



WHO/BS/2011.2163
ENGLISH ONLY

EXPERT COMMITTEE ON BIOLOGICAL STANDARDIZATION
Geneva, 17 to 21 October 2011

**Report of a Collaborative study for Proposed 1st International
standard for Transforming Growth Factor- β 3 (TGF- β 3)**

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Summary

One candidate preparation of human sequence recombinant Transforming growth factor- β 3 (TGF- β 3) was formulated and lyophilized at NIBSC prior to evaluation in a collaborative study for its suitability to serve as an international standard. The preparation was tested by 8 laboratories using in vitro bioassays and immunoassays. The candidate preparation 09/234 was judged suitable to serve as an international standard based on the data obtained for biological activity and stability (predicted % loss per year of less than 0.01% at the recommended storage temperature of -20°C). On the basis of the results reported here, it is proposed that the preparation coded 09/234 be accepted as the WHO 1st IS for human TGF- β 3 with an assigned value for TGF- β 3 activity of 19, 000 IU/ampoule.

Responses from study participants

Responses have been obtained from six of the eight study participants. All were in agreement with the proposal that the preparation coded 09/234 is suitable as the WHO 1st IS for human TGF- β 3 with an assigned value for TGF- β 3 activity of 19, 000 IU/ampoule

Introduction

Transforming growth factor beta-3 (TGF- β 3) is a member of the TGF- β superfamily (30 proteins) which includes activins, bone morphogenetic proteins and growth and differentiation factors. Three closely related isoforms exist in mammals - TGF- β 1, - β 2 and - β 3, each derived from a distinct gene but exhibit greater than 70% sequence homology and are similar in functional responses in vitro (1,2). They are produced as precursor proteins composed of a signal peptide, a 75kd dimeric latency associated glycoprotein and a 112 amino acid mature TGF- β and undergo proteolytic activation to form the active 25 kd homodimer via a disulfide-rich core consisting of a characteristic 'cysteine knot' (3,4).

Produced by several immune and non-immune cell types such as fibroblasts, endothelium, smooth muscle cells, TGF- β acts in an autocrine and paracrine manner to regulate multiple cellular processes, such as immune function, proliferation, differentiation, extracellular matrix production, migration and survival. These responses, however, are highly dependent on the target cell/site, the concentration of TGF- β and the presence or absence of other growth factors in the local environment (2,5).

The biological functions are mediated by binding of TGF- β to a complex of the accessory receptor betaglycan (or TGF- β RIII) and a type II serine/threonine kinase receptor, TGF- β RII which then phosphorylates and activates a type I serine/threonine kinase receptor, TGF- β RI (also called ALK-5). The activated type I receptor phosphorylates and, in turn activate Smads (which regulate transcription) and other intercellular proteins leading to altered expression of target genes (5).

Despite their highly pleiotropic nature, the TGF- β isoforms elicit distinct non-redundant functions *in vivo*. For example, studies in animal models and human patients have shown that TGF- β 1 has a critical function in regulating leukocyte functions in autoimmune diseases. A disruption of TGF- β signalling or its production (due to loss of T regulatory cells which are the primary source) in mouse models causes autoimmune disease (6) whereas overexpression protects from autoimmune diseases. Targeted deletions of the genes for the TGF- β 2 and - β 3 isoforms in mice have shown the importance of TGF- β 2 in the development of cardiac, lung, craniofacial, limb, eye, ear, and urogenital systems while TGF- β 3 has a significant influence on cellular adhesion and extracellular matrix formation during palatogenesis and pulmonary development (7). Without TGF- β 3, the deformity known as a 'cleft palate' is caused due to failure of epithelial cells in both sides of the developing palate to fuse (7,8). TGF- β 3 also controls wound healing by regulating the movements of epidermal and dermal cells in injured skin.

The therapeutic potential of TGF- β 3 in the treatment of oral mucositis and in the prevention and reduction of scarring in skin following surgical procedures and wound repair of wounds has been shown in various phase I/II clinical trials (9). The opportunity to extend this to more clinically significant human scar models e.g., keloids and hypertrophic scars and to develop new methods of delivery of the TGF- β 3 protein or its gene for treatment of fibrotic or other disorders is also very promising (10). Additionally, TGF- β 3 appears to be a potentially useful growth factor in engineered organogenesis and in the context of regenerative medicine (11). Therefore, various therapeutic interventions are currently being evaluated such as cartilage development from mesenchymal stem cell differentiated chondrocytes, regeneration of periodontal tissue, palate development and neurogenesis (11).

Based on its activities (pro-inflammatory, immunosuppressive), TGF- β has been implicated in many human diseases, including certain cancers, onset of several autoimmune diseases including joint destruction in arthritis and in pathogenesis of fibrotic diseases associated with skin, lung, liver and eye (cornea) (2,12). Therefore, inhibitors of TGF- β (some of these appear to target all 3 isoforms) such as ligand traps, other antagonists - antibodies, antisense technologies or small molecules (to inhibit the receptor or signalling pathway) are currently being exploited in certain cancers (13).

A reference standard for TGF- β 3 could facilitate measurement of the potency and stability of therapeutic preparations or for preparations in use in regenerative medicine, its antagonists and in addition, measurement of TGF- β 3 levels for research purposes.

Currently, a reference reagent for TGF- β 3 (98/608) is available from NIBSC; this has an arbitrary unitage of 10,000 U/ampoule. The objective of the current exercise, therefore, is the potency evaluation of candidate preparations relative to the current reference reagent for TGF- β 3 (98/608) in an international collaborative study. Calibration of the proposed WHO 1st IS is primarily based on the assays in use in various laboratories and relies entirely on the estimates calculated relative to the reference reagent for TGF- β 3 (98/608) to ensure continuity. Since TGF- β 3 is only intended for use therapeutically or in the context of regenerative medicine (and not diagnostically), the commutability issue has not been addressed in this study.

This project was endorsed by the WHO Expert Committee on Biological Standardisation at its meeting in October 2009.

Aims of the Study

The purpose of the study was to characterize a candidate WHO 1st IS for the bioassay of human TGF- β 3 and assign a unitage for TGF- β 3 activity. To achieve this, the study sought

1. To assess the suitability of ampouled preparations of human TGF- β 3 to serve as the 1st WHO IS for the bioassay of human sequence TGF- β 3 by assaying their biological activity in a range of routine, 'in-house' bioassays.
2. To assess the relative activity of the ampouled preparations in different bioassays in current use for these materials and to calibrate the candidate IS against the NIBSC reference reagent (98/608).

Materials and Methods

Two preparations of recombinant human sequence TGF- β 3 were kindly donated to WHO (see Acknowledgement). One preparation was expressed in E coli while the other preparation was sf-21 insect cell expressed (available currently as NIBSC reference reagent, code 98/608). Trial fills were conducted and the biological activity of the lyophilized preparations compared with the bulk material in a bioassay based on the inhibitory effect of TGF- β on IL-5 induced proliferation of a human erythroleukaemic cell-line, TF-1 (14). This bioassay, developed at NIBSC is also used in other laboratories for evaluation of the biological activity of TGF- β . As the trial lyophilizations performed appropriately in the bioassay, the preparations were filled into ampoules and final lyophilisation was carried out at NIBSC as per the procedures used for International Biological Standards (ECBS guidelines - WHO Technical Report Series 932, 2006).

Buffers, final compositions as shown in Table 1, were prepared using nonpyrogenic water and depyrogenated glassware. Buffer solutions were filtered using sterile nonpyrogenic filters (0.22 μ M Stericup filter system, Millipore, USA) where appropriate.

For the study, the two preparations were coded as described in Table 1. The mass content of the preparations was determined by the manufacturers. 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 μ g".

For the two preparations, the appropriate volume was added to the buffer to provide a solution of concentration predicted as 1 μ g/ml TGF- β 3 for 98/608 and 09/234. For these coded preparations, the TGF- β 3 solution was distributed in 1.0ml aliquots, giving the theoretical protein content per ampoule shown in Table 1.

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

Participants

Samples were despatched in October 2010 to eight laboratories in three countries. The participants comprised 2 control laboratories, 3 manufacturers' laboratories, 1 contract research laboratory and 2 academic scientists. All 8 participants submitted data and are listed in Appendix 1. Participants are referred to by a code number allocated at random and not representing the order of listing in Appendix 1.

Assay Methods and Study Design

A summary of the bioassay methods used by the individual laboratories in the study is given in Table 3. Proliferation assays which were either based on the stimulatory or inhibitory effect of TGF- β on the target cell/cell-line and employed different readouts for measuring cellular proliferation were mainly used by participants. For example, laboratory 1 measured stimulation of proliferation of dermal fibroblasts while laboratory 5 measured the inhibitory effect on proliferation of the TF-1 cell-line. Both laboratories used a colorimetric formazan based dye for assay readout but laboratory 1 used the soluble form, MTS as opposed to the insoluble form, MTT favoured by laboratory 5 (Table 3). Three participants used the TF-1 assay but employed different readouts for assessing the proliferation while in two laboratories, reporter gene assays were used (16,17). ELISAs, using commercially available reagents/kits were performed in addition to cell-based assays in two laboratories (Table 3).

Participants were asked to assay all samples, which includes the current NIBSC reference reagent (98/608) concurrently on a minimum of three separate occasions using their own routine bioassay methods within a specified layout which allocated the samples across 4 plates and allowed testing of replicates as per the study protocol (Appendix 2). It was requested that participants perform eight dilutions of each preparation using freshly reconstituted ampoules for each assay. Where available they were asked to include their own in-house reference material in their assay.

Participating laboratories were sent 6 ampoules each of study samples coded A-C and 3 ampoules of an excipient, coded D as detailed in Table 1. Samples A and C were coded duplicate samples of the same material (candidate standard 09/234). Sample B was the NIBSC reference reagent (coded 98/608).

Participants were requested to return their raw assay data, using spreadsheet templates provided, and also their own calculations of potency of the study samples relative to the current IS.

Statistical Analysis

The potencies of the study samples were calculated relative to NIBSC Reference Reagent (98/608) by analysis of the raw assay data at NIBSC. The assays were analysed using a weighted logistic parallel line model, using the full dose-response curve, using the European Directorate for Quality of Medicines and Healthcare (EDQM) assay analysis software, Combistats (18). In some instances, the assays were analysed using a simple parallel-line model based on a linear portion of the dose response curve (19). Assay validity was assessed by the usual Analysis of Variance (ANOVA) tests for linearity and parallelism and by visual inspection of the plotted dose-response curve.

Potencies within laboratories were combined using geometric means, and intra-laboratory variability was expressed as geometric coefficients of variation (%GCV) (20). Overall potencies were calculated as geometric means of the individual laboratory means, and inter-laboratory variability was expressed as %GCVs between laboratory means.

The agreement between duplicate samples was assessed by calculating the difference in log potency estimates of samples A and C for each assay relative to sample B, calculating the mean of the squared difference for each laboratory, taking the square root to give a root mean square (RMS) value, and expressing this as an average percentage difference.

Stability Studies

Accelerated Degradation Studies

Samples of the candidate standard 09/234 (study samples A & C) were stored at elevated temperatures (+4°C, 20°C, 37°C and 45°C) for 14 -16 months and assayed in two laboratories, laboratory codes 3 and 6 using a similar layout but different cell-based bioassays. Samples were tested concurrently with those stored at the recommended storage temperature of -20°C, and baseline samples stored at -70°C. For each material, 3 assays were performed with each temperature replicated across 3 plates within each assay. The potencies of all samples were expressed relative to the appropriate -70°C baseline samples.

Stability after Reconstitution

Samples of the candidate standard 09/234 were reconstituted and left at 4°C and 20°C for periods of 4 hours, 24 hours, and 1 week. The reconstitutions were timed to allow all samples to be assayed concurrently. Three independent bioassays (using the TF-1 cell-line and ³H-thymidine as readout) were performed (on samples that had been reconstituted from three separate ampoules), with each sample replicated across 2 plates within each assay.

Stability on Freeze-Thaw

Samples of the candidate standard 09/234 were reconstituted and subjected to a series of freeze-thaw cycles (1 up to 3). They were then assayed concurrently with a freshly reconstituted ampoule. Two independent bioassays (using the TF-1 cell-line and ³H-thymidine as readout) were performed (on samples that had been reconstituted from two separate ampoules), with each sample replicated across 4 plates within each assay.

Results

Data Received and Assay validity

All eight laboratories who were sent the coded samples for the collaborative study contributed data which were derived using different assay methods (Table 3).

Laboratory 3 contributed three sets of data from use of three different methods, which have been analysed separately as if from different laboratories, and are referred to as laboratories 3A, 3B and 3C. Laboratory 6 submitted two sets of assays from two different methods, which have been analysed separately and referred to as 6A and 6B. Data from 3C and 6B is derived from ELISAs while data from all other laboratories is derived from bioassays.

The majority of laboratories contributed data from three independent bioassays, each with at least 3 plates as requested. Laboratory 7 returned data from two assays each with 3 plates per assay. Laboratory 2 submitted data from 4 separate assays with 1 plate per assay while laboratory 8 sent data from 3 assays with 1 plate per assay. For laboratory 5, one plate was excluded from the second assay due to excessive variability compared with the other plates in the assay.

For laboratory 7, analysis was restricted to three doses on the linear portion of the curve due to excessive variability at the bottom and top doses. As a result, analysis was performed using a simple parallel-line approach on the untransformed responses.

For laboratory 2, analysis was restricted to the top 3 or 4 doses, as lower doses were giving responses close to the background. Analysis was performed using a simple parallel line approach on the log transformed responses.

For laboratory 3C, responses covered only the bottom section of the full dose response curve and so were analysed using a simple parallel line approach with a log transformation.

Apart from the exclusions detailed above, all assays were valid using the ANOVA criteria (methods) and were included in subsequent analysis. In some cases however, individual data points or dose levels were removed at the extremes of the dose-response curves, to improve the fit of the parallel line model.

Excipient sample D

In all assays for all laboratories, the excipient (sample D) did not give any dose response.

Potencies of samples A and C relative to B (coded 98/608)

The laboratory geometric mean potencies for samples A and C relative to sample B (currently available as a reference reagent from NIBSC, code 98/608 - assigned unitage of 10^4 U/ampoule) are shown in Table 4, along with the intra-laboratory (between assay) %GCV. The laboratory mean potency estimates are also shown in histogram form in Figures 1 – 3. Each box represents a laboratory geometric mean estimate, and the boxes are labelled with the laboratory code. The laboratories performing an ELISA are shaded in grey. Figure 3 is a combination of Figures 1 and 2, combining results for the two duplicate samples (A and C) of the candidate standard.

There is generally good agreement between the mean potency estimates for the duplicate samples A and C for each laboratory (Table 4).

The intra-laboratory variability, as measured by the within-laboratory %GCV differs between laboratories, ranging from below 5% to around 30%. The value for laboratory 7 is based on only 2 assays. For each laboratory, the agreement between duplicate samples A and C within individual assays was also assessed by calculating percent differences in potency estimates which were averaged across assays, as described in the statistical methods section. The results are shown in table 5. The within assay variability in estimates differs between laboratories, ranging from around 5% to 35%. This is of a similar order to the between assay variability noted above.

From the Figures and Table 4, it appears that the results from the two laboratories performing ELISAs (3C and 6B) are a little lower than the results from bioassays performed in other laboratories. This is marginally statistically significant ($p = 0.045$ using Mann-Whiney test), however, from figures 1 to 3, the results from the ELISA assays still appear to be consistent with the overall distribution of results.

The overall geometric mean potency estimates are shown in Table 4, along with the inter-laboratory (between-laboratory) %GCVs for each sample. The means for laboratories using bioassays and using ELISA are shown separately, and an overall mean of all laboratories is also shown. The means derived from bioassay data were 1.96 and 1.94×10^4 IU/ml for samples A and C respectively. For ELISAs, the means were 1.64 and 1.68×10^4 IU/ml. The overall means for A and C including both methods were in close agreement as expected (1.90 and 1.89×10^4 IU/ml respectively). The overall between-laboratory %GCV's were low at approximately 10% for both A and C. This represents good agreement between laboratories.

Stability Studies**Accelerated Degradation Studies**

Geometric mean potency estimates of the samples A and C (coded 09/234) stored at different temperatures (expressed as a percentage of the -70°C sample) based on assays carried out at NIBSC are shown in Table 6. There is very little observed degradation, with the mean potency of the samples stored at 45°C being 79% relative to the -70°C samples after 14

months of storage. The usual Arrhenius model for accelerated degradation was applied (21) to obtain a predicted % loss per year or per month at the different temperatures. For the candidate standard 09/234 the predicted % losses are shown in Table 7. Three additional assays were performed by a second laboratory relative to a -70°C baseline following storage for up to 16 months. Results are shown in Table 8. A duplicate -70°C sample was included in the assays. The variability between the duplicate -70°C samples was large (potency of 89% for one against the other) but as the potencies observed at the higher temperatures are well within this observed assay variability, there is no evidence of degradation. Because of the high intra-assay variability demonstrated by the differences between the duplicate -70°C samples, data from these assays were not included in the Arrhenius modelling. Overall, the material appears very stable, with a predicted % loss per year of less than 0.001% at the recommended storage temperature of -20°C. The predicted loss at +37°C is 0.52% per month. These figures indicate that the material is stable for long term storage at -20°C and for limited excursions at higher temperature during transportation. It is sufficiently stable to serve as an International Standard.

Stability after Reconstitution

The potencies of the reconstituted ampoules of the candidate (09/234) are shown in Table 9 (expressed as a percentage of the freshly reconstituted ampoule), along with the %GCV between individual assay estimates. The results are quite variable with a mean of 89% after storage at 20°C for 4 hours compared with the freshly reconstituted ampoule, and a mean of 110% after storage at 20°C for 24 hours. At 4°C, identical average potencies were observed after 4 hours and after 1 week. For both temperatures, there is no indication of a decrease in potency after 1 week (means of 98% for 4°C and 104% for 20°C) and the variability in observed potencies is within the limits of the assay.

Stability on Freeze-Thaw

The potencies of samples after 1-3 freeze-thaw cycles are shown in Table 10 (expressed as a percentage of the freshly reconstituted ampoule), along with the %GCV between individual assay estimates. From the tables, it is clear that the potency of the candidate (09/234) does not decrease with an increasing number of freeze-thaw cycles (the observed potencies after 2 and 3 cycles are marginally higher than after 1 cycle) and all cycles are within the limits of variability of the assay.

Discussion

From results derived from this study, it is evident that there is good agreement between the potency estimates for the duplicate samples A and C (code 09/234) for each laboratory irrespective of the assay used. The mean potency derived for samples A and C relative to the reference reagent (coded B, 98/608) from bioassay data were 1.96 and 1.94 x10⁴ U/ml while those obtained from ELISAs were slightly lower at 1.64 and 1.68 x10⁴ U/ml respectively (Table 4).

A majority of laboratories performed bioassays based on inhibitory effect of TGF- β 3 using either the MLEC (Mink lung epithelial) or the TF-1 cell-line (using either a radioactive label or colorimetric/fluorescence dye for detection) while others used either primary cells or reporter gene assays. For all assays, the potencies were predominantly clustered around a value of 1.7-2.3 (relative to Sample B or NIBSC reference reagent, 98/608).

For the bioassays used in the study, data was generally consistent and demonstrated a low intra-laboratory and inter-laboratory variability. For samples A and C, therefore, the intra-laboratory variability, as measured by the within-laboratory % GCV differs between laboratories, ranging from below 5% to around 30%. In a majority of laboratories, the % GCV was within 20%. The inter-laboratory variability for bioassays was approximately 9% while that for the ELISAs was 5-7%. While a low variability is expected with ELISAs, bioassays are often inherently highly variable. However, given that data from all assays including bioassays is very consistent, the potency for the candidate preparation has been derived using the mean from all assays. The overall mean for both samples, A and C using both types of assays is 1.90 and 1.89 $\times 10^4$ U/ml. Overall, the inter-laboratory variability was low at approximately 10%.

Stability studies indicated that candidate preparation (code 09/234) is stable for long term storage at -20°C and the potency is not diminished after 1 week of storage at either 4°C or 20°C following reconstitution or after repeated freeze-thaw cycles. Data derived for the NIBSC reference reagent (coded 98/608) after 8 years of storage has shown no loss of biological activity. For evaluation of real time stability of 09/234, therefore, we will monitor stability of ampoules stored at -20 against a -70 baseline after 5 and 10 years, although from results of accelerated degradation studies so far, there is no indication that more frequent monitoring is needed.

These results clearly indicate that candidate preparation (code 09/234) is suitable for use as an international standard for TGF- β 3. It is proposed that the new international unit should preserve continuity with the units of the existing NIBSC reference reagent (98/608). It is therefore proposed that a value of 19,000 IU/ampoule is assigned to the candidate international standard for TGF- β 3.

Conclusions and Proposal

Based on the results of this study, it is clear that the TGF- β 3 candidate (sample A/C, coded 09/234) is suitable to serve as the WHO 1st IS for TGF- β 3 for assessing potency of TGF- β 3 therapeutic products. There are 4,600 ampoules of this standard available from NIBSC. It is proposed that the candidate preparation 09/234 be accepted as the WHO 1st IS for TGF- β 3 with an assigned value for TGF- β 3 activity of 19,000 IU/ampoule.

Acknowledgements

We are very grateful to the manufacturers (Renovo, UK and R&D Systems, USA) for the supply of candidate materials and to the participating laboratories for performing the

laboratory tests. We are grateful to Paul Matejtschuk and Kiran Malik for assistance with pilot fills of TGF- β 3 preparations and staff of SPD for lyophilizing and despatching the candidate materials of the study.

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List of Appendices

Appendix 1 – List of participants

Appendix 2 – Protocol for Collaborative Study for 1st IS for human TGF- β 3

Appendix 3 – Draft Instructions for Use for the proposed WHO 1st IS for TGF- β 3

Table 1: Materials used in study

Ampoule Code	Date of Fill	Study Code		Ampoule Contents					C
				No Of Ampoules in Stock	TGF- β 3 (Predicted Mass - μ g)	Expression System	Excipients	Reconstitution Volume	
09/234	13/11/09	A, C		4,500~	1	E.coli	0.1% Acetic acid, 1% HSA*, WFI	1 ml 0.1% Acetic acid	S
98/608	17/9/98	B		3,100~	1	Insect			
Excipient	19/11/09	D		100~	0	N/A			

* HSA – Human serum albumin; WFI – water for irrigation

Table 2 – Mean fill weights and residual moisture content of candidate preparation

Ampoule Code	Study Code	Mean Fill weight (g)	CV Fill weight %	Mean Residual Moisture %	CV Residual Moisture %	Mean Headspace Oxygen %	CV Headspace Oxygen %
09/234	A, C	1.0073(178)	0.148	0.404 (12)	34.81	0.21(12)	55.36
98/608	B	1.0102 (58)	0.130	0.084 (3)	35.41	0.78(6)	69.2%

The numbers in parentheses indicate the number of determinations; n/a – not available. Residual moisture of each preparation was measured by the coulometric Karl-Fischer method (Mitsubishi CA100). Headspace oxygen content was determined by frequency modulated spectroscopy (Lighthouse FMS-760).

Table 3A: Individual Laboratory Codes and Assays used by Study Participants

Laboratory Code	Cells/ Cell Line	Assay Type	Assay Duration (hrs)	Read-out	Reference
1	Human dermal fibroblasts	Proliferation	96	Colorimetric (Cell Titer 96 AQueous One solution, MTS)	-
2	MFB-11	Reporter-gene (SEAP)	24	Chemiluminescence (SEAP)	17
3A	MLEC	Inhibition of Proliferation	96	Colorimetric (p-NPP hydrolysis)	15
3B	TF-1	Inhibition of IL-5 induced proliferation	72	Fluorescence (Cell Titer Blue, Resazurin)	14
4	MLEC	Inhibition of Proliferation	96	Colorimetric (p-NPP hydrolysis)	15
5	TF-1	Inhibition of IL-5 induced proliferation	72	Colorimetric (MTT)	14
6A	TF-1	Inhibition of IL-5 induced proliferation	48	³ H Thymidine	14
7	PAI-1-MLEC	Reporter-gene (luciferase)	24	Luminescence (firefly Luciferase)	16
8	HT-2	Inhibition of IL-4 induced proliferation	48	Fluorescence Resazurin	-

Table 3B: Other assays contributed to the study

Laboratory Code	Assay Type
3C	ELISA
6B	R&D Duoset ELISA

Table 4: Relative potencies of samples A and C (09/234) relative to sample B (98/608) (Results x10⁴ U/ml)

		Sample			
		A		C	
		Potency	%GCV	Potency	%GCV
Lab	Method ^{\$}				
1	B	1.83	20.6	2.08	9.5
2	B	1.93	11.2	1.86	8.3
3A	B	1.82	17.3	1.90	8.7
3B	B	1.99	12.6	1.92	30.3
4	B	2.28	4.7	2.10	8.9
5	B	1.77	32.7	1.70	14.5
6A	B	1.95	2.1	2.17	3.6
7	B	1.95	10.6	1.77	22.7
8	B	2.15	6.2	2.04	6.7
Geomean (Bioassay)		1.96		1.94	
%GCV		8.4		8.6	
3C	E	1.57	4.1	1.62	2.2
6B	E	1.72	5.4	1.74	2.1
Geomean (ELISA)		1.64		1.68	
%GCV		6.7		5.2	
Overall Mean		1.90		1.89	
Overall %GCV		10.9		10.0	

\$ B – bioassay, E – ELISA

Table 5: Average differences between Samples A and C within assay of each Laboratory

Lab	Method	% Difference
1	B	29.5
2	B	6.4
3A	B	13.1
3B	B	21.4
3C	E	5.9
4	B	14.2
5	B	34.4
6A	B	12.2
6B	E	5.5
7	B	25.1
8	B	9.3

Table 6: Accelerated degradation – Potencies of preparation 09/234 at higher temperatures relative to ampoules at -70⁰ C baseline after 14-15 months.

Assay	Temperature ⁰ C				
	-20	+4	+20	+37	+45
1	112	102	111	94	77
2	96	87	123	83	78
3	106	111	102	93	83
Geomean	104	99	112	90	79
%GCV	8	13	10	7	4

Table 7: % Predicted loss of potency per month and year at each temperature.

Temperature ⁰ C	% Predicted loss/month	% Predicted loss/year
-20	0.000	0.000
+4	0.002	0.026
+20	0.036	0.432
+37	0.521	6.070

Table 8: Potencies of accelerated degradation samples of 09/234 relative to -70⁰ C baseline (data from Laboratory 3).

Temperature ⁰ C	Potency (%)
-70	89
-20	101
+4	103
+20	100
+37	95
+45	95

Table 9: Stability of 09/234 after reconstitution at +4°C and +20°C for different time periods expressed as % of freshly reconstituted ampoule.

	Temperature and Time period					
Assay	20°C 4hr	20°C 1day	20°C 7day	4°C 4hr	4°C 1day	4°C 7day
1	82	128	101	112	101	119
2	77	100	93	80	83	90
3	111	103	101	127	90	105
Geomean	89	110	98	104	91	104
%GCV	22	14	5	27	10	15

Table 10: Stability of 09/234 after 1-3 freeze thaw cycles expressed as % of freshly reconstituted ampoule.

	Freeze-thaw cycle		
Assay	x1	x2	x3
1	89	99	97
2	111	120	112
Geomean	99	109	104
%GCV	17	15	11

Figure 1: Potencies of Sample A (09/234) relative to Sample B (98/608) by method and laboratory (x10⁴U).

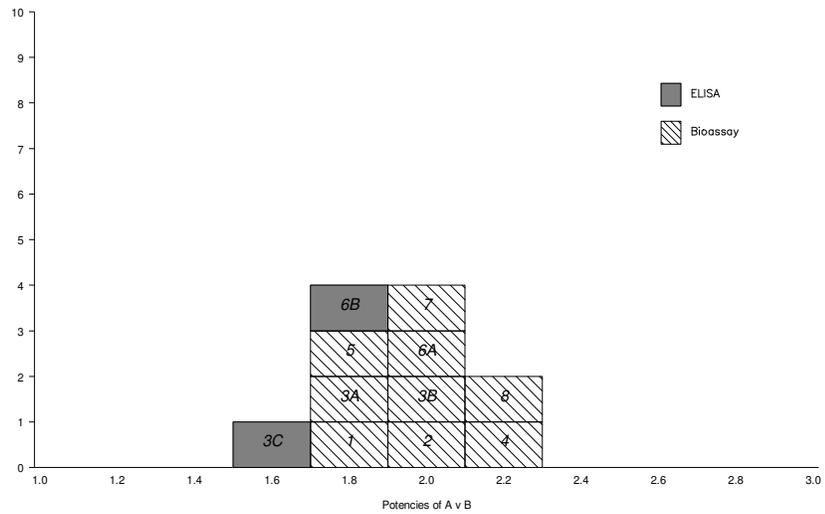


Figure 2: Potencies of Sample C (09/234) relative to Sample B (98/608) by method and laboratory (x10⁴U).

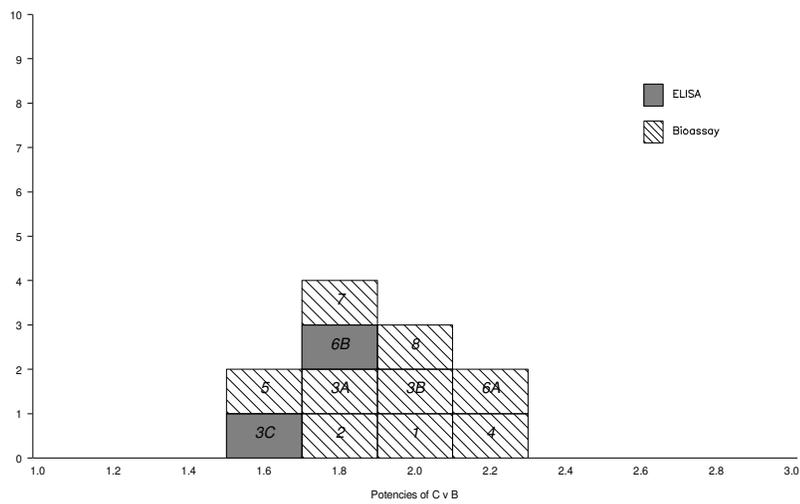
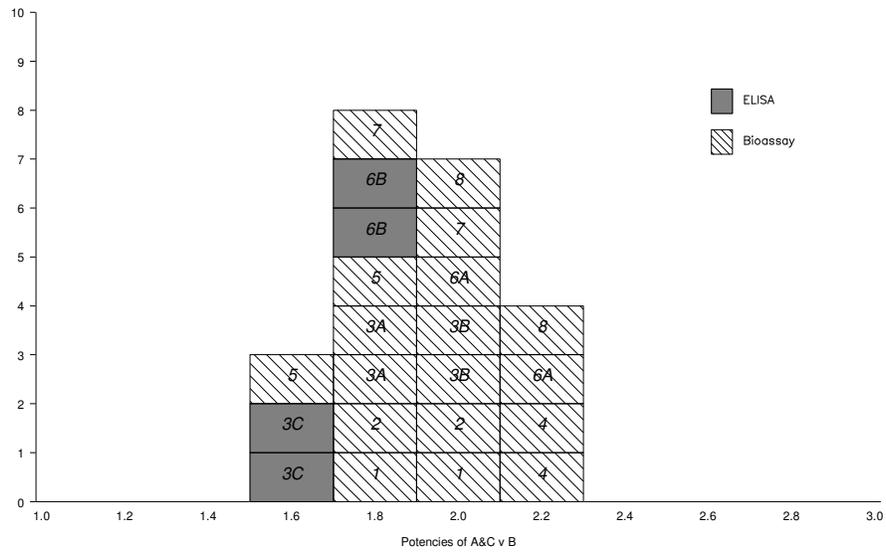


Figure 3: Potencies of both A and C (09/234) relative to Sample B (98/608) by method and laboratory (x10⁴U).



Appendix 1

List of Participants

The following participants contributed data to the study. In this report, each laboratory has been identified by a number from 1 to 8 that is not related to this order of listing.

- Jeff Moore and Jo Read, Novel Therapies Division, Epistem Holdings Plc , 48 Grafton Street, Manchester M13 9XX, UK
- Tony Wyss-Coray & Hui Zhang, Department of Neurology and Neurological Sciences, Stanford Univ School of Medicine, Stanford, CA 94305, USA
- John Little and Kate Getliffe, Renovo Group plc, 46 Grafton Street, Manchester, M13 9XX, UK
- Weihong Wang and David Bender, Molecular and Cell Biology Services, Lancaster Laboratories, 2425 New Holland Pike, Lancaster, PA 17605, USA
- Gao Kai, Deputy Director, Division of Biopharmaceuticals, NICPBP, NO2.Tiantan Xili, Beijing 100050, P.R.China
- Ann Cooke and Paola Zaccone, Department of Pathology, University of Cambridge, Tennis Court Rd, Cambridge CB2 1QP, UK
- Guoping Wu and Aaron Boeckermann, R&D Systems Inc, 614 McKinley Place NE Minneapolis, MN 55413, USA
- Chris Bird and Paula Dilger, Cytokines and Growth Factors Section, Biotherapeutics Group, NIBSC, Blanche Lane, South Mimms, Potters Bar, HERTS, EN6 3QG, UK

Appendix 2

COLLABORATIVE STUDY FOR THE 1st WHO INTERNATIONAL STANDARD for HUMAN TRANSFORMING GROWTH FACTOR-BETA 3

Study Protocol for TGF-beta 3 Assay

1. AIMS OF THE STUDY

- To assess the suitability of ampouled preparations of human Transforming Growth Factor-Beta 3 (TGF- β 3) to serve as the WHO International Standard for the bioassay of human TGF- β 3 by assaying their biological activity in a range of routine, 'in-house' bioassays or immunoassays.
- To assess the relative activity of the ampouled preparations in different assays in current use for these materials.
- To compare the ampouled preparations with characterised 'in-house' laboratory standards where these are available.

2. MATERIALS INCLUDED IN THE STUDY

Participants will be sent

- A set of samples coded by letter **A, B, C**, (6 ampoules for each preparation of A, B, C) for testing in TGF- β 3 bioassays.
- The ampoules contain approximately 1 μ g (microgram) of TGF- β 3.
- 3 ampoules containing excipient, coded **D**, are included in the set to assess any effects of excipient in the assays.

3. RECONSTITUTION AND STORAGE OF PREPARATIONS

Prior to initiating the study, please read the Instructions for Use provided with the collaborative study. 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.

All preparations A - D should be reconstituted with 1ml of sterile 0.1% Acetic Acid and used immediately.

4. ASSAY STRUCTURE

- Participants are asked to include all samples in each TGF- β 3 assay. In addition, we request that participants include their own in-house standard in each assay, where available.
- **Please use a freshly prepared ampoule of each preparation, in each of the assays.** An assay is considered independent if the assay is carried out on different days/occasions.

- For each assay method used, participants are asked to perform an initial assay (a pilot assay) to ensure that all preparations are diluted such that the concentration range falls within the working range of the assay. **Please include dilution series of all preparations (A to D*, and in-house standard) in the assay.** * If sample D is shown to be inactive after an initial assay it may be excluded from subsequent assay runs.
- Following the pilot assay (as in step 3 above), **perform at least 3 independent assays for each of the preparations using the most appropriate dilutions (those giving responses in the linear portion of the dose response curve) derived from the pilot assay for the different preparations tested.**
- **Participants are requested to include at least three dilution series for each preparation in each assay. An example assay layout is provided (separate excel file) which can be amended according to individual assay layouts. Each plate should (where possible) include all the samples* in replicate dilution series. Ideally the position of the samples on the plates should be varied for each assay. Include blank control wells (cells with culture medium but no TGF- β 3) as indicated.**

*Sample D can be assayed on a separate plate if there is not enough room for all the samples on a single plate.

5. INFORMATION TO BE SUPPLIED AND PRESENTATION OF RESULTS

- We have provided an **Excel template (separate excel file) for returning the data obtained for all the samples tested from all the assays. Please amend this template according to your assay layout for positions and dilutions of samples.**
- Please let us know, as clearly as possible, how the assay was carried out, especially how the stock solutions were diluted and what dilutions were entered into the assay (and at what positions, if microtitre plates were used). We have provided an example for a microtitre plate format data sheet on page 6 for diagrammatically illustrating an example assay format, dilutions and results.
- **IT IS VITAL TO INDICATE THE PRE DILUTIONS (off plate starting dilutions) OF THE ORIGINAL PREPARATION IN EACH ASSAY, along with the working dilutions on the plate.**
- **Please PROVIDE ALL RAW DATA** (microtitre plate readout CPM/OD, Response Units etc) as direct analysis of the raw data provided by the assays permits data from all participants to be handled, as far as possible by uniform procedures.
 - a) We request participants to follow the example provided and enter data as indicated in the Excel template.
 - b) Please return all data relating to the assays electronically in the same format as the Excel template provided.
- Please provide information regarding your local in-house standard on the sheet provided.
- Please provide information regarding your assay on the sheet provided.

PLEASE PROVIDE ALL INFORMATION REQUESTED AS THIS IS NEEDED FOR COMPILATION OF THE STUDY REPORT AND SEND TO:

Meenu.Wadhwa@nibsc.hpa.org.uk

6. CALCULATION OF RESULTS BY PARTICIPATING LABORATORY

Although NIBSC will calculate relative potencies from the raw data provided by the participants, participants are requested to calculate the contents of each preparation using their own in-house methods relative to the in-house standard.

Please provide information of all methods used to calculate results.

7. REPORTING OF RESULTS

A draft report of the results will be sent to participants so that they will have an opportunity to comment on it. Participants in the collaborative study are asked to note that they do so with the understanding that they agree not to publish or circulate information concerning the materials sent to them without the prior consent of the organisers.

COLLABORATIVE STUDY FOR HUMAN TGF- β 3

Laboratory identification.....

Local standard information

1. What is the nature of your local standard?

Please state expression system _____

2. How did you obtain the standard?

Bought _____ Source _____

Made in-house _____ (please give reference if available)

3. What units do you use with the standard?

Mass _____

Units _____

2. If units, please provide information on how it was derived

Assay information

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

Appendix 3

Draft Instructions for Use for the proposed WHO 1st IS for TGF-β3



**WHO International Standard
Transforming Growth Factor Beta-3 (Human rDNA derived).
NIBSC code: 09/234
Instructions for use
(Version 1.00, Dated)**

1. INTENDED USE

This preparation coded 09/234 is the primary biological standard for Transforming Growth Factor Beta-3 (TGF-β3).

2. CAUTION

This preparation is not for administration to humans.

The preparation contains material of human origin, and either the final product or the source materials, from which it is derived, have been tested and found negative for HBsAg, anti-HIV and HCV RNA. 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

The assigned potency agreed on the basis of an International Collaborative Study is 19,000 International Units of biological activity per ampoule.

4. CONTENTS

Country of origin of biological material: United Kingdom.
Each ampoule contains the residue after freeze-drying of 1.0 ml of 0.1% acetic acid that contained:

TGF-β3, approximately 1.0 microgram
10mg human serum albumin

The TGF-β3 protein was expressed in E.coli.

5. STORAGE

For economy of use, it is recommended that the solution be sub divided into several small aliquots and stored at -40°C or below. Avoid repeated thawing/freezing. Unopened ampoules should be stored at -20°C.

6. DIRECTIONS FOR OPENING

DIN ampoules have an 'easy-open' coloured stress point, where the narrow ampoule stem joins the wider ampoule body.

Tap the ampoule gently to collect the material at the bottom (labeled) end. Ensure that the disposable ampoule safety breaker provided is pushed down on the stem of the ampoule and against the shoulder of the ampoule body. Hold the body of the ampoule in one hand and the disposable ampoule breaker covering the ampoule stem between the thumb and first finger of the other hand. Apply a bending force to open the ampoule at the coloured stress point, primarily using the hand holding the plastic collar.

Care should be taken to avoid cuts and projectile glass fragments that might enter the eyes, for example, by the use of suitable gloves and an eye shield. Take care that no material is lost from the ampoule and no glass falls into the ampoule. Within the ampoule is dry nitrogen gas at slightly less than atmospheric pressure. A new disposable ampoule breaker is provided with each DIN ampoule.

7. USE OF MATERIAL

No attempt should be made to weigh out any portion of the freeze-dried material prior to reconstitution. Dissolve the total contents of the ampoule

in 1.0ml of 0.1% acetic acid. The solution will contain TGF-β3 at a concentration of 19,000 IU/ml. Use carrier protein where extensive dilution is required.

8. STABILITY

Reference materials are held at NIBSC within assured, temperature-controlled storage facilities. Reference Materials should be stored on receipt as indicated on the label.

NIBSC follows the policy of WHO with respect to its reference materials.

9. REFERENCES

This standard was produced under WHO guidelines cited in the WHO Technical Reports Series, No. 932, 2006, Annex 2.

10. ACKNOWLEDGEMENTS

We are thankful to the manufacturers for their generous donations of TGF-β3 preparations used in the collaborative study, and to the study participants for their contributions in evaluating the preparations.

11. FURTHER INFORMATION

Further information can be obtained as follows;

This material:

enquiries@nibsc.hpa.org.uk

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.who.int/biologicals/reference_preparations/en/

Ordering standards from NIBSC:

http://www.nibsc.ac.uk/products/ordering_information/frequently_asked_questions.aspx

NIBSC Terms & Conditions:

http://www.nibsc.ac.uk/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.hpa.org.uk

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.

14. MATERIAL SAFETY SHEET

Physical and Chemical properties	
Physical appearance: Freeze dried powder	Corrosive: No
Stable: Yes	Oxidising: No
Hygroscopic: No	Irritant: No
Flammable: No	Handling: See caution, Section 2
Other (specify): Contains material of human origin	
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 dependant 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: 4.6g
Toxicity Statement: Toxicity not assessed
Veterinary certificate or other statement if applicable.
Attached: No

