report. Where a laboratory returned data from more than one method, the different assay methods were analysed and reported separately and coded, for example, laboratory 1a and 1b. Overall, study data included results from eight different assays as shown in Table 4.

Table 3: Study Participants

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Table 4: Assays contributed to the study

Laboratory Code	Bioassay Cell Line	Assay Type	Assay Incubation with TNF (hrs)	Assay Readout
1a	**HEK-Blue™ CD40L**	**Reporter-gene**	**20-24**	**Quanti-Blue**
1b	**L929**	**Cytotoxicity**	**20-24**	**Alamar Blue**
2	**WEHI-164**	**Cytotoxicity**	**21**	**(Cell Titer 96 AQ_{ueous} One, MTS)**
3	**L929**	**Cytotoxicity**	**14-16**	**CCK-8**
4	**WEHI-164**	**Cytotoxicity**	**20-24**	**MTS**
5	**WEHI-164**	**Cytotoxicity**	**20-24**	**MTS**
6	**WEHI-164**	**Cytotoxicity**	**20-24**	**MTS**
7	**L929**	**Cytotoxicity**	**72**	**Crystal Violet**

Statistical Analysis

Analysis of dose-response curve data was performed using a four-parameter logistic model

$$y = \alpha - \frac{\delta}{1 + 10^{\beta(\log_{10}x - \log_{10}\gamma)}}$$

where y denotes the assay response, x is the concentration, α is the upper asymptote, δ is the difference between upper and lower asymptotes, β is the slope factor and γ is the EC_{50} (50% effective concentration). Models were fitted using the R package 'drc' [1,2].

Parallelism for a pair of dose-response curves was concluded by demonstrating equivalence of the parameters α, β and δ. For the α and δ parameters, the differences in estimates obtained for the two samples under consideration were used as measures of curve similarity. For the β parameter, the ratio of estimates for the two samples under consideration was used. Methods used for determining equivalence ranges and the values obtained are discussed in the Results section of this report. Where satisfactory parallelism was concluded for two samples, the model was fitted to both samples with common values of α, β and δ in order to directly estimate their relative potency.

The final relative potency estimate for each plate was taken as the geometric mean of the valid estimates obtained for samples A and B on the plate. If the relative potency of A to B was not in the range [0.80,1.25] the plate was considered invalid and excluded from further calculations. All relative potency estimates were combined to generate unweighted geometric mean (GM) potencies for each laboratory and these laboratory means were used to calculate overall unweighted geometric mean potencies. Individual assay estimates of relative potency were log transformed and a mixed effects model used to determine intra-laboratory and inter-laboratory variance components [1,3] which were expressed as geometric coefficients of variation (GCV = $\{10^{s}-1\}\times100\%$ where s is the standard deviation of the $\log_{10}$ transformed potencies).

Results and Discussion

Parallelism of dose-response curves

As the model parameters are expected to be equivalent when testing the same sample against itself, equivalence ranges for concluding parallelism were based on the results obtained for coded duplicate samples A and B. Values for each curve similarity measure ($|\alpha_A - \alpha_B|$, $|\delta_A - \delta_B|$, $\max[\beta_A/\beta_B, \beta_B/\beta_A]$) were calculated for each plate (n=81) and non-parametric upper tolerance bounds (90% coverage, 90% confidence) were calculated using these values. These were used to define equivalence ranges for parallelism, as shown in Table 5, which were then used to assess the parallelism of samples A and B with IS 12/154. It should be noted that these ranges were intended for use in the analysis of data from this study only, so that consistent criteria were applied to all laboratories. They should not be interpreted as suitable values for routine use in the assessment of assay validity within the collaborating laboratories and may in some cases be overly stringent or inappropriate. The resulting numbers of cases concluded as non-parallel using these criteria are summarised in Table 6.

Table 5. Equivalence ranges used to conclude parallelism of dose response curves

Similarity Measure	Equivalence Range
Difference in α parameters	-0.063 to 0.063
Difference in δ parameters	-0.067 to 0.067
Ratio of β parameters	0.883 to 1.132

Table 6. Summary of parallelism conclusions (samples A and B, both coded 17/232 relative to IS 12/154) by laboratory

Lab	Assay	Plate	Sample	Similarity measure not in range
1a	1	1	B	α
1a	1	3	B	α and δ
1a	3	1	B	α and δ
1a	4	2	A	α and δ
1a	4	3	A	δ
1b	4	3	A	β
1b	4	3	B	β
3	1	2	A	β
3	2	1	A	β
3	2	3	A	β
3	2	3	B	α and δ
6	1	1	A	α and δ
6	1	1	B	α and δ
7	1	1	B	β
7	1	3	B	β
7	2	3	A	β and δ
7	2	3	B	β
7	3	1	A	β
7	3	2	A	α and δ
7	3	2	B	α and δ
7	3	3	A	β

Relative potency estimates

Potency estimates for the candidate standard relative to IS 12/154, calculated using the geometric mean potency of A and B on each plate, are summarised in Table 7 and Figure 2. All individual plate estimates are shown in Table 9. Only one plate (laboratory 1b, assay 5, plate 3) was excluded due to the relative potency of A to B being outside the range [0.80,1.25].

Table 8 shows the overall estimate for the candidate standard relative to the current IS 12/154. Multiplying by the assigned value for 12/154 (43,000 IU/ampoule) gives a final estimate of 42,607 IU/ampoule (expanded uncertainty 40,720 – 44,582 calculated on a log scale with coverage factor k=2.36 corresponding to a 95% level of confidence).

Table 7. Summary of laboratory mean potency estimates for candidate standard relative to IS 12/154

Lab	GM	%GCV			N
		Intra-assay	Inter-assay	Total	
1a	0.986	2.55	4.93	5.58	12
1b	1.037	5.15	3.19	6.10	13
2	0.943	5.16	4.48	6.90	9
3	0.933	9.74	3.51	10.42	8
4	1.093	12.28	7.01	14.36	9
5	1.008	8.29	0.00	8.29	9
6	0.941	9.98	0.00	9.98	8
7	0.989	2.18	3.01	3.73	7

GM: Geometric Mean; GCV: Geometric Coefficient of Variation (%); N: Number of plates with valid estimate

Table 8. Overall study potency estimates for candidate standard relative to IS 12/154

GM	%GCV			LCL	UCL	N
	Intra-lab	Inter-lab	Total			
0.991	8.27	4.85	9.69	0.947	1.037	8

GM: Geometric Mean; GCV: Geometric Coefficient of Variation (%); LCL: 95% Lower Confidence Limit; UCL: 95% Upper Confidence Limit; N: Number of labs

Stability Studies

Given the short time since lyophilization (7 months), no stability studies have been undertaken to date. However, considering the characteristics of this working standard in terms of the low moisture content, low headspace oxygen in the ampoule (Table 2) and that the protein in the working standard is of the same lot as the IS, there is no reason to believe that the stability of this standard will be significantly different from that of the current IS which has a predicted loss of potency of < 0.001% per year when stored at -20°C. Nevertheless, ampoules have been stored at different temperatures for a stability study to be undertaken at a later time-point.

Conclusions

There was good agreement between laboratories with geometric mean potency estimates for 17/232, ranging from 0.93 to 1.09, relative to 12/154 and intra-lab and inter-lab variability of 8.27% and 4.85% respectively. Based on these estimates, the overall potency estimate of 17/132 was calculated to be 0.991 of the assigned value of 12/154 (43, 000 IU/ampoule) which is 42, 607 IU/ampoule (expanded uncertainty 40,720 – 44,582 calculated on a log scale with coverage factor k=2.36 corresponding to a 95% level of confidence).

Study data shows that the candidate standard 17/232 is suitable to serve as the working standard for TNF-α. Since material for this standard is sourced from the same lot as the current TNF-α IS (also serves as a critical reagent in bioassays for anti-TNF-α products), it is expected to behave in a comparable manner to the current IS and is therefore suitable for use in assays which utilise the current TNF-α IS.

It is proposed that the standard 17/232 be established as the NIBSC working standard for TNF-α with an assigned value of 42,607 IU/ampoule (expanded uncertainty 40,720 – 44,582 calculated on a log scale with coverage factor k=2.36 corresponding to a 95% level of confidence).

Figure 2. Potency estimates for candidate standard relative to IS 12/154

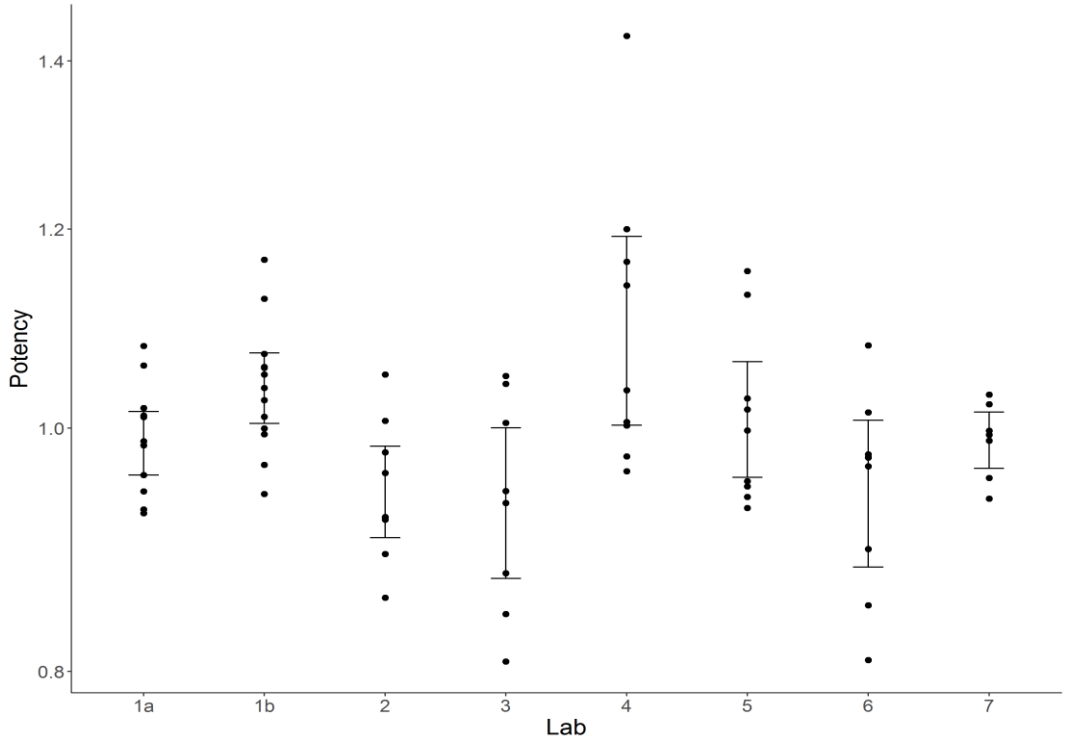

Table 9. Potency estimates relative to IS 12/154 from all individual plates

Lab	Assay	Plate	Sample A	Sample B	GM (A,B)	A:B
1a	1	1	0.928	NP	0.928	n/a
1a	1	2	0.992	0.925	0.958	1.07
1a	1	3	0.925	NP	0.925	n/a
1a	2	1	0.952	0.935	0.943	1.02
1a	2	2	0.974	0.915	0.944	1.06
1a	2	3	1.011	0.966	0.988	1.05
1a	3	1	1.078	NP	1.078	n/a
1a	3	2	1.088	1.030	1.059	1.06
1a	3	3	1.013	1.007	1.010	1.01
1a	4	1	0.968	1.072	1.019	0.90
1a	4	2	NP	1.012	1.012	n/a
1a	4	3	NP	0.984	0.984	n/a
1b	1	1	1.063	1.050	1.057	1.01
1b	1	2	0.862	1.084	0.967	0.80
1b	1	3	1.165	0.960	1.058	1.21
1b	2	1	1.001	0.988	0.994	1.01
1b	2	2	0.866	1.023	0.941	0.85
1b	2	3	1.022	0.978	1.000	1.04
1b	3	1	1.224	1.112	1.167	1.10
1b	3	2	0.941	1.085	1.010	0.87
1b	3	3	1.159	0.989	1.070	1.17
1b	4	1	0.928	1.134	1.026	0.82
1b	4	2	0.970	1.110	1.037	0.87
1b	4	3	NP	NP	n/a	n/a
1b	5	1	1.000	1.103	1.050	0.91
1b	5	2	1.167	1.086	1.126	1.07
1b	5	3	1.261	0.950	Excluded	1.33
2	1	1	0.928	0.913	0.921	1.02
2	1	2	1.018	1.084	1.050	0.94
2	1	3	0.876	0.969	0.922	0.90
2	2	1	0.982	1.032	1.006	0.95
2	2	2	0.957	0.962	0.959	0.99
2	2	3	0.948	1.009	0.978	0.94
2	3	1	0.771	0.950	0.856	0.81
2	3	2	0.841	0.944	0.891	0.89
2	3	3	0.858	0.985	0.919	0.87
3	1	1	0.955	0.932	0.944	1.02
3	1	2	NP	1.041	1.041	n/a
3	1	3	1.022	0.988	1.005	1.04
3	2	1	NP	0.807	0.807	n/a
3	2	2	0.897	0.971	0.933	0.92
3	2	3	NP	NP	n/a	n/a
3	3	1	1.145	0.961	1.049	1.19

3	3	2	0.852	0.900	0.875	0.95
3	3	3	0.916	0.776	0.843	1.18
4	1	1	1.319	1.556	1.433	0.85
4	1	2	0.941	1.139	1.035	0.83
4	1	3	0.951	1.056	1.002	0.90
4	2	1	1.129	1.201	1.165	0.94
4	2	2	1.119	1.161	1.140	0.96
4	2	3	1.194	1.206	1.200	0.99
4	3	1	0.988	1.023	1.006	0.97
4	3	2	0.941	0.981	0.961	0.96
4	3	3	0.955	0.994	0.974	0.96
5	1	1	1.076	0.961	1.017	1.12
5	1	2	0.892	0.968	0.929	0.92
5	1	3	1.007	1.049	1.027	0.96
5	2	1	0.972	0.933	0.952	1.04
5	2	2	0.998	0.883	0.939	1.13
5	2	3	1.161	1.148	1.155	1.01
5	3	1	1.177	1.085	1.130	1.08
5	3	2	0.928	0.968	0.948	0.96
5	3	3	0.998	0.997	0.998	1.00
6	1	1	NP	NP	n/a	n/a
6	1	2	0.831	0.870	0.850	0.96
6	1	3	0.989	0.942	0.965	1.05
6	2	1	1.120	1.039	1.079	1.08
6	2	2	0.955	0.992	0.973	0.96
6	2	3	0.901	0.889	0.895	1.01
6	3	1	1.031	0.998	1.015	1.03
6	3	2	0.793	0.825	0.808	0.96
6	3	3	0.873	1.091	0.976	0.80
7	1	1	0.937	NP	0.937	n/a
7	1	2	0.963	0.947	0.955	1.02
7	1	3	0.994	NP	0.994	n/a
7	2	1	1.022	NL	1.022	n/a
7	2	2	1.003	1.060	1.031	0.95
7	2	3	NP	NP	n/a	n/a
7	3	1	NP	0.997	0.997	n/a
7	3	2	NP	NP	n/a	n/a
7	3	3	NP	0.988	0.988	n/a

GM: Geometric Mean; NP: Non-parallel to IS 12/154 (see Table 2); NL: Non-linear

Acknowledgements

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Appendix

Collaborative study for an NIBSC TNF-alpha working standard

Study Protocol

1. AIMS OF THE STUDY

1. To assess the suitability of an ampouled preparation of human TNF-α to serve as a working standard for the bioassay of human TNF-α by assaying its biological activity in a range of bioassays.

2. To assess the activity of the ampouled preparation in different TNF-α bioassays in current use and to calibrate the candidate working standard relative to the 3rd International standard (IS) for TNF-alpha (NIBSC code 12/154).

2. MATERIALS INCLUDED IN THE STUDY

- A set of samples coded by letter **A & B** (5 ampoules for each preparation) for testing in TNF-α bioassays. Each sample contains approximately 1 μg of TNF-α.
- 5 ampoules of the current IS for TNF-alpha (12/154). The current IS contains 43,000IU (approximately 1 μg) of TNF-α.

3. RECONSTITUTION AND STORAGE OF PREPARATIONS

Prior to initiating the study, please read the Instructions for Use provided with the samples. Please note the statements regarding safety and that these preparations are not for human use.

Lyophilized preparations provided should be stored at -20°C or below until used.

- Samples A and B should be reconstituted with 1ml of sterile distilled water. Mix GENTLY and ensure contents are fully dissolved prior to use. Use carrier protein where extensive dilution is required.
- The IS coded 12/154 should also be reconstituted with 1ml of sterile distilled water. Mix GENTLY and ensure contents are fully dissolved prior to use. This

  solution will contain TNF-α at a concentration of 43,000 International Units/ml. Use carrier protein where extensive dilution is required.

4. ASSAY STRUCTURE

PLEASE NOTE: All concentrations are reported as the final dilution in the assay after all components and cells have been added. We ask all participants to report concentrations and doses in this format.

1. Please include all samples A, B and the current IS (12/154) in each TNF-α assay.

2. Please use a freshly prepared ampoule of each preparation, A, B and the current IS (12/154) in each of the assays. An assay is considered independent if the assay is carried out on different days/occasions. Each assay will consist of 3 plates, see step 5 below.

3. For each assay method used, participants are asked to perform an assay initially (a pilot assay) to ensure that all preparations (A, B, 12/154) are diluted such that the concentration range falls within the working range of the assay. Please include dilution series for all preparations (A, B, 12/154) in the assay.

4. Following the pilot assay (as in step 3 above), perform at least 3 independent assays for the preparations (A, B, 12/154) using the most appropriate dilutions, derived from the pilot assay, for the different preparations in order to obtain suitable dose response curves.

5. **For each independent assay each plate should include at least 1 independent dilution series for each preparation (A, B and 12/154) in duplicate.** This should be repeated 3 times across 3 different plates for each independent assay as illustrated in the example assay plate layouts at the end of the protocol. This will provide a total of 9 independent estimates from 3 assays in total. Please try to vary the positions of the samples on different plates to ensure that assays are not susceptible to edge or positional effects.

Please follow one of the suggested bioassay layouts provided if possible. Include blank control wells (cells with culture medium but no TNF-α).
The layouts can be amended to suit in house methods if preferred, however, it is important to ensure that each plate includes at least 1 dilution series for each of the samples (A, B and 12/154).

5. INFORMATION TO BE SUPPLIED AND PRESENTATION OF RESULTS

1. We have included an assay method sheet and example microtiter plate layouts for

   illustrating the layout and dilutions for the samples in the plates. Excel templates (separate excel file) are included for returning the data obtained from 3 bioassays for the samples tested.

2. Please let us know, as clearly as possible, how the assay was carried out, especially how the stock solutions were diluted and what the final dilutions were in the assay plates after all the components have been added. We have provided **example microtiter plate format data sheets at the end of this protocol** for diagrammatically illustrating the assay layout, dilutions and results.

PLEASE NOTE: report all dilutions (candidate samples A, B and TNF- α IS 12/154) as the final dilution in the assay after all components and cells have been added.

3. Please PROVIDE ALL RAW DATA (microtiter plate readouts e.g. OD, Luminescence/Fluorescence Units etc) as direct analysis of the raw data provided from the assays permits data from all participants to be handled consistently as far as possible.

4. We request participants to follow one of the examples provided and enter data as indicated in the Excel template (that has been provided separately). Please return all data relating to the 3 assays electronically in the same format as on the Excel template.

5. Please provide information regarding your assay methodology on the sheet provided.

6. Although NIBSC will calculate relative potencies from the raw data provided by the participants, participants are requested (if possible) to calculate the contents of samples A and B relative to the IS (12/154) using their own in-house methods. Please provide information of all methods used for calculating results**.**

Please provide all information requested to chris.bird@nibsc.org

COLLABORATIVE STUDY FOR HUMAN TNF-ALPHA WORKING STANDARD

Laboratory identification

Assay methodology information

Briefly outline the assay methods used (provide full protocol on separate sheets if available):

Please record all information on concentrations and dilutions of samples relating to each assay on the results spreadsheets provided as separate Excel templates.

a) Please report details of the cell line, source, seeding density and passage number of cells used in each assay, if applicable:

b) Please report details of any pre-treatment of the cells before the assay (e.g. removal of growth factors):

c) Details of the ampoule reconstitution, dilution steps and dilution buffer used:

TNF-α IS (12/154)

Sample A

Sample B

d) Dose range and dilutions for samples A, B and TNF-α IS used in the assay, and the plate layout for the samples (please also provide separately in the Excel spreadsheet).

e) Length of incubation of the cells with TNF-α:

f) Length of incubation of the cells with readout reagent:

g) Report details of the readout of the assay (e.g. Absorbance/ Luminescence) and the equipment used to obtain this readout:

h) Additional comments

COLLABORATIVE STUDY FOR HUMAN TNF-ALPHA WORKING STANDARD

Example Layout 1. With dilution series in a horizontal orientation.

Plate 1. Sample Layout:

	1	2	3	4	5	6	7	8	9	10	11	12
A	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank
B	IS	IS	IS	IS	IS	IS	IS	IS	IS	IS	IS	IS
C	IS	IS	IS	IS	IS	IS	IS	IS	IS	IS	IS	IS
D	A	A	A	A	A	A	A	A	A	A	A	A
E	A	A	A	A	A	A	A	A	A	A	A	A
F	B	B	B	B	B	B	B	B	B	B	B	B
G	B	B	B	B	B	B	B	B	B	B	B	B
H	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank

Optional Sample Pre-dilution: reciprocal e.g. 10 for 1/10 or 100 for 1/100.

IS		A		B					

Final sample dilution/concentration after cells added (reciprocal e.g. 2 for 1 /2,10 for 1/10 etc).

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C												
D												
E												
F												
G												
H												

Response e.g. OD (with duplicates listed horizontally)

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C												
D												
E												
F												
G												
H												

IS = International Standard Blank=Blank Control Wells

Plate 2. Sample Layout:

	1	2	3	4	5	6	7	8	9	10	11	12
A	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank
B	A	A	A	A	A	A	A	A	A	A	A	A
C	A	A	A	A	A	A	A	A	A	A	A	A
D	B	B	B	B	B	B	B	B	B	B	B	B
E	B	B	B	B	B	B	B	B	B	B	B	B
F	IS	IS	IS	IS	IS	IS	IS	IS	IS	IS	IS	IS
G	IS	IS	IS	IS	IS	IS	IS	IS	IS	IS	IS	IS
H	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank

Sample Pre-dilution: reciprocal e.g. 10 for 1/10, 100 for 1/100 etc.

IS		A		B					

Final sample dilution/concentration after cells added (reciprocal e.g. 2 for 1 /2,10 for 1/10 etc).

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C												
D												
E												
F												
G												
H												

Response e.g. OD (with duplicates listed horizontally)

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C												
D												
E												
F												
G												
H												

IS = International Standard Blank=cells only control wells

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Plate 3. Sample Layout:

	1	2	3	4	5	6	7	8	9	10	11	12
A	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank
B	B	B	B	B	B	B	B	B	B	B	B	B
C	B	B	B	B	B	B	B	B	B	B	B	B
D	IS	IS	IS	IS	IS	IS	IS	IS	IS	IS	IS	IS
E	IS	IS	IS	IS	IS	IS	IS	IS	IS	IS	IS	IS
F	A	A	A	A	A	A	A	A	A	A	A	A
G	A	A	A	A	A	A	A	A	A	A	A	A
H	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank

Sample Pre-dilution: reciprocal e.g. 10 for 1/10, 100 for 1/100 etc.

IS		A		B					

Final sample dilution/concentration after cells added (reciprocal e.g. 2 for 1 /2,10 for 1/10 etc).

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C												
D												
E												
F												
G												
H												

Response e.g. OD (with duplicates listed horizontally)

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C												
D												
E												
F												
G												
H												

IS = International Standard Blank=Blank Control Wells

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COLLABORATIVE STUDY FOR HUMAN TNF-ALPHA WORKING STANDARD

Example Layout 2. With dilution series in a vertical orientation.

Plate 1. Sample Layout:

	1	2	3	4	5	6	7	8	9	10	11	12
A	Blank	IS	IS	A	A	B	B	IS	IS	A	A	Blank
B	Blank	IS	IS	A	A	B	B	IS	IS	A	A	Blank
C	Blank	IS	IS	A	A	B	B	IS	IS	A	A	Blank
D	Blank	IS	IS	A	A	B	B	IS	IS	A	A	Blank
E	Blank	IS	IS	A	A	B	B	IS	IS	A	A	Blank
F	Blank	IS	IS	A	A	B	B	IS	IS	A	A	Blank
G	Blank	IS	IS	A	A	B	B	IS	IS	A	A	Blank
H	Blank	IS	IS	A	A	B	B	IS	IS	A	A	Blank

Optional Sample Pre-dilution: reciprocal e.g. 10 for 1/10 or 100 for 1/100.

IS		A		B					

Final sample dilution/concentration after cells added (reciprocal e.g. 2 for 1 /2,10 for 1/10 etc).

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C												
D												
E												
F												
G												
H												

Response e.g. OD (with duplicates listed vertically)

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C												
D												
E												
F												
G												
H												

IS = International Standard Blank=Blank Control Wells

Plate 2. Sample Layout:

	1	2	3	4	5	6	7	8	9	10	11	12
A	Blank	B	B	IS	IS	A	A	B	B	IS	IS	Blank
B	Blank	B	B	IS	IS	A	A	B	B	IS	IS	Blank
C	Blank	B	B	IS	IS	A	A	B	B	IS	IS	Blank
D	Blank	B	B	IS	IS	A	A	B	B	IS	IS	Blank
E	Blank	B	B	IS	IS	A	A	B	B	IS	IS	Blank
F	Blank	B	B	IS	IS	A	A	B	B	IS	IS	Blank
G	Blank	B	B	IS	IS	A	A	B	B	IS	IS	Blank
H	Blank	B	B	IS	IS	A	A	B	B	IS	IS	Blank

Sample Pre-dilution: reciprocal e.g. 10 for 1/10, 100 for 1/100 etc.

IS		A		B					

Final sample dilution/concentration after cells added (reciprocal e.g. 2 for 1 /2,10 for 1/10 etc).

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C												
D												
E												
F												
G												
H												

Response e.g. OD (with duplicates listed vertically)

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C												
D												
E												
F												
G												
H												

IS = International Standard Blank=cells only control wells

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