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A new bioassay for the immunocytokine L19-IL2 for simultaneous analysis of both functional moieties

Jonas Winter[a,*], Karin Barbin[a], Camilla Bacci[b], Thomas Bunte[a]

[a] Analytical Development, Bayer Schering Pharma AG, Müllerstr. 178, 13353 Berlin, Germany
[b] Philogen S.p.A, Località Montarioso 11, Monteriggioni (Si), Italy

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ABSTRACT

Currently, cancer directed new biological entities (NBEs) in the pharmaceutical R&D pipelines are derived from monoclonal antibodies in various formats, such as immunocytokines. Generally, immunocytokines are bi-functional molecules that consist of a specific targeting antibody-based portion and a linked cytokine. To confirm the quality of the drug product both moieties have to be characterized using appropriate techniques. Until now, the binding capacity of antibodies is usually examined by ligand binding assays whereas the biological activity of the linked cytokine is determined by cell-based potency assays. However, the simultaneous analysis of both functional moieties in a single assay format has not been described so far.

In this paper we present a newly designed bioassay format for the anti-cancer immunocytokine L19-IL2, comprising of the human vascular targeting single-chain Fv L19 and human interleukin 2 (IL2). This new potency assay allows simultaneous analysis of both moieties, thus specific L19 binding capacity and the ability of IL2 to induce the proliferation of the detector cytotoxic T-cell line CTLL-2.

Assay development was performed with special focus on application of different fitting models for the sigmoid dose–response curves to evaluate the influence of model optimization on the validity of assay results.

For assay validation generally accepted characteristics were determined. Assay specificity was shown by testing L19-IL2 related compounds. All other validation parameters were derived from 25 batch runs using five nominal L19-IL2 concentrations, covering a range from 60% to 140% of the standard's potency. Accuracy ranged from −3.4% to −6.9% relative error (%RE). Interbatch precision ranged from 6.1% to 10.6% coefficient of variation (%CV). For assay linearity a coefficient of determination (R^2) of 0.9992 was found. Assay robustness was shown with L19-IL2 samples after three freeze–thaw cycles and also with different cell passages of the used cytotoxic T-cell line.

Based on the data, we conclude that this assay is valid for potency estimation of the immunocytokine L19-IL2. Moreover, this format represents a major improvement compared to other approaches which only allow potency evaluation of both functional moieties in separate assays. In general the underlying assay principle described seems suitable for potency determination of other immunocytokines.

1. Introduction

The first monoclonal antibodies (mAbs) from mice had been generated in 1975 [1] and have been studied in the clinic for nearly three decades now. Owing to the ability of these agents to selectively target tumor-associated antigens, cancer has been a major focus of development programs for unmodified mAbs and modified mAbs such as immunoconjugates [2,3]. Immunoconjugates are bi-functional molecules that combine the specificity of mAbs or antibody fragments to antigens with the extraordinary potency of a conjugated moiety, which can be a small molecular drug, a protein toxin, a radioisotope or a cytokine [4,5]. Antibody–cytokine immunoconjugates, designated immunocytokines, have become of increasing interest for tumor immunotherapy, since they direct immunomodulatory cytokines into the tumor microenvironment [6,7]. L19-IL2 is a promising immunocytokine, currently under clinical testing. It comprises of the targeting antibody L19 fused via a peptide linker to human IL2. The human single-chain Fv (scFv) L19 is highly specific for the tumor extracellular matrix (ECM) component Extra Domain-B (EDB) of fibronectin [8], a marker of angiogenesis [9]. Due to the ability of L19 to bind EDB, L19-IL2 mediates the selective delivery and concentration of IL2 to tumor vasculature, thereby leading to a dramatic enhancement of the therapeutic effects of the cytokine [10,11].

* Corresponding author. Tel.: +49 30468193746; fax: +49 30468192457.
E-mail address: jonas.winter@bayerhealthcare.com (J. Winter).

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Assessment of the biological properties of L19-IL2 is necessary to ensure safety and efficacy of the product. An important property is the biological activity that describes the specific ability of a product to achieve a defined biological effect. The biological activity of L19-IL2 is based on the functional activity of both moieties of the immunocytokine, thus L19 binding capacity and IL2-mediated activation of effector cells. For the determination of the biological activity it is therefore essential to develop a bioassay which considers both moieties of L19-IL2.

However, such an assay has not been described so far. To date, the ability of antibodies to bind to its antigen is usually analyzed in ligand binding assays. In contrast to that the biological activity of cytokines can be determined in cell-based potency assays that allow the determination of cytokine mediated effects on cells, including stimulation of cell proliferation, cytotoxicity/apoptosis, antiviral activity as well as up-regulation of surface membrane proteins [12]. Several bioassays for the determination of IL2 activity have been described, which measure IL2 dependent proliferation of different cell lines including HT-2 (murine T-helper cell-derived) [13], Kit-225 (human T-cell chronic lymphocytic leukemia-derived) [14], NKC3 (murine natural killer cell line) [15] as well as the most commonly used T-cell line CTLL-2 [16–18]. However, such assays are unsuitable for the determination of the biological activity of immunocytokines, because they are limited to the evaluation of IL2 activity and do not consider that the function of an immunocytokine also depends on the ability of the antibody portion to bind to the respective antigen. As all conventional IL2-assay formats are unable to distinguish whether the IL2 activity is mediated by IL2 alone or by the complete immunocytokine the true potency of the immunocytokine may not determined correctly.

In order to overcome this problem and to address the true biological properties of L19-IL2 it is necessary to analyze both functional moieties of the immunocytokine simultaneously. Here, we present a new valid cell-based potency assay for L19-IL2, allowing the simultaneous analysis of both functional moieties in one assay format.

2. Materials and methods

2.1. Cells and culture conditions

For all experiments the murine cytotoxic T-cell line CTLL-2 purchased from ATCC was used. These cells are dependent upon IL2 for growth, and can be used to assay for IL2 activity. Cells were cultured in complete medium, containing RPMI 1640 + L-glutamine–$NaHCO_3$ (GIBCO, Grand Island, NY, USA), supplemented with L-glutamine (2 nM, GIBCO, Grand Island, NY, USA), sodium pyruvate (2 nM, GIBCO, Grand Island, NY, USA), sodium bicarbonate (0.15%, GIBCO, Grand Island, NY, USA), HEPES (pH 7.4, 10 nM, Sigma–Aldrich, St. Louis, MO, USA), d-glucose (0.25%, GIBCO, Grand Island, NY, USA), and adjusted to a final concentration of 10% fetal bovine serum (Hyclone, Logan, UT, USA). IL2 (R&D Systems, Minneapolis, MN, USA) was added to a final concentration of 4 ng/ml to obtain a complete medium. Cells were seeded at a density of 5×10^3 to 1.5×10^4 cells/ml in a T-25 flask (Corning, NY, USA) and incubated in a humidified incubator at 37 °C, 5% CO_2. Subcultivating was done at a cell density of approximately 2×10^5 cells/ml three times a week.

2.2. Reagents

L19-IL2 comprises of the scFv L19, which is specific for the EDB domain of fibronectin and human IL2 and has been described in detail previously [10]. For all experiments an in-house standard of L19-IL2 manufactured by Philogen S.p.A. (Siena, Italy) was used. Biological activity of L19-IL2 with regard to the conjugated IL2 was almost identical compared to the activity of the rDNA derived International Standard for human IL2 (code: 86/564, NIBSC, Potters Bar, UK), which was shown in an IL2 potency assay as described elsewhere [10].

For specificity experiments L19-SIP was used, comprising of two L19 scFv fragments fused by the CH4 domain of human IgE, which mediates its homodimerization [19,20]. The unlabeled scFv AP39 is a genetically modified derivative of L19 that includes a C-terminal $(Gly)_3$-Cys-Ala sequence. AP39 is also specific for EDB and has been characterized recently [21]. Moreover, recombinant human IL2 (R&D Systems, Minneapolis, MN, USA) was used for cell culturing and assay control. The protein 7B89 was used as antigen for the antibody portion of all scFv constructs. This protein consisting of EDB and its surrounding domains 7, 8 and 9 was produced in *E. coli* strain M15 harboring an expression plasmid for 7B89. Bacteria were disrupted and the 7B89 protein was purified via its C-terminal hexahistidine tag by immobilized metal affinity chromatography (IMAC). In addition, biotinylation of this 7B89 fragment was performed using biotin-ε aminocaproic ester (La Roche AG, Basel, Switzerland) in accordance with the manufacturer's protocol.

2.3. Solid phase potency assay

The L19-IL2 solid phase potency assay was performed on a 96-well plate. Only the inner 60 wells were used and 150 μl of phosphate buffered saline (PBS) were added to edge wells to reduce evaporation effects.

The biotinylated antigen 7B89 was diluted to a concentration of 1000 ng/ml in PBS, containing 0.05% Tween 20 (PBST) and 50 μl of this solution was transferred to each well of a flat bottom streptavidin-coated 96-well plate (Immobilizer F96 Micro Well Plate, Nunc, Langenselbold, Germany).

L19-IL2 standard preparations were also diluted in PBST to obtain a dose–response curve with nine calibrator concentrations. For this purpose L19-IL2 stock solution was diluted to a concentration of 170 ng/ml. In addition, 7 twofold dilutions and a final sixfold dilution step were performed and 50 μl of each L19-IL2 calibrator concentration was transferred in duplicate to the plate and incubated together with 7B89 dilution on a rotary shaker (900 rpm) for 2 h.

The plate was washed twice with PBST and complete medium without IL2, respectively. Then, 50 μl of complete medium without IL2 was added to each well. An equal volume of CTLL-2 cells, which had been washed in medium without IL2 and adjusted to a density of 6×10^5 to 8×10^5 cells/ml was added and mixed very carefully afterwards. This led to a final cell density of approximately 3×10^4 to 4×10^4 cells/well. For control purpose three wells, which only contained 7B89 and cells but no L19-IL2 served as blank value. Moreover, three wells, which contained 50 μl of medium with 8 ng/ml IL2 served as an estimate of the maximum proliferation of the indicator cells. The plate was incubated in a humidified incubator at 37 °C, 5% CO_2 for 20 h. After adding 20 μl CellTiter 96® AQueous One Solution (Promega, Madison, USA) the plate was incubated for additional 4 h at 37 °C, 5% CO_2. Following intensive shaking to homogenize the reaction solution and cells in the wells, absorbance at a wavelength of 490 nm was measured using the multi-spectrometer KC4 Power Wave X Select (BIO-TEK Instruments Inc, Winooski, VT, USA).

2.4. Dose–response curve analysis

In order to evaluate competing fitting models of the dose–response curve, 16 assays were performed as described above, using nine L19-IL2 calibrator concentrations in duplicate. The resulting dose–response values, covering the proliferative

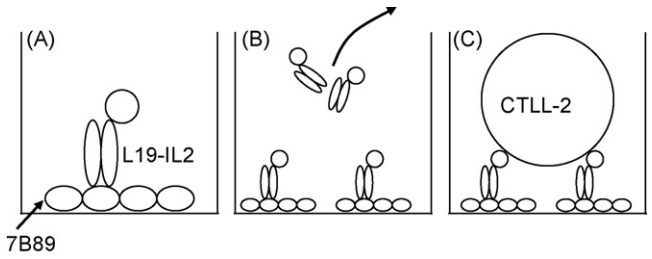

Fig. 1. Schematically illustrated principle of the solid phase potency assay for the immunocytokine L19-IL2. (A) L19-IL2 binds its antigen (7B89). (B) Unbound protein is removed by washing. (C) CTLL-2 cells are added to the wells and incubated for 24 h. The read-out is performed using the colorimetric cell proliferation assay CellTiter 96® AQueous One Solution.

active range of CTLL-2 cells were fitted with a four-parameter logistic model (4PL) and a five-parameter logistic model (5PL) using PLA 2.0 software (Stegmann Systems GmbH, Rodgau, Germany).

2.5. Data processing

Relative potency values of L19-IL2 sample preparations were calculated against the in-house reference standard and were assayed as described above. Resulting dose–response data were fitted with different fitting models using PLA 2.0 software which fulfills the requirement of the European Pharmacopoeia. Validity of each assay was evaluated by the analysis of variances (*F*-test) to show the significance of slope as well as significant deviation of non-linearity and non-parallelism, respectively. For assay acceptance calculated probability (p) had to fulfill the following criteria: test for slope ($p < 0.05$) test of non-linearity ($p > 0.05$) and test of non-parallelism ($p > 0.05$).

3. Results and discussion

3.1. Assay design

The method includes three main steps, the first of which represents binding of the scFv L19 to its antigen 7B89 and a second washing step that removes non-binding antibody fragments. Until this step the assay is quite similar to widely used ligand binding assays. However, the biological activity of L19-IL2 with regard to the conjugated IL2 is then tested in a third step. For this purpose CTLL-2 cells are added to the wells and their viability is measured after an incubation period of 24 h using the colorimetric cell proliferation assay CellTiter 96® AQueous One Solution (Fig. 1). The solid phase potency assay for L19-IL2 represents a major improvement in bioassay design for immunocytokines as it combines a binding assay for the antibody portion L19 and a cell-based potency assay for IL2, thereby allowing simultaneous analysis of both functional moieties.

3.2. Dose–response relationship and curve fitting

The use of nine calibrator concentrations of L19-IL2 covering a range from 0.2 ng/ml to 170 ng/ml resulted in a sigmoid dose–response curve on a log scale, which is characteristic for bioassays [22,23]. To identify the best fitting algorithm, the mean response values were fitted using a four-parameter logistic model (4PL) and a five-parameter logistic model (5PL), respectively. In both cases the coefficient of determination (R^2) for all 16 fitted calibration curves was >0.99. However, the R^2 is not per se a measure of goodness-of-fit, because it is possible to have a high R^2 and yet unacceptable bias [24,25]. Indeed, the 4PL fitted curve showed deviation from measured values in some parts of the curve, whereas the

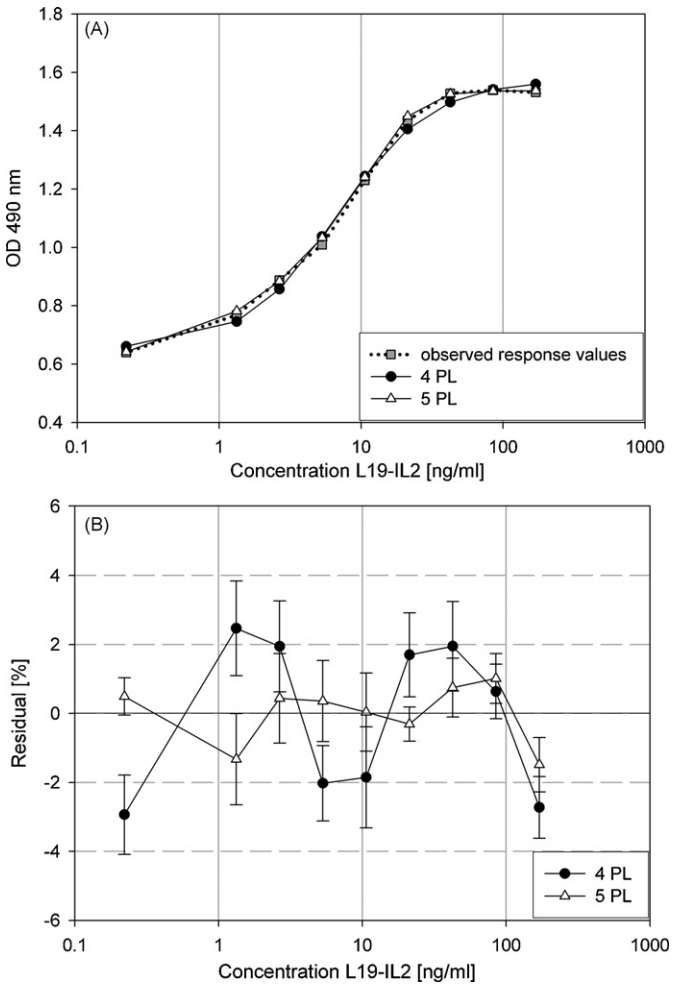

Fig. 2. Comparison of different standard curve fitting models. (A) One representative of 16 dose–response curves with nine L19-IL2 calibrator concentrations. Regression curves for a four-parameter logistic model (4PL) and a five-parameter logistic model (5PL) are depicted. (B) Percent difference (residuals) between the observed calibrator response values and values predicted by the 4PL and 5PL, respectively. Error bars indicate the standard deviation of 16 standard curves.

five-parameter curve was almost superimposable with the measured values (Fig. 2A). Therefore, the residuals between observed response values and predicted values were used to evaluate the different fitting models more appropriately. Over the whole L19-IL2 concentration range the 5PL fitted curves displayed residuals ranging from −1% to 1%, whereas residuals of 4PL fitted curves ranged from −3% to 3%, showing that the 5PL fits the measured response values more precisely (Fig. 2B). Moreover, the residuals of the 4PL showed a specific wavelike pattern over the concentration range of the analyte. This deviation from homogeneous pattern around zero can be understood as an additional indicator for lack-of-fit of the model [26]. Taken together, the residual plot showed, that the addition of a fifth parameter helped to optimize fitting, which is often the case when asymmetry of the calibration curve is observed [27]. Consequently, the 5PL fit was used for further investigations and the determination of accuracy and precision.

3.3. Specificity

Specificity of the assay format was demonstrated using different immunoproteins or unconjugated IL2, respectively. Both, the small immunoprotein L19-SIP and AP39 include the same targeting scFv as L19-IL2 but lack IL2. Using high, equimolar amounts (4000 pM) of these three proteins, viability of L19-SIP and AP39

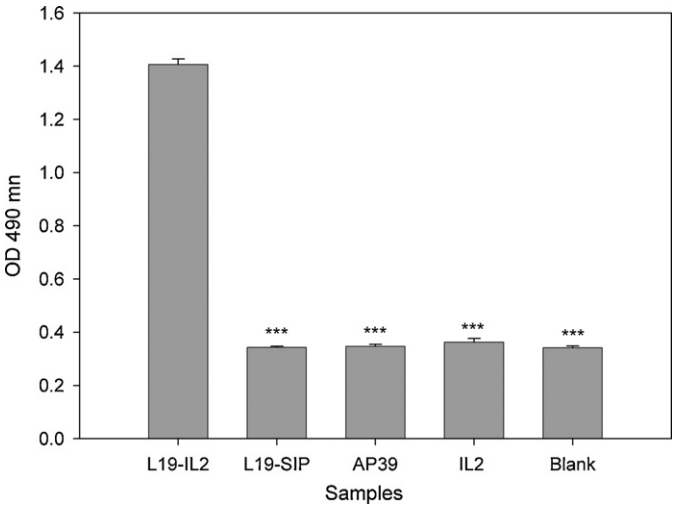

Fig. 3. CTLL-2 viability after 24 h stimulation with 4000 pM L19-IL2 or equimolar amounts of IL2, L19-SIP and AP39 or complete medium without IL2 (blank). Viability of cells treated with L19-SIP, AP39 and IL2 were comparable to blank values and significant lower compared to L19-IL2, thereby demonstrating assay specificity. Error bars indicate the standard deviation of six replicates. Asterisks (***) denote significant difference of values vs. L19-IL2 in paired *t*-test ($p < 0.001$).

treated cells were comparable to blank values and significant lower compared to L19-IL2 ($p < 0.001$ vs. L19-IL2; paired *t*-test). Furthermore, no proliferative effect was observed when cells were treated with unconjugated IL2 alone (Fig. 3). From these results we conclude that the assay measures specifically the potency of the intact immunocytokine L19-IL2 consisting of the antibody fragment L19 and the conjugated cytokine IL2. This is a major advantage compared to former IL2 bioassays, which cannot distinguish between IL2 bioactivity mediated by integer immunocytokines or IL2 alone.

3.4. Precision, accuracy and linearity

In order to estimate accuracy and precision of the assay, L19-IL2 standard was diluted to final concentrations with nominal relative potencies of 60%, 80%, 100%, 120%, and 140%. These samples were assayed as if they contain an equal amount of L19-IL2 compared to the standard preparation. Each nominal potency was tested in five separate batch runs on different days. Within each batch run samples were independently tested twice. This experimental design led to ten independent potency determinations for each relative nominal potency level.

In order to calculate intrabatch precision for each nominal relative potency level (e.g. 100%), an analysis of variance (ANOVA) was performed. The intrabatch standard deviation was given by the square root of the intrabatch variance component obtained in the ANOVA.

Interbatch precision was estimated by the standard deviation of all ten potency values from the total mean of the five batch runs with the same nominal potency (e.g. 100%). In both cases precision was reported as percent coefficient of variation (%CV) by dividing the respective standard deviation by the nominal potency value and multiplying the quotient by 100. As an example of precision calculation, results of samples with a nominal potency of 100% are shown in Table 1. In these runs intrabatch standard deviation was 5.8 and the overall standard deviation was 7.0 which resulted in an intrabatch precision of 5.8%CV and an interbatch precision of 7.0%CV. Intrabatch and interbatch precision for all runs summarized in Table 2 ranged from 1.0 to 8.6%CV and 6.1 to 10.6%CV, respectively. All values were smaller then the widely accepted limit for bioanalytical methods of 20%CV [28–30] and were even lower than the acceptance limit of 15%CV suggested by the FDA Guidance for Industry [31]. Thus, we state that the method is precise over the whole anticipated assay range from 60% to 140%.

Table 1
Determination of intrabatch and interbatch precision reported as percent coefficient of variation (%CV) as well as accuracy expressed as percent relative error (%RE) of the L19-IL2 solid phase potency assay in five batch runs.

Batch run	Replicate results		Statistics			
	1	2	Total mean	SD	%CV	%RE
1	91.8	95.3				
2	84.2	87.3				
3	91.2	96.8				
4	108.0	93.9				
5	94.1	103.2				
Intrabatch (within run) ANOVA			94.6	5.8	5.8	
Interbatch (between run)			94.6	7.0	7.0	−5.4

Replicate results represents estimated potency values for samples diluted to a nominal potency of 100%.

Accuracy for each nominal relative potency level (e.g. 100%) was determined by the difference between the observed total mean of the five batch runs with the same nominal potency and the respective nominal value (equal to 100% in Table 1). It was expressed as percent relative error (%RE) and determined by subtracting the nominal potency from the total mean and dividing the difference by the nominal potency. This term was then multiplied by 100. The %RE was low with a range from −3.4 to −6.9 (Table 2). Although, the assay tended to somewhat underestimate the nominal potencies in some batch runs, all obtained %RE values fall under the limit of 20%RE suggested in published literature in the field of bioassay development [22,23,29,30], thereby showing that the assay gives accurate results.

Linearity of the assay was evaluated by focusing on the relationship between estimated potency values and nominal potency values. An excellent linearity was demonstrated over the anticipated assay range from 60% to 140% with a R^2 value of 0.9992 for the linear regression line (Fig. 4).

As an overall benchmark for assay error the total error was calculated by the addition of the systematic error (mean bias) and the random error (precision) [23]. A limit of 30%RE has been reported to be acceptable for bioanalytical methods [22,32]. Total error values of the described assay ranged from 12.4% to 17.5%,

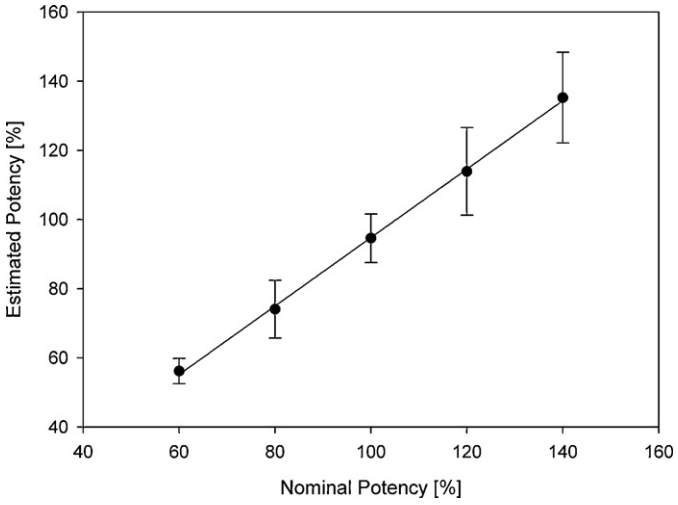

Fig. 4. Assay linearity in a range from 60% to 140% relative potency of a reference standard. The coefficient of determination (R^2) for the linear regression between estimated potency values and nominal potency values was 0.9992 with a slope of 0.9903 and an *y*-intercept of −4.3. Error bars indicate the standard deviation between five batch runs.

Table 2
Summary table of intrabatch and interbatch precision reported as percent coefficient of variation (%CV), accuracy expressed as percent relative error (%RE) and total error (%RE) of the assay.

Characteristic	Statistic	Nominal potencies (%)				
		60	80	100	120	140
Accuracy	Mean bias (%RE)	−6.4	−5.1	−5.4	−6.9	−3.4
Precision	Intrabatch (%CV)	1.0	7.5	5.8	8.6	6.1
	Interbatch (%CV)	6.1	10.4	7.0	10.6	9.4
Total error	IAccuracyI + Interbatch (%RE)	12.5	15.5	12.4	17.5	12.8

Each of the five samples (60–140%) was analysed in five separate batch runs.

Table 3
Results obtained from assays using CTLL-2 cells with different passage numbers and L19-IL2 samples after three freeze–thaw cycles (FT) at −20 °C and −80 °C, respectively.

Sample	Replicate results		Mean EC_{50} (ng/ml)
	1	2	
Passage 142/149	94.7	96.4	18.6
Passage 83/90	97.1	102.9	6.6
Passage 56/63	91.8	100.6	13.0
Passage 17/24	109.0	100.4	9.8
FT −20 °C	99.7	93.3	14.0
FT −80 °C	91.4	96.1	12.8

Replicate results represent estimated potency values for samples diluted to a nominal potency of 100%. EC_{50} values are calculated on the basis of the respective standard curves.

emphasizing the excellent accuracy and precision of the method (Table 2).

Taken together, we conclude that the described assay gives precise and reliable results for the relative potency assessment of L19-IL2.

3.5. Robustness/stability of the L19-IL2

Bioassays are subject to many sources of variability. The response of an in vitro bioassay may be affected by differences in, e.g., batches of media, speed of reagent additions, shear forces during mixing or age of cells [33]. For this reason the influence of cell passage numbers ranging from 17 to 149 of the used CTLL-2 cell line on assay results was tested. Solid phase potency assays were performed at two different days using sample preparations with nominal relative potencies of 100%. The shape of the obtained standard dose–response curves differed from one another resulting in EC_{50} values, ranging from 6.6 ng/ml to 18.6 ng/ml L19-IL2 depending on the age of the cells. Nevertheless, standard and sample curves within each assay run were superimposable, leading to potency values of 91.8 to 109.0% (Table 3). Thus, although absolute response values were influenced by the age of cells, relative potency values were determined accurately. Furthermore, this example highlights the advantage of relative potency estimation of a sample preparation relative to a standard, instead of reporting absolute values like the EC_{50} which are much more affected by different sources of assay variability.

To analyze if L19-IL2 stored in assay buffer at −20 °C or −80 °C can be used without a significant loss in activity after repeated freeze–thaw cycles, stability samples were diluted to a nominal potency of 100% and compared to freshly prepared standard in two independent assay runs. The relative potencies of the −20 °C and -80 °C samples ranged from 93.3 to 99.7 and 91.4% to 96.1%, respectively (Table 3). For all assays performed, using different cell passage numbers and temperature stressed samples, potency results were within the total error of the method (Table 2), showing that the assay is robust with regard to the age of cells and stability of the L19-IL2 standard.

3.6. Validity of potency estimations

F-tests were performed for slope, non-linearity and non-parallelism in order to evaluate the validity of assay results. In all assays the test for slope was passed. However, only 23 of 50 assays passed the test of non-linearity, if the 4PL was applied (Table 4). In contrast, 90% of all assays passed, if the 5PL was used, reflecting the minor lack-of-fit pattern of the 5PL and emphasizing the importance to choose an appropriate fitting model. If all assays were analyzed as typical three dose parallel-line assays almost all tests passed. However, in contrast to the 4PL and 5PL this analysis is limited to the linear part of the dose–response curves and does not take into account important regions of the curves, e.g. the asymptotes, therefore providing only a poor description of the inherently nonlinear dose–response relationship of a bioassay [28].

Similarity between standard and sample is a fundamental requirement for valid potency estimation and can be examined by testing the deviation from generalized parallelism [34,35]. The test of parallelism revealed no differences with regard to assay validity in dependence of the used 4PL or 5PL at the same *p*-level. Only by reducing probability for non-parallelism testing from $p > 0.05$ to >0.02 assay acceptance was improved from 88% to 96% for the 5PL. A likely reason for *F*-test failure of some assays is marginal variability between measured replicate response values. The fact that perfectly acceptable assay results may fail due to good precision and that obviously faulty assay results may pass due to poor preci-

Table 4
Number of accepted, valid assays after test for slope, non-linearity and non-parallelism with different probabilities (p) of the performed *F*-tests.

Model	Probability (p)	*F*-test		
		Slope	Non-linearity	Non-parallelism
4PL	0.05	50 of 50 passed (100%)	23 of 50 passed (46%)	44 of 50 passed (88%)
5PL	0.05	50 of 50 passed (100%)	45 of 50 passed (90%)	44 of 50 passed (88%)
5PL	0.02	50 of 50 passed (100%)	49 of 50 passed (98%)	48 of 50 passed (96%)
PLA	0.05	50 of 50 passed (100%)	47 of 50 passed (94%)	50 of 50 passed (100%)

Different models for data fitting and potency estimation were compared: four-parameter logistic model (4PL), five-parameter logistic model (5PL) and parallel-line model (PLA).

sion because of the used statistical technique (*F*-test) was pointed out recently by the revision committee of chapter ⟨1 1 1⟩ of the US Pharmacopeia [36]. One possible approach, which is currently discussed to overcome this problem is equivalence testing allowing negligible degree of non-parallelism, because it proposes different statistical hypothesis. Instead of the null hypothesis of parallelism and the alternative hypothesis of non-parallelism used in the *F*-test, equivalence testing proposes non-parallelism as the null hypothesis and "sufficiently parallel" as alternative statistical hypothesis. Thus, this approach takes into account that the parameter of standard and sample curves may differ by some specified amount but that this difference is trivial and that they may considered equivalent [33].

4. Conclusions

The solid phase potency assay for the L19-IL2 described here allows simultaneous analysis of both functional moieties of the immunocytokine, thus specific antigen binding of L19 and the ability of IL2 to induce T-cell proliferation. Moreover, the assay is highly specific as neither L19 nor IL2 alone induces a signal. An excellent linearity of the assay was observed over the anticipated range from 60% to 140% nominal potency of a reference standard. Within this range precision and accuracy met the acceptance criteria in accordance to the common guidelines. Furthermore, almost all assays were valid when analyzed with a 5PL. In addition, assay results were robust and comparable if high cell passage numbers or temperature stressed L19-IL2 samples were used. Taken together, we conclude that the described bioassay gives precise and reliable results for the relative potency assessment of L19-IL2 and that the assay principle may be adopted for other immunocytokines.

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