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Research paper

MTS dye based colorimetric CTLL-2 cell proliferation assay for product release and stability monitoring of Interleukin-15: Assay qualification, standardization and statistical analysis

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ABSTRACT

A colorimetric cell proliferation assay using soluble tetrazolium salt [(CellTiter 96® Aqueous One Solution) cell proliferation reagent, containing the (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt) and an electron coupling reagent phenazine ethosulfate], was optimized and qualified for quantitative determination of IL-15 dependent CTLL-2 cell proliferation activity. An in-house recombinant Human (rHu)IL-15 reference lot was standardized (IU/mg) against an international reference standard. Specificity of the assay for IL-15 was documented by illustrating the ability of neutralizing anti-IL-15 antibodies to block the product specific CTLL-2 cell proliferation and the lack of blocking effect with anti-IL-2 antibodies. Under the defined assay conditions, the linear dose–response concentration range was between 0.04 and 0.17 ng/ml of the rHuIL-15 produced in-house and 0.5–3.0 IU/ml for the international standard. Statistical analysis of the data was performed with the use of scripts written in the R Statistical Language and Environment utilizing a four-parameter logistic regression fit analysis procedure. The overall variation in the ED_{50} values for the in-house reference standard from 55 independent estimates performed over the period of 1 year was 12.3% of the average. Excellent intra-plate and within-day/inter-plate consistency was observed for all four parameter estimates in the model. Different preparations of rHuIL-15 showed excellent intra-plate consistency in the parameter estimates corresponding to the lower and upper asymptotes as well as to the 'slope' factor at the mid-point. The ED_{50} values showed statistically significant differences for different lots and for control versus stressed samples. Three R-scripts improve data analysis capabilities allowing one to describe assay variations, to draw inferences between data sets from formal statistical tests, and to set up improved assay acceptance criteria based on comparability and consistency in the four parameters of the model. The assay is precise, accurate and robust and can be fully

Abbreviations: DCs, dendritic cells;NIBSC, National Institute of Biological Standards and Controls;BDP, Biopharmaceutical Development Program;MTS, (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt);PES, phenazine ethosulfate;HRP, horse-raddish peroxidase; HBSS, Hank's balanced salt solution;FCS, Fetal Calf Serum;rHuIL-15, recombinant human IL-15;cGMP, current Good Manufacturing Practice;GLP, Good Laboratory Practice;CIs, confidence intervals;WHO, World Health Organization;4PNLLRM, 4-parameter nonlinear logistic regression method;ELISA, enzyme linked immunosorbant assay;IL-2, interleukin-2;IL-15, interleukin-15

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validated. Applications of the assay were established including process development support, release of the rHuIL-15 product for pre-clinical and clinical studies, and for monitoring storage stability.

1. Introduction

Several cytokines and/or cytokine derivatives have proven to be successful in the clinic as biotherapeutics. Interlukin-15 (IL-15) is a cytokine that plays a significant role in lymphocyte homeostatic and NK cell development. IL-15 is a 14–15 kDa member of the four α-helix cytokine family with structural similarities to IL-2 (Rosenberg et al., 1994; Bamford et al., 1994). Like IL-2, IL-15 stimulates the proliferation of $CD4^+$ and $CD8^+$ T cells, immunoglobulin M (IgM) or CD40L-treated B cells, as well as the generation and persistence of NK cells (Burton et al., 1994; Grabstein et al., 1994; Waldmann et al., 2001; Waldmann and Tagaya, 1999; Fehniger and Caligiuri, 2001a,b; Fehniger et al., 2002; Carson et al., 1997; Waldmann, 2006). Mice deficient in IL-15 exhibit depleted NK, NKT, Tγδ, CD8+ and memory phenotype T-cell numbers, emphasizing the important role of IL-15 in lymphocyte subset development (Lodolce et al., 2002). IL-15 also plays a critical role in the functional maturation of both macrophages and dendritic cells DCs[1] (Ohteki et al., 2001). IL-15 enhances the phagocytic activity of monocytes and macrophages and induces the production of pro-inflammatory factors such as IL-8 and MCP-1 (Badolato et al., 1997). In DCs, IL-15 up-regulates the expression of co-stimulatory molecules and IFN-γ, enhancing the ability of DCs to activate $CD8^+$ cells and NK cells (Mattei et al., 2001; Jinshui et al., 2003). In addition, reduced numbers of peripheral DCs have been observed in IL-$15^{-/-}$ and IL-15R$\alpha^{-/-}$ mice suggesting a role for IL-15 in DC survival (Dubois et al., 2005). IL-15 is an immunoregulatory cytokine that exhibits pro-inflammatory activity by acting on a wide variety of cell types. Some of these effects are direct and include Th1 and Th17 polarization as well as activation of effector cells such as B, NK, mast cells and neutrophils. Other effects are indirect and involve the production of other pro-inflammatory cytokines such as IFN-γ and IL-17 as well as IL-18.

Waldmann (2002) demonstrated that, in contrast to IL-2, IL-15 does not contribute to AICD or the maintenance of T_{reg} cells. In light of these functional differences, IL-15 may be superior to IL-2 in the therapy of cancer and as an agent for use in the treatment of patients with AIDS receiving HAART therapy. A number of studies in murine models have suggested that IL-15 may prove to be of value in the therapy of neoplasia (Fehniger and Caligiuri, 2001a; Munger et al., 1995; Kobayashi et al., 2005; Klebanoff et al., 2004; Fehniger and Caligiuri, 2001b; Carroll et al., 2008). A recombinant form of IL-15 [rHuIL-15] is expressed in *E. coli* inclusion body and solubilized, refolded and purified within NCI and other divisions of NIH. The purified rHuIL-15 is undergoing pre-clinical investigation in preparation for a Phase I clinical study of intravenous administration in patients with refractory metastatic malignant cell cancer.

Measurement of biological activity (potency) of the rHuIL-15 product, and monitoring the stability and lot–lot consistency in biological activity is a critical component for product release for clinical investigation studies. For many cytokines, *in vitro* cell proliferation activity on susceptible cells is used as a surrogate potency assay. Available cell-based bioassays for cytokines and the theory and applications of the bioassays are summarized in two review articles (Mire-Sluis et al., 1995; Meager, 2005). The most widely used type of *in vitro* cell proliferation assay is based on the detectible increase or decrease in DNA synthesis as measured by tritiated (^{3}H) thymidine incorporation. Though somewhat tedious, the method is easily automated and provides a high signal to background ratio. The use of radioactive material and the regulatory constraints associated with many clinical manufacturing facilities puts restrictions on the use of this assay in some testing laboratories. Following the report (Mossman, 1983) of the use of the redox sensitive formazan [3-(4-5) dimethylthiazol-2yl)-2,5-diphenyl tetrazolium bromide] (MTT) forming dark blue/black crystals that can be solubilized and quantified by colorimetric methods, micro plate reader based colorimetric methods have been developed that utilize MTT or other aqueous soluble derivatives of MTT such as XTT and MTS (Roehm et al., 1991; Buttke, et al., 1993). Assays based on fluorometric methods (Jones et al., 2001; Wan et al., 1994; Nociari et al., 1998) are also used currently. Though these assays are commonly employed and adapted in a variety of basic research laboratories worldwide, specific applications may require the development, optimization, qualification and validation of these assays. For release of products for clinical investigation and marketing for human and veterinary applications, these bioassays (for determining product potency) need to be well defined, qualified for initial phases of clinical investigation, and fully validated for late phase clinical studies and marketing.

IL-15 induces a proliferative response on a number of cell lines such as CTLL-2, HT-2 etc. ^{3}H thymidine incorporation and MTT (or analogous colorimetric) as well as fluorometric assays are used in several laboratories. An international standard with defined activity is available from the National Institute of Biological Standards and Controls (NIBSC) for standardization of IL-15 from different sources and Laboratories. In this report we summarize the in-house optimization and qualification of tetrazolium dye based colorimetric cell proliferation assay of CTLL-2 cells using soluble CellTiter96® Aqueous One reagent from Promega Corporation for the quantitative estimation of the biological activity of rHuIL-15. Statistical analyses of the assay variations/consistencies are performed with a four-parameter logistic regression model (DeLean et al., 1978) employing three different scripts written in the R Statistical Language and Environment (R Development Core Team, 2008).

2. Materials and methods

2.1. Cell lines and reagents

Interleukin-15 (human rDNA derived), International Standard reagent, NIBSC Code: 95/554 was obtained from National

Institute of Biological Standards and Controls (NIBSC)[1], UK. Purified recombinant Human IL-15 (rHuIL-15) expressed in *E. coli* system was obtained from the Development Laboratories of the Biopharmaceutical Development Program (BDP), SAIC Frederick Inc., within National Cancer Institute, USA. Antibodies to IL-15 and IL-2 (anti-human IL-2 antibody (Cat#MAB202), anti-human IL-15 antibody (Cat#MAB647, anti-human IL-15 antibody (Cat#MAB2471), biotinylated goat anti-human IL-15 (Cat# BAF247), biotinylated goat anti-human IL-2 (R&D, BAF202) and streptavidin HRP (Cat#890803) and IL-2 (Lot AE4208021) were from R&D systems, Inc., Minneapolis, MN.

The CTLL-2 cell bank was generated by the BDP Cell Culture Lab from cells obtained from ATTC (TIB-214). CellTiter 96® Aqueous One Solution Cell Proliferation Assay containing the (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS) and an electron coupling reagent phenazine ethosulfate (PES), Promega (Cat #G3580,) was from Promega Corporation, Madison, WI.

2.2. CTLL-2 cell proliferation assay

Briefly, CTLL-2 cells were cultured in RPMI 1640 supplemented with 10% fetal bovine serum (FBS), and 200 U/ml IL-2. The cells were harvested in their logarithmic phase (Cell passage 5 or more after thawing; Cell viability: ≥95%) and washed two times with the initial volume of Hanks' balanced salt solution (HBSS) (by centrifugation at 1000 rpm, 5 min) and incubated for 4 h in assay medium (RPMI 1640 supplement with 10% FBS without IL-2) at 37 °C, 5% CO_2. During this period, a 96-well tissue culture plate was set up. The IL-15 samples were diluted to an initial concentration of 2 ng/ml (or 20 IU/ml for NIBSC standard) in the assay medium and followed by serial two-fold dilutions (additional points at 0.4 and 0.2 ng/ml were also included) and added to the wells in 100 µl of the assay medium in triplicates. After the four h incubation, the prepared cell suspension was transferred to a sterile reservoir and seeded immediately in the wells of the 96-well plate (containing 100 µl of rhuIL-15 at different concentrations) in 100 µl of the assay medium [final cell density: (5×10^4) cells/well] and incubated at 37 °C, 5% CO_2 for 48 h. After the 48 h incubation period, CellTiter96® Aqueous One Solution was added (20 µl/well) and incubated for another 4 h at 37 ºC and 5% CO_2 then 25 µl/well of 10% SDS was added. The plate was then read at 490 nm. The background readings in the wells with medium were subtracted from the sample well read outs.

For measuring the neutralizing activity of the different antibodies, rHuIL-15 (2 ng/ml) was incubated with various concentrations of the antibody (anti-IL-15 or anti-IL-2) in assay medium for 1 h at 37 °C in a 96-well plate before adding CTLL-2 cells. At the end of incubation, the prepared cell suspension was transferred to a sterile reservoir and seeded immediately in the wells of the 96-well plate (containing 100 µl of rhuIL-15 and antibodies at different concentrations) in 100 µl of the assay medium [final cell density: (5×10^4) cells/well, rHuIL-15 concentration 1 ng/ml and antibody concentration specified in the titration curves] and incubated at 37 °C, 5% CO_2 for 48 h. Proliferation measurement was as described above.

2.3. Two-step capture ELISA for measuring the cytokine binding to antibodies

A 96-well plate was coated with the anti-IL-15 antibodies (0.5 µg/well in 100 µl) overnight at 4 °C. The antibody coated plate was washed with PBS-Tween, and then blocked with 1% BSA in PBS-Tween. rHuIL-15 (0.01–50,000 ng/ml) was added in triplicate wells of the antibody coated plate and incubated for 1 h at 37 °C, The unbound rHuIL-15 was removed by washing with PBS-Tween three times. Biotin labeled anti-IL-15 (Cat # BAF247, R&D) was added to the wells (100 µl of 50 ng/ml) and incubated for 1 h at 37 °C. The unreacted biotinylated antibody was removed by washing with PBS-Tween three times. Streptavidin HRP (Cat # 890803, R&D) was added to the wells and incubated 1 h at 37 °C. The plate wells were washed 3 times in PBS-Tween and then TMB substrate was added. The plate was incubated for 12 min, and the reaction arrested by adding 100 µl of 2 N H_2SO_4. The plate was read at 450 nm.

2.4. Data analysis — statistical methods

Data were analyzed using the following four-parameter nonlinear logistic regression model (4PNLLRM) (DeLean et al., 1978)

$$Y\sim((A-D)/(1+(X/C)^B))+D$$

where Y = the response, OD (optical density); X = the arithmetic dose, concentration in ng/ml; A = the response when $X=0$, the lower asymptote; D = the response when X is 'infinite', the upper asymptote; C = the X value, concentration, resulting in a response halfway between A and D, referred to as ED_{50}; and B = a 'slope' factor, that corresponds to the slope of the logit–log plot, when X, the concentration, is portrayed in terms of natural logarithms; specifically,

$$dY/dln(X)=[(D-A)*B]/4 \qquad \text{when } X=C, \text{ or}$$

$$d\,\text{logit}\{(Y-D)/(A-D)\}/dln(X)=B.$$

Some results were obtained by fitting the 4PNLLRM using SoftMax Pro software program {(SOFTmaxPRO® Users Manual (2000)}. More refined and extensive analytic results were obtained through the use of three scripts, written by WGA[2] and OQ, in the R Statistical Language (version 2.6.1). Complete source code, scripts and instructions for the use of these scripts may be downloaded from our web source (http://css.ncifcrf.gov/services/download/soman/4PNLLRM.zip). The primary function employed in the scripts, ABCD.4.parm.logistic.fun, is a self-starter function that obviates the necessity of the user to supply start values for the nonlinear regression algorithm (Pinheiro et al., 2008; Pinheiro and Bates, 2000).

Briefly the three R-scripts perform the following functions. Script 1, the 'Individual Case Fit Script' constructs the 'ABCD.4.parm.logistic.fun' self-starter function, applies the function to an input data set, estimates the parameters and their standard errors, and performs additional calculations such as computing the 'activity value' and the 95% confidence bounds for the important ED_{50} parameter estimate and corresponding

activity value. It also provides a plot of the data and a smooth fitted curve through the data points (on a common logarithmic scale), projects the ED_{50} value (C parameter estimate) and its 95% confidence bounds onto the plot, and outputs a summary report containing pertinent information to an external file regarding the fit of the model and data input.

Script 2, the 'Confidence Intervals Determination and Inferences for Multiple Data Sets script' applies the 'ABCD.4.parm.logistic.fun' self-starter function to an arbitrary number of separate data sets, calculates 95% confidence intervals for the four (A, B, C and D) parameter estimates for each data set and produces a trellis plot displaying the 95% confidence intervals for the parameter estimates for visualization of similarities and differences among the input data sets. A report containing the 95% confidence intervals for the parameter estimates and additional pertinent information is produced and output to an external file.

Script 3, the 'Formal Tests for Significance Between Two Data Sets Script' applies the 'ABCD.4.parm.logistic.fun' self-starter function and additional nonlinear mixed effects models and computes formal statistical tests of significance for comparison of parameter estimates for two alternative data sets of arbitrary size. Results are output to an external file.

2.5. Calculation of specific activity and relative activity

One unit of activity is defined as the concentration of IL-15 required to induce the half maximal stimulation. This value is referred to as ED_{50} and corresponds to the C parameter estimate of the four-parameter logistic curve fit. Relative activity referenced to the standard lot was estimated by: (1) using the ED_{50} values for the standard and test sample (Relative activity of test sample = ED_{50} of Reference standard ÷ ED_{50} of test sample), or: (2) using test samples as unknowns, extrapolating the absorbance readings that fall within the standard absorbance readings in the steep part of the four-parameter curve to estimate the concentration of a given test sample. After adjustments for the dilution factor, the estimated values for all test sample dilutions with absorbance in the 'linear' or steep part of the standard curve are averaged. The relative activity for test samples with known protein concentration are then calculated using the formula: Relative activity of test sample = Estimated activity of test sample ÷ protein concentration.

3. Results

3.1. Assay optimization

^{3}H thymidine incorporation and tetrazolium dye based colorimetric assays using CTLL-2 cells are widely employed for the measurement of biological activity of a number of cytokines including IL-15. We chose to optimize and qualify the colorimetric cell proliferation assay using MTS (aqueous soluble form of the tetrazolium salt based assay) with the CellTiter96® Aqueous One assay kit from Promega Corporation. The major factors attributing to the assay variations during initial assay development were cell related. CTLL-2 cells grown in different Laboratories within the program showed significant differences. These cells were obtained from different sources and maintained in separate laboratories for extended time periods. These observed variations are presumably consistent with the reported variations in the cell proliferation of CTLL-2 cells routinely maintained in IL-2 as a function of length of continuous culture and to culture medium related changes (Khatri et al., 2007; Meager 2005).

Subsequently, CTLL-2 cells were obtained from ATCC, and expanded into a research cell bank within the Cell Culture Development Laboratory, and the cell bank was tested for sterility and mycoplasma. The vials are stored within the NCI Frederick Repository under controlled conditions. Cells from the bank, thawed and cultured within different Laboratories of the program gave comparable results with acceptable laboratory to laboratory and analyst to analyst variation. A series of initial experiments were performed to optimize the assay conditions and to define the cell seed density and passage range, IL-15 concentration range for satisfactory dose response curve, incubation time, Aqueous One 96® reagent volume, incubation period, etc. These optimized assay conditions are described in Materials and methods above (Section 2.2).

3.2. Standardization of E. coli expressed rHuIL-15 against International Standard of IL-15

To enable accurate calibration of potency assignations, it is essential that bioassays are monitored for sensitivity on an 'assay-to-assay' basis. This is best done by the inclusion, with test samples, of an appropriate, well-characterized, biological reference standard. However, this can lead to the adoption by individual laboratories of different unitages of biological activity for the same active protein and the reporting of potency/activity in non-comparable units. This problem has significantly grown in importance as active proteins, including certain cytokines, have entered the clinic. To address this issue, the World Health Organization (WHO) has instigated and coordinated International efforts to evaluate the suitability of well-characterized proteins (including cytokines) that serve as International standards (IS) or reference reagents (RR) for individual biologically active proteins (WHO, 1995). The global aim of such 'biological standardization' is to permit the reliable comparison of the results of bioassays performed by different operators with different cells and reagents and at different times (Bangham, 1983; Meager, 2005).

After optimization of the assay conditions for rHuIL-15 induced proliferation of CTLL-2 cells, we standardized the relative activity of an in-house reference Lot of rHuIL-15 against the NIBSC International standard. The NIBSC international standard was shipped to the laboratory under controlled conditions by overnight delivery in a dry-ice packaged box, and transferred to −70 °C (±10 °C) calibrated and controlled freezers. The freeze dried material in the vial was reconstituted and aliquoted and stored at −20 °C as recommended in the NIBSC product description manual. A total of sixteen assays on different plates (5 day triplicate plates and one day single plate) were performed over a one-month period. Freshly thawed aliquots of NIBSC standard from −20 °C freezer were used in each experiment. The cell passage numbers for these six independent experiments differed (5–32 passages after thaw and seed). All other conditions remained identical.

Fig. 1A shows a typical standard curve using NIBSC International Standard and Fig. 1B shows a typical standard curve using the internal reference control. The ED_{50} values for the NIBSC standard and the internal reference control from the different experiments are summarized in Tables 1A and 1B.

Examination of Fig. 1A and B reveals excellent four-parameter non-linear logistic curve fits over the concentration range of 0.5–20.0 IU/ml in the assay for NIBSC International standard and over 0.02–1.00 ng/ml for *E. coli* expressed rHuIL-15. The effective response range for the IL-15 on CTLL-2 cell proliferation was narrow (0.6–5.0 IU/ml for NIBSC standard [Fig. 1A] and 0.02–0.2 ng/ml for reference control [Fig. 1B]). The linearity ranges were from 1 to 4 IU/ml for NIBSC standard and 0.04–0.17 ng/ml for the reference control. The approximate linear response curve for the standard curves as determined by the comparison of the back calculated estimates for the different OD readings to standard curve distribution were also in the 1–4 IU/ml for NIBSC standard and 0.04–0.17 ng/ml for the reference control showing good agreement.

3.3. Assay specificity for IL-15

CTLL-2 cell proliferation is induced by a variety of cytokines and growth factors. Hence, for cytokine specific proliferation effects, the product specificity and the interference from other cytokines in the dose response range must be examined. Experiments were designed to address IL-15 specificity of the product induced cell proliferation under the defined assay conditions.

For defining the product specificity for IL-15, two sets of experiments using IL-15 and IL-2 specific antibodies were performed. Because of its similar biological effects, IL-2 was chosen as a potential interfering cytokine to illustrate the assay specificity for IL-15.

The antibodies for IL-15 and IL-2 were obtained from R & D Systems. The specificity of the reagents is defined by the reagent supplier (http:www.rndsystems.com) and other laboratories (Carvalho et al., 2007; Silva et al., 2005). Fig. 1C shows the effect of two α-IL-15s and one α-IL-2 on the CTLL-2 cell proliferation by rHuIL-15 product. Both the α-IL-15s neutralized the IL-15 induced proliferation response in a concentration dependent manner. Fig. 1D shows the binding of the α-IL-15 antibodies to IL-15 as probed by sandwich ELISA using appropriate reagents. As evident from Fig. 1C and D, only IL-15 binds to the α-IL-15 capture antibody to be detected with biotinylated α-IL-15 and HRP-streptavidin. Similarly, only IL-2 bound to anti-IL-2 coated plate as detected by biotin-anti-IL-2/StreptavidinHRP (not shown). There was no detection with biotin-anti-IL-15/streptavidin

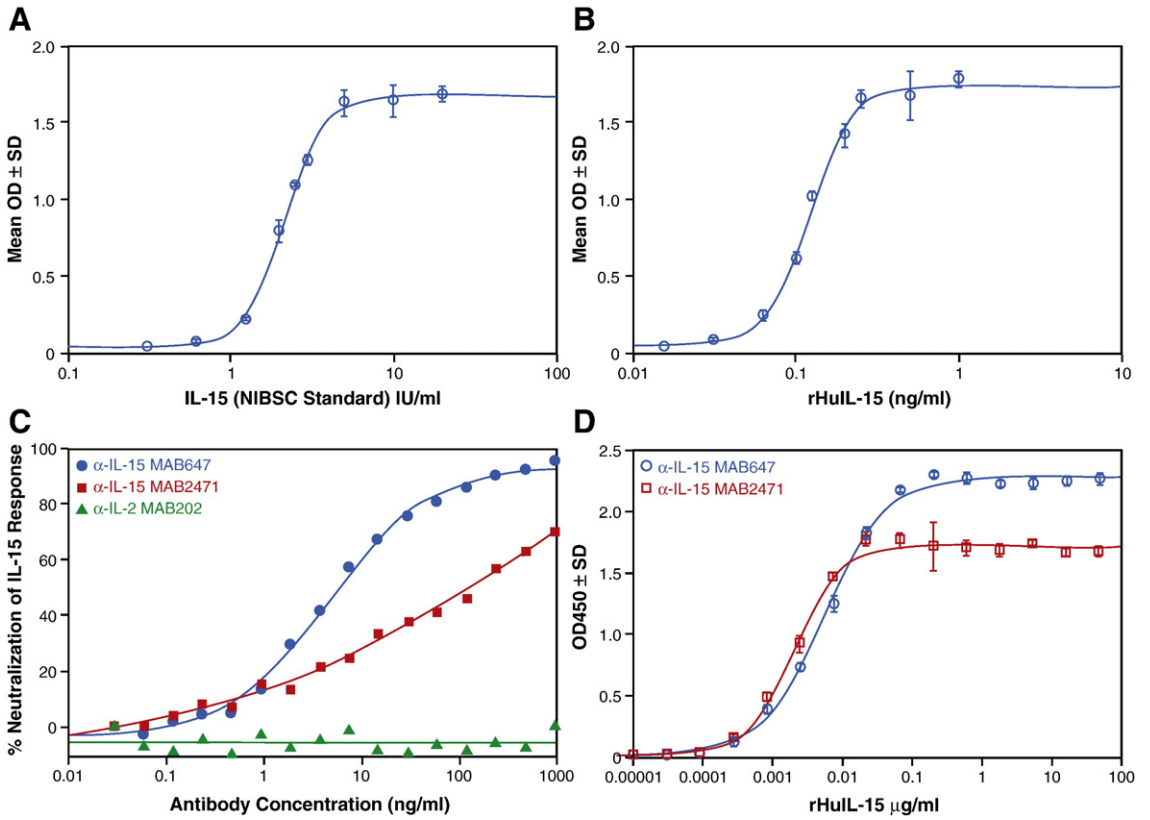


Fig. 1. IL-15-induced specific CTLL-2 cell proliferation. A: rHuIL-15 international reference standard (NIBSC code 95/554) concentration (IU/mL) dependent CTLL-2 cell proliferation. B: rHuIL-15 (BDP/SAIC/NCI) concentration (ng/mL) dependent proliferation of CTLL-2 cells. Details of the assay conditions are described in Materials and Methods Section 2.2. C: Neutralization of rHuIL-15 bioactivity by the anti-IL-15 and anti-IL-2 antibodies. The CTLL-2 cell proliferation responses induced by rHuIL-15 (2 ng/ml) were measured in the presence of various concentrations of the antibody (anti-IL-15 or anti-IL-2). Details are described in Materials and methods Section 2.2. D: IL-15 binding to anti-IL-15 detected by ELISA. Binding ELISA was performed as described in Materials and methods Section 2.2.

Table 1A
ED_{50} values of NIBSC Reference Control and ED_{50} values and Specific activity of BDP Reference Standard.

Expt. #	Cell Passage	NIBSC Std.	BDP Ref. Std.	BDP Ref. Std Activity (IU/mg)	
		ED_{50} (IU/ml)	ED_{50} (ng/ml)	Based on ED_{50} Ratio	Based on extrapolation of absorbance readings to Standard curve
1	P27	2.32	0.134	17.33×10^6	16.94×10^6
		2.15	0.129	16.67×10^6	16.52×10^6
		2.26	0.135	16.75×10^6	16.23×10^6
2	P5	1.81	0.095	19.03×10^6	19.49×10^6
		1.81	0.096	18.90×10^6	19.70×10^6
		1.84	0.096	19.20×10^6	20.24×10^6
3	P6	1.97	0.114	17.31×10^6	18.68×10^6
		1.98	0.117	16.88×10^6	16.06×10^6
		2.01	0.118	17.05×10^6	18.24×10^6
4	P7	1.92	0.101	19.01×10^6	19.98×10^6
		1.91	0.101	18.87×10^6	19.50×10^6
		1.92	0.105	18.24×10^6	19.14×10^6
5	P32	2.11	0.120	17.58×10^6	18.56×10^6
		2.10	0.121	17.37×10^6	18.43×10^6
		2.13	0.119	17.92×10^6	18.24×10^6
6	P13	2.10	0.106	19.84×10^6	18.70×10^6
Mean		2.02	0.113	18.00×10^6	18.42×10^6
S.D.		0.15	0.013	1.02×10^6	1.33×10^6
CV%		7.64	11.82	5.65	7.23

ED_{50} values correspond to the C parameter estimates of the four-parameter curve. Specific activity is estimated (1) by using the formula specific activity (IU/mg) = (ED_{50} of NIBSC Standard, IU/ml) ÷ ED_{50} of BDP Ref. Std, ng/ml) × 10^6 or (2) extrapolation of absorbance readout (absorbance readings of BDP Ref. Std at different dilutions which are within the steep part of NIBSC standard curve) and adjustment to dilution factor.

HRP (not shown). These experiments clearly demonstrate specificity of the rHuIL-15 product.

We also looked at potential interference of IL-2 in the IL-15 specific proliferation response by spiking IL-2 in the assay. The IL-2 used in this study was obtained from R & D Systems. Under the assay conditions, IL-15 responses were in the picogram range whereas the IL-2 responses were in the ng range. Under the assay conditions described, IL-2 behaved as a non-interfering protein impurity in the assay (data not shown). These results suggest that the IL-2 molecule, which has no CTLL-2 cell proliferation effect at <1 ng/ml, does not influence the cell proliferation effect of IL-15, and in fact simply acts only as a non-interfering protein impurity under the assay conditions. In body fluids IL-15 levels are usually in the picogram levels and IL-2 concentrations may reach nanogram levels. Because the neutralizing antibody to IL-2 does not interfere with IL-15 specific cell proliferation, addition of antibodies for IL-2 in reaction system may enable one to assess the IL-15 specific activity in the body fluids.

3.4. Assay consistency and variations

Consistent and proceduralized data analysis is a critical component of assay qualification under cGMP and GLP, especially for product release. For *in vitro* cell proliferation assays, dose response curves are typically sigmoid-shaped. Our laboratory routinely employs the non-linear four-parameter logistic model described in the Data analysis — statistical methods Section 2.4.

The samples were analyzed in triplicate wells; the coefficient of variation (CV) for the raw absorbance values was <5% for all the dilutions. The CVs for the replicates of the blank corrected absorbance readings were <10% with very few exceptions. These exceptions occurred at low concentration values of IL-15, where the absorbance readings for test wells were close to zero. The curve-fit R^2 values were ≥0.99, indicating extremely good fit of the four-parameter logistic model to the data.

3.5. Curve linearity

The ED_{50} ratio of two lots (reference standard and test) gives a fairly accurate comparison of relative activities. However, curve linearity of the estimated values of activity of product lots over a range of concentration or dilution range should also be evaluated to address comparability of different product lots. Fig. 2A shows linear curve fits of the estimated concentration values to the % maximal stimulation from an

Table 1B
Intra-day (inter-plate within day) and Inter-day assay variations in the ED_{50} values and Specific activity.

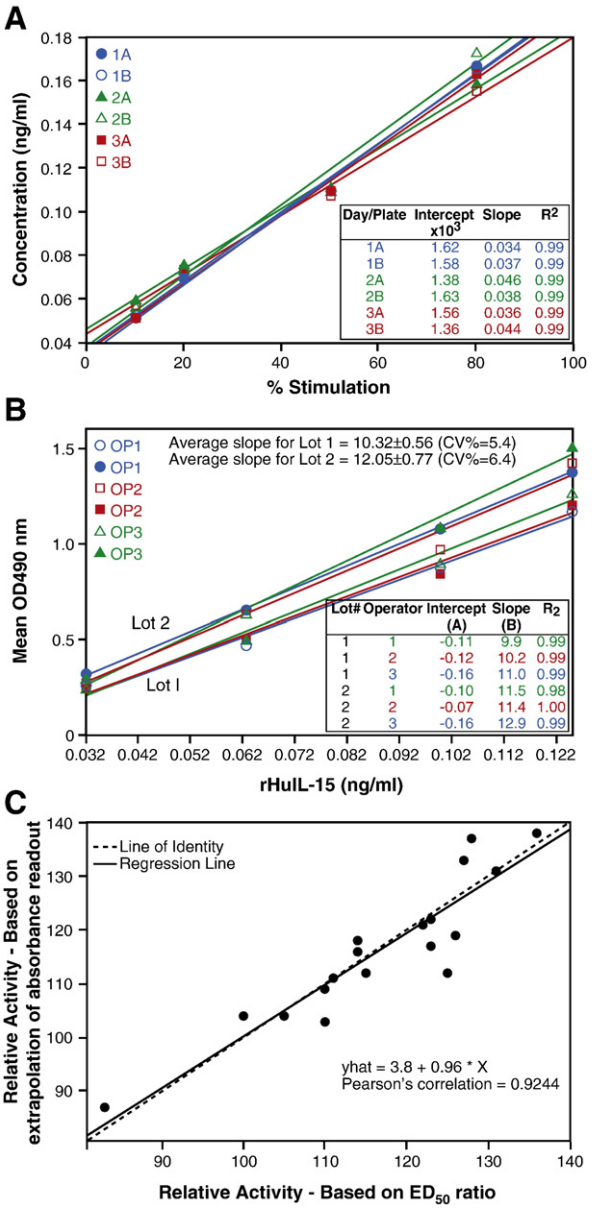
Expt. #	Cell passage #	NIBSC Std.		BDP Ref. Std. Lot					
		[a]Mean ED_{50}	[b]Intra day	[a]Mean ED_{50}	[b]Intra day	[a]Activity ED_{50}-based	[b]Intra day	[a]Activity Back calculated by extrapolation of absorbance readings	[b]Intra day
		IU/ml	CV%	ng/ml	CV%	IU/mg	CV%	IU/mg	CV%
1	P27	2.25	3.86	0.133	2.42	16.92×10^6	2.13	16.56×10^6	2.16
2	P5	1.82	1.03	0.096	0.60	19.04×10^6	0.79	19.81×10^6	7.94
3	P6	1.99	1.11	0.116	1.79	17.08×10^6	1.27	17.66×10^6	2.16
4	P7	1.90	0.37	0.102	2.26	18.71×10^6	2.19	19.54×10^6	–
5	P32	2.12	0.73	0.120	2.42	19.84×10^6	–	18.70×10^6	2.16
6	P13	2.10	–	0.106	–	17.62×10^6	1.57	18.41×10^6	0.87
[c]Mean		2.03		0.112		18.20×10^6		18.45×10^6	
[c]S.D.		0.15		0.014		1.17×10^6		1.21×10^6	
[c]Inter-day variation (CV %)		7.54		12.05		6.45		6.55	

[a] Mean from three different plates (triplicate wells in each plate).
[b] Calculated from triplicate values obtained from three different plates.
[c] Mean, S.D. and CV% of the results from the six different days (inter-day assay variation).

experiment in which the same sample was run in duplicate on three different days. The inset table shows the intercepts, slopes and R^2 values for the six plots.

The results in Fig. 2A and the inset table indicates that the best linearity range for the quantitatively accurate estimates of the rHu-IL15 are in the range of 0.04–0.17 ng/ml. The lower limit of detection is approximately 0.02 ng/ml. Fig. 2B shows linear dose–response fits from another experiment performed for two different Lots (1 and 2) of rHu-IL15 from three different operators. Again, the inset table shows the intercepts, slopes and R^2 values for the six plots.

Table 2 shows the relative activity of different preparations of rHuIL-15 calculated using the ED_{50} ratio or extrapolation of absorbance readings of different dilutions in the linear range (steep part of the four-parameter curve) of the reference Standard. Fig. 2C shows a scatter plot of relative activity based on the extrapolation of the absorbance readout versus the relative activity based on the ED_{50} ratio values from Table 2. The relative activity values obtained by these two methods show excellent agreement in that the regression line (solid) is statistically equivalent to the line of identity (dashed); i.e., the 95% confidence interval for the intercept includes zero and the 95% confidence interval for the slope includes 1.00.



3.6. Statistical analysis of ED_{50} parameter estimates

For purposes of assay validation, the most important parameter in the four parameter logistic regression model is the C parameter, referred to as ED_{50}. It represents the half-maximal stimulation concentration. Fifty five ED_{50} values were collected over a one year period with the reference material and were analyzed for trend. The ED_{50} values were computed using the 'Individual Case Fit Script' (Script 1). The plot in Fig. 3 shows the ED_{50} values with their mean (solid line) and upper and lower (dashed) lines that are 1.96 times the standard deviation of the data. The mean of the 55 ED_{50} values is 0.108 ng/ml. Of the 55 values, 2 fell outside 1.96 standard deviations. (Under the assumption of normality, one would expect approximately 5%, or approximately 3, of the values to fall outside 1.96 standard deviations of the mean). The ED_{50} values are homogeneously distributed about the mean. The analysis revealed that there is no trend in the data over time.

3.7. Intra and inter assay variations

Data generated with the same lot of rHuIL-15 on five different days in triplicate plates were analyzed using Script 2, the 'Confidence Intervals Determination and Inferences for Multiple Data Sets' script. Fig. 4 displays a trellis plot of the parameter estimates along with their 95% confidence inter-

Fig. 2. Linear range of rHuIL-15 dependent cell proliferation. A: Linear correlation of estimated concentration versus the % stimulation. Cell proliferation data generated on three different plates (with two triplicate well sets in each plate) was reanalyzed to calculate % stimulation at each concentration. Concentrations corresponding to 10%, 20%, 50%, and 80% stimulation were determined for each of the six sets of data. Linear fits of concentration versus % stimulation were calculated. The inset table shows the intercepts, slopes and R^2 values for the six plots. 1, 2, and 3 designates plate numbers and A and B designates the duplicate sets in each plate. B: Linear dose response range of rHuIL-15 dependent cell proliferation performed by three different operators on two different Lots. The proliferation data generated was filtered to get the best linear curve fit. The best linear curve-fits are in the concentration range of ~0.03–0.125 ng/ml rHuIL-15. The slope values (B), the intercept values (A) and R^2 values are shown in the inset Table. C: Comparison of relative activity based on ED_{50} ratio vs. relative activity based on extrapolation of absorbance readout. Relative activity based on the ED_{50} ratio is compared to the relative activity based on extrapolation of absorbance readout by means of fitting a regression line (solid) to the data. A line of identity is also plotted (hatched). The intercept parameter, 3.8, and slope parameter, 0.96, are provided on the graph, as well as Pearson's correlation coefficient, 0.92. The regression line obtained from the fit is statistically equivalent to the line of identity with the intercept parameter, (3.8 ± 11.09), being equivalent to zero and the slope parameter, (0.96 ± 0.09), being equivalent to 1.00. Pearson's correlation coefficient of 0.92 is indicative of a strong, positive linear relationship between the data, $p<0.0001$.

Table 2
Comparison of the Relative activities (%Reference Standard) of different preparations calculated by ED_{50} ratio or absorbance reading extrapolation to standard curve.

[a]Test article	Relative activity (%Reference Standard)	
	[b]Based on ED_{50} ratio	[c]Based on extrapolation of absorbance readout
A	136	138
B	123	117
C	122	121
D	131	131
E	126	119
F	125	112
G	100	104
H	115	112
I	110	109
J	128	137
K	114	116
L	83	87
M	123	122
N	115	112
O	110	103
P	114	118
Q	111	111
R	105	104
S	127	133
T	136	138

[a] Designations A–T are arbitrary and simply indicate samples from different fermentation and/or purification process.

[b] Relative activity calculated using the formula, relative activity = (ED_{50} of Reference Standard ÷ ED_{50} of test sample) × 100.

[c] Activity of test sample is calculated by extrapolating the test sample dilution absorbance readings to the standard curve generated using reference standard. The estimated values of those sample dilutions with absorbance readings in the steep or linear part of the standard curve is adjusted for sample dilution by multiplying the values with sample dilution factor. The adjusted values are averaged and taken as the activity. Relative activity is calculated using the formula: relative activity (5 Reference standard) = (estimated activity ÷ reported protein concentration) × 100.

vals (CI's) for these data. When the 95% CI's for a particular parameter estimate overlap, one is 95% confident that the parameter estimates are statistically equivalent. Fig. 4 shows that there was excellent intraday/inter plate consistency, due to the fact that the 95% CIs for each parameter, within each day, overlap for all 5 days. Similar intraday/inter-plate consistency was observed with other data sets (not shown). Fig. 4 also shows that some inter-day variability was present. For example, the 95% CIs for the C values for the triplicate plates on days D2 and D3 do not overlap with the 95% CIs for days D1 and D4. Similarly, the 95% CIs of the D values for days D2 and D3 did not overlap with the 95% CI's for day D4.

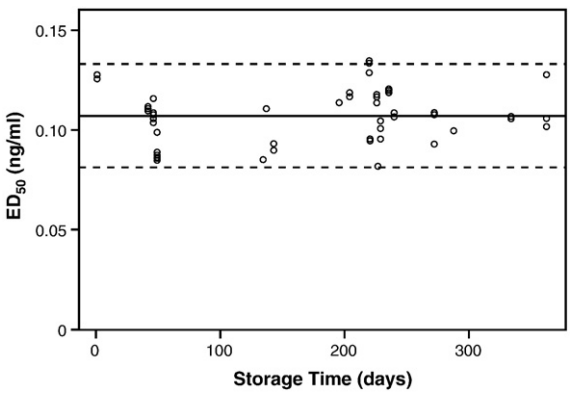


Fig. 3. Distribution of ED_{50} values from fifty five different experiments performed on 20 different days. Predicted ED_{50} values obtained from the plots using Script 1, the 'Individual Case Fit Script' are plotted. The ± 1.96 S.D. are also shown (dashed lines). ED_{50} values are the predicted values obtained from analysis of the data using the Script 1, the 'Individual Case Fit Script'.

3.8. Operator to operator consistency

Robustness of an assay is defined as the consistency in the results when the assays are performed by different operators and also in different laboratories. Two different preparations of rHuIL-15 were run simultaneously in the same lab by three independent operators. Fig. 5A shows fitted 4-parameter curves for two different lots 1 and 2) of rHu-IL15 performed by three different operators (total of six curves). Fig. 5B shows a trellis plot of the parameter estimates along with their 95% CI's for the six fitted curves. If the 95% CI's of the parameter estimates overlap, for different operators within a given lot, we say that the results are consistent. Fig. 5B shows that within each lot, the A, B, C and D parameter estimates obtained from the three operators overlap, demonstrating consistency for this set of experiments.

3.9. Detailed statistical comparison of two different data sets

Two major goals of this assay development effort are: (1) to quantify and compare the biological activity of products lots release for clinical trials, and (2) to monitor the stability of the product. Script 3, the 'Formal Tests for Significance Between Two Data Sets Script', provides formal statistical tests of parameter estimates for two alternative data sets of arbitrary size. Fig. 6A shows dose response curves for three different rHuIL-15 samples. The inset table in Fig. 6A shows exact probabilities obtained from analyses comparing datasets a versus b (a vs. b), a versus c (a vs. c), and b versus c and (b vs. c), respectively. In a comparison of two parameter estimates, a probability value less than 0.05 ($p \leq 0.05$) is considered significant. The inset table in Fig. 6A suggests that the A, B and D parameter estimates are statistically equivalent as their probability values are greater than 0.05 ($p > 0.05$). The p values for the C parameter estimates (i.e., ED_{50}), however, are less than 0.05 ($p < 0.05$) for the pairs designated as a vs. c and b vs. c, but greater than 0.05 ($p > 0.05$) for a vs. b. Collectively, the comparative analyses for these dose response curves for a vs. b, b vs. c, and a vs. c suggest qualitatively comparable dose response patterns for lots a, b, and c with quantitative differences in activity. That is, the activity for lot c is lower than the activities for lots a and b. However, the respective activities for lots a and b do not differ from each other.

We have also used Script 3 to perform formal statistical tests of parameter estimates between two sets of data to investigate within-plate assay variations. To evaluate the within-plate assay variations we analyzed two sets of data (Set I and Set II) within the same plate for nine different plates (Table 3A). Table 3B shows probability values obtained for comparisons of the A, B, C and D parameter estimates on the two sets of data within each plate. Of the total nine plates, statistical equivalence for all four parameter estimates (A, B, C

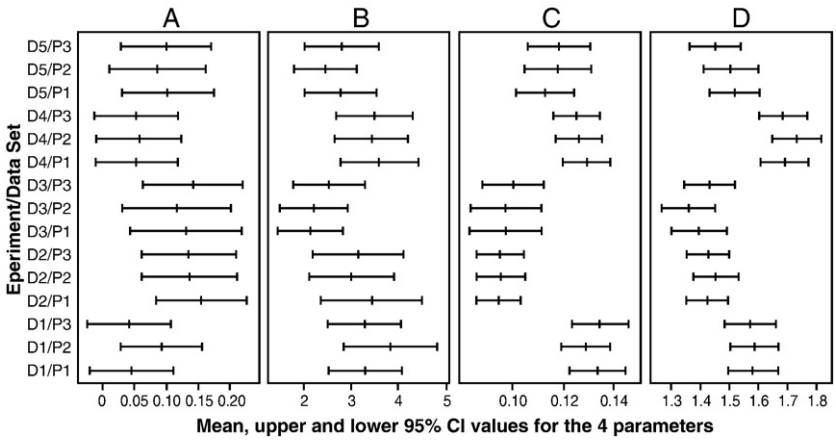


Fig. 4. Trellis-plot of ED_{50} values and the upper and lower 95% confidence intervals for data generated on five different days in triplicate plates. Data generated on five different days, designated as D1, D2, D3, D4 and D5, were analyzed. Trellis plot shows A, B, C (ED_{50}) and D parameter estimates (center) along with their upper (right) and lower (left) 95% CIs. ED_{50} (C parameter) is displayed in ng/ml.

and D) were observed in Set I and Set II for eight of the nine plates ($p \geq 0.05$). [Similar consistency was detected for many other within-plate comparisons performed (data not shown).]

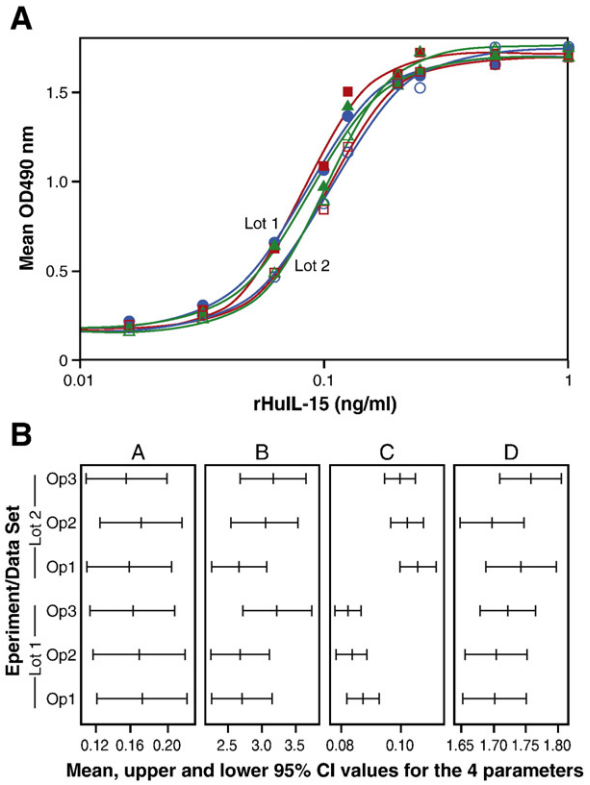


Fig. 5. Operator to operator variations. A: Comparison of the dose response curves for two different lots of rHuIL-15 performed by three different operators. The values for the four parameter coefficients and curve-fit correlation coefficients are shown. B: Trellis plot generated using Script 2, the 'Confidence Intervals Determination and Inferences for Multiple Data Sets script'. The Trellis plot shows the mean (center), and the upper (right) and lower (left) bounds of the 95% CIs for all four parameter estimates. Op1, op2 and op3 refers to operator 1, operator 2 and operator 3, respectively. ED_{50} (C parameter) is displayed in ng/ml.

One of the nine plates (P7) did not show statistical equivalence in the B and D values ($p<0.05$). Our methods of comparison conform to accepted norms for verification of within assay variations (Meager, 2005). Consistency in the four-parameter estimates of the same preparation in different sets within the same plate was observed (within 95% CIs) when samples were run either in sets of horizontal or vertical blocks. This observed consistency suggests that no biases have occurred or have been introduced into analyses of this bioassay, and thus, validate the accuracy and precision in the relative activity estimates (Meager et al., 2001; Meager and Gaines Das, 2005; Gaines Das and Meager, 1995).

We further used Script 3 to perform formal statistical tests of parameter estimates to investigate differences in activity between control and heat-treated samples. Fig. 6B shows a trellis plot of the parameter estimates along with their 95% CI's for two experiments comparing control and heat treated samples. For these two experiments, heat treatment resulted in an approximate 20% decrease in biological activity, indicated by higher ED_{50} values (C parameter estimates, $p<0.0001$). The equivalency in the A, B, and D parameter estimates ($p>0.05$), respectively, for the heated and control samples, suggests that the loss of activity due to heating is attributable to the loss of active components rather than due to molecular changes. Analytical size-exclusion HPLC revealed approximately 25% aggregates in the heat treated sample (data not shown) suggesting that the aggregates are presumably not active.

4. Discussion

The MTS dye based colorimetric assay using Aqueous One 96® cell titer assay kit from Promega Corporation is qualified for quantitative estimation of IL-15 dependent cell proliferation activity. There is linearity in the dose response curve over the range of 30–125 pg/ml rHuIL-15 in the assay. The lower limit of detection is ~20 pg/ml in the assay. The assay is specific for IL-15 as revealed by neutralization or blocking of activity by α-IL-15 antibodies and not with α-IL-2 antibodies. IL-2, another major cytokine that stimulates CTLL-2 proliferation functions

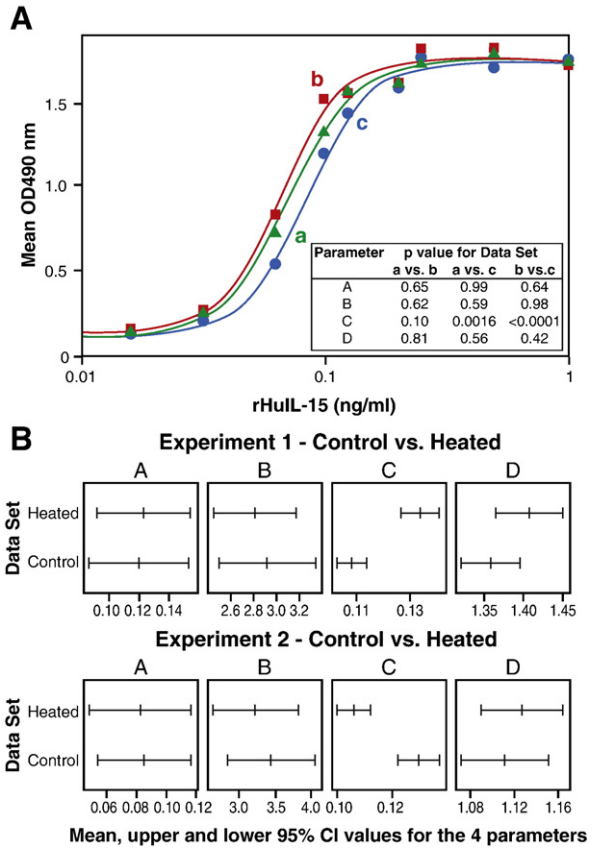


Fig. 6. Statistical analysis of activity of different rHuIL-15 lots or heat-treated rHuIL-15. A: Statistical comparison of three different IL-15 lots. Four-parameter curve fit plot of the rHuIL-15 dose response proliferation of CTLL-2 cells is shown along with the statistical comparison of the four parameter estimates for pairs of plots using Script 3, the 'Formal Tests for Significance Between Two Data Sets Script'. The *p* values for pair comparisons of the three lots of data sets are tabulated in the inset table. B: Effect of heat treatment on rHu-IL15 activity. The rHuIL-15 sample was heated at 65 °C for 6 h. Dose–response CTLL-2 cell proliferation of the control (not-heated) and the heated samples were performed following the procedure described in Materials and methods. Data were analyzed using Script 2, the 'Confidence Intervals Determination and Inferences for Multiple Data Sets script'. Mean (center), and the upper (right) and lower (left) 95% CIs for all four parameter estimates are shown. The two plots correspond to two independent experiments performed on two different days. ED_{50} (C parameter) is displayed in ng/ml.

at a concentration level two orders of magnitude higher than the dose–response range of rHuIL-15 and does not interfere with the measurement of rHuIL-15 activity under the specified assay conditions (data not shown).

A valid comparison of two different preparations of the same protein requires that they behave identically in the bioassay. Reproducibility is also a critical element (Mire-Sluis et al., 1995; Meager, 2005). The comparison of the same Lot of rHuIL-15 in the same plate, using it for both the standard and test article is an ideal case to establish the assay validity as it is analogous to running the same protein preparation in the same plate by the same operator under identical conditions. The results from a large number of experiments where the same material is run on intra-plate or inter-plate on the same day show comparable results.

Table 3A
Four-parameter curve-fit coefficients of IL-15 dependent CTLL-2 proliferation data generated on three different days.

Expt. # [a]			Four-parameter Curve-Fit Parameters				
			A	B	C	D	R^2
1 [Passage #4 (95%)]	Plate #1	Set I	0.19	3.23	0.110	1.78	0.997
		Set II	0.20	3.42	0.112	1.77	0.996
	Plate #2	Set I	0.20	4.02	0.110	1.64	0.996
		Set II	0.13	3.51	0.110	1.65	0.993
	Plate #3	Set I	0.22	3.61	0.111	1.69	0.994
		Set II	0.18	3.34	0.110	1.69	0.996
2 [Passage #5 (97%)]	Plate #4	Set I	0.20	3.41	0.109	1.91	0.997
		Set II	0.19	3.62	0.106	1.88	0.997
	Plate #5	Set I	0.21	3.47	0.107	1.81	0.995
		Set II	0.21	2.98	0.109	1.86	0.992
	Plate #6	Set I	0.19	3.62	0.109	1.86	0.997
		Set II	0.22	3.68	0.108	1.83	0.996
3 [Passage #6 (96%)]	Plate #7	Set I	0.18	2.80	0.092	1.83	0.994
		Set II	0.20	3.66	0.087	1.69	0.996
	Plate #8	Set I	0.16	2.67	0.089	1.78	0.994
		Set II	0.16	2.83	0.085	1.75	0.993
	Plate #9	Set I	0.18	3.34	0.086	1.71	0.995
		Set II	0.19	3.37	0.087	1.72	0.997
Mean Value			0.19	3.37	0.102	1.77	
S.D.			0.02	0.35	0.011	0.08	
CV%			8.29	10.40	10.43	4.64	

The A, B, C, and D parameters are defined in the data analysis section. R^2 is the square of the sample correlation coefficient between the outcomes and their predicted values.
Assay is performed in three different plates on each day, six different wells in each plate. The data set in the six replicates are divided into two sets (Set I and Set II) of triplicates. The variations in Set I and Set II of each plate indicates the regional effect or intra-plate variation.
[a] Cell passage # and cell viability are also shown in parentheses.

For establishment of the assay precision, with respect to absolute activity defined by ED_{50} or units/mg or International Units/mg, one should look at the degree of reproducibility; i.e. consistency of the agreement of repeated values which is usually expressed as CV%. The CV% for the estimated ED_{50} values for the initial reference material from fifty five different experiments was ~12.3%. Collective studies by WHO have shown that for the biological assay, absolute values generally show much wider distribution (variation) than the relative values compared to a reference standard (Mire-Sluis et al., 1995; Meager, 2005). Parallelism in the linear dose–response curves is a critical requirement for establishing similarity and

Table 3B
Comparison of the Intra-plate (Set I: Set II) curve-fit using the Script 3, the 'Formal Tests for Significance between Two Data Sets Script'.

Parameter	Day 1			Day 2			Day 3		
	P1	P2	P3	P4	P5	P6	P7	P8	P9
A	0.62	0.62	0.22	0.44	1.00	0.72	0.47	0.99	0.69
B	0.54	0.26	0.82	0.43	0.23	0.87	0.008 *	0.65	0.93
C	0.55	0.97	0.55	0.21	0.50	0.60	0.075	0.33	0.67
D	0.89	0.86	0.50	0.38	0.40	0.35	0.001 *	0.59	0.93

Experiment was performed on three different days. Each day same Lot of rHuIL-15 was run in three different plates and each plate had two sets (Set1 and Set 2) of triplicate wells. P1–P9 refers to plate numbers 1–9. *p* values correspond to comparison of data sets of Set 1 and Set 2 within the same plate. *p* values are commuted using the statistical program of Script 3, the 'Formal Tests for Significance Between Two Data Sets Script'.
* *p* values <0.05.

comparability of different product lots. In most cases where the dose response is sigmoid and log–dose response curves are used, the establishment of curve parallelism is a difficult task. For rHuIL-15, there is an apparent linear response range (covering the ED_{10}–ED_{80} response region) and the slopes of these linear response curves were comparable for the same lot of material run in the same plate as well as inter-plate within day analysis. A similar observation was made for operator to operator consistency (same day using same lot). These results established parallelism in the linear response range and suggested that the linear response curve could be used for measurement of the relative activity of two lots. The linear dose response curve consistency can be further improved by optimization of the dose response curve concentration and/or dilution by adding more points in the linear range and cutting of the asymptote region. The assay described here is designed to use a four-parameter non-linear response curve fit and the data analyses and statistical evaluation are focused on that.

For establishment of the validity of the assay results, consistency in the obtained estimates of the assay parameters is critical. In order to gain full appreciation of the curve fit comparability it is desirable to define the assay variations in all four parameters. Script 2, the 'Confidence Intervals Determination and Inferences for Multiple Data Sets Scrip' and Script 3, the 'Formal Tests for Significance Between Two Data Sets Script' enable one to estimate the individual parameter estimates along with their upper and lower 95% CIs. The data analyses performed established statistical equivalence for the 95% CIs in all four parameter estimates when the same preparation was tested in the same plate by the same operator under identical conditions. This was also true for the same sample run on the same day, by the same operator on different plates (inter-plate within day consistencies, Fig. 4). Similarly, statistical equivalence for the 95% CIs was also detected for the same sample run by different operators on the same day (Fig. 5B). The consistency in the four parameter estimates for the same sample run in different sets in the same plate under identical conditions may be regarded as proof for the validity of the assay for establishing product equivalence. This could be used as criteria for establishment of product equivalence analogous to the use of parallelism for linear dose response curves. Script 3 computes a formal statistical test of significance for all parameter estimates. The script can be modified to set the confidence interval limits to any confidence level and would enable one to set an assay acceptance criteria based on pre-set confidence intervals. In this example, a p value ≤ 0.05 generally means statistically significant differences in the parameter estimates for the data sets being compared whereas a $p > 0.05$ suggests no statistically significant difference (at the 95% confidence level) in the parameter estimates for the two data sets being compared. In all the experiments comparing intra-plate or inter-plate within day, in general, the p values for the four parameter estimates for two identical sample sets were > 0.05 (Table 3B). There were a few exceptions in which the p values were < 0.05, suggesting the possibility of an assay failure or experimental (operator) error. These statistical methods for data set comparison help us identify situations where assay failure may occur, and thereby enabling one to repeat an assay to obtain valid test results by presetting rules governing the investigation of Out-of-Specification results.

A number of different rHuIL-15 preparations produced by different methods during the early phase of process development were tested for biological activity (Table 2). The different preparations showed differences in the absolute activity as well as relative activity compared to a reference lot. Data analysis using scripts 2 and 3 showed statistical equivalence in the A, B, and D parameters (not shown). This consistency in A, B, and D parameter estimates within 95% CIs may be considered as an alternative test for curve parallelism to establish product comparability. The only parameter estimate that showed statistically significant differences (non-overlapping 95% CIs) was the C parameter that defines the absolute activity or ED_{50}. This difference in ED_{50} value paralleled the difference in relative activity computed from extrapolation of absorbance readings of the test lot dilutions to the standard curve limited to the linear response range (Fig. 3A). Process changes upstream and downstream that resulted in differences in the degree of refolding, post-translational changes such as amino acid substitution, differences in oxidation and deamidation as detected by analytical characterization were demonstrated (not shown). Any biological assay used for product release should also be capable of monitoring the stability of product during storage. The results from analysis of heat stressed samples indicate that the CTLL-2 cell proliferation assay could be used to monitor stability side by side with stability indicating physico-chemical assays such as analytical SEC.

The three new scripts, in particular Scripts 2 and 3, provide us with a novel approach of data analysis by providing us tools to perform more sophisticated statistical tests in order to determine product comparability, product equivalence and assay validity.

Another area of high interest is the applicability of the methodology for estimation of the cytokine in body fluids such as serum, plasma, etc., in pre-clinical and clinical samples. This is an area that needs to be explored separately with emphasis on assay interference by other cellular components in the body fluid. The presence of any neutralizing anti-IL-15 may function as inhibitors by effective complexion with IL-15. Similarly, the presence of other cytokines such as lL-2, IL-6, TGFβ1, TGFβ2, etc., may interfere with CTLL-2 proliferation by inducing proliferation response (Khatri et al., 2007; Meager 2005). The potential interfering components have to be identified and sample treatment procedures need to be developed to avoid or minimize the interference. The interference from other cytokines obviously depends on the dose response range and concentration level in the body fluid. The concentration of many of the responsive cytokines in the blood samples are less than 1 pg/ml (Khatri et al., 2007) except under exaggerated immune responses and in certain treatment conditions. Apart from the interference from other cytokines, antibodies and cytokine inhibitors in the body fluids may also interfere with the cell proliferation. In addition, the cell line characteristic associated changes in the proliferation response, is an important factor to be considered and controlled.

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Inquiries or questions regarding the Statistical Scripts used in the data analysis may be directed to Dr. W. Gregory Alvord, Director of Statistical Consulting Services, Data Management Systems, NCI Frederick, Frederick, MD 21702, Telephone 301-846-5101, e-mail: greg.alvord@nih.gov.

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