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A novel reporter gene assay for Recombinant Human Erythropoietin (rHuEPO) pharmaceutical products

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ABSTRACT

Accurate determination of in vitro biological activity of therapeutic erythropoietin is essential in quality control of recombinant human erythropoietin (rHuEPO) pharmaceutical products. However, most of currently-used methods leave much to be desired so that a simpler, quicker and more accurate method is urgently needed. The bioassay described here utilizes a sub clone of UT-7/epo cell line stably transfected with luciferase gene under the control of sis inducible element and interferon γ-activated sequence element promoter. Active erythropoietin could induce the expression of luciferase by signaling through the erythropoietin receptor and the dose-response curve showed good linearity, yielding a coefficient of determination of 0.99 or higher. The optimized assay was simpler with the operation completed within 24 h and more sensitive with EC_{50} being 0.077 IU/mL. The accuracy estimates ranged from 81.7% to 102.4%, and both intra-assay and inter-assay precision was below 15.0%. The robustness of the assay was demonstrated by no effect of passage levels of the cells on the performance of the assay (*p* values: 0.772 for sample 1 and 0.943 for sample 2). Besides, Bland–Altman analysis showed a high consistency of the new assay with in vivo reticulocyte assay in results. These results suggested that the new reporter gene assay can be a viable supplement to the traditional reticulocyte assay and employed in potency determination of rHuEPO pharmaceutical products.

1. Introduction

Erythropoietin (EPO) is mainly secreted by the kidney and involved in the growth and maturation of erythroid cells from precursors. Decreased bioactive production of EPO could result in anemia [1–3]. Since recombinant human erythropoietin (rHuEPO) was successfully developed in the 1980s, it has been widely used in the treatment of anemia caused by chronic kidney disease, blood loss anemia, and myelodysplasia induced by chemoradiotherapy of cancer as the first hematopoietic growth factor applied clinically [4–7].

Accurate determination of in vivo and in vitro biological activities of therapeutic EPO is crucial to quality control of rHuEPO pharmaceutical products [8,9]. In vivo bioactivity of EPO is closely related to its serum half-life, which is dependent on carbohydrate structure, especially on the number of sialic acid residues at the termini of the tri- and tetra-antennary sugar chains [10,11]. Traditional ^{59}Fe incorporation assay is considered the gold standard assay for EPO, but the procedure has such drawbacks as the use of a radioisotope and elaborate animal preparation [8]. Recently, reticulocyte assay based on the proliferation of reticulocytes in normocythemic mice has been widely applied in standardization studies and potency evaluation of rHuEPO pharmaceutical preparations [12,13]. In vitro bioassay could estimate EPO's receptor-activating ability that is associated with the protein structure of EPO molecules [14]. The various in vitro bioassays that have been developed so far, including immunoassays, affinity assays and cell proliferation assays, have some limitations: immunoassays and affinity assays can confirm nothing more than antigenic sites and binding activities respectively while cell proliferation assays such as murine 32D cell line, NFS60, TF1, UT-7 and UT-7/EPO usually require extended experimental cycles [15–19]. Considering the huge market demand for rHuEPO products, a more accurate and convenient in vitro assay needs to be explored to facilitate the rapid batch release of rHuEPO formulations.

The reporter gene assay (RGA) has been increasingly used to determine the bioactivities of cytokines based on their

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individual signal pathway for its simplicity, reliability and high efficiency [20–23]. EPO signal pathway has been extensively explored over the past decades. It has been confirmed that EPO works by binding to its receptor EpoR which will undergo a conformational change that activates Janus kinase 2 (JAK2). The activated JAK2 will in turn activate tyrosine phosphorylation of signal transducer and activator of transcription 5 (STAT5) that will bind to the sis inducible element (SIE) and the interferon γ-activated sequence (GAS) element, activate the expression of downstream genes and finally promote erythropoiesis [24–26]. But till today, there has been no report on the potency determination of EPO based on its signal pathway using RGA.

In this report, a novel RGA for EPO was developed based on a UT-7/epo cell line stably transfected with luciferase gene under the control of SIE and GAS promoter. The new RGA was optimized and fully validated in accordance with the Guidelines of the International Conference on Harmonization (ICH) using good laboratory practices (GLPs), followed by comparison with in vivo reticulocyte assay in terms of results.

2. Materials and methods

2.1. Reagents

IMDM (12440), RPMI 1640 (11835) and fetal bovine serum (10099) were purchased from Gibco, Bright Glo luciferase assay reagent (E2620) from Promega, and hygromycin B (K547) from Amersco.

2.2. RHuEPO and other cytokines

National Standard for rHuEPO was obtained from National Standardization Study Center of Pharmaceutical and Biological Products. RHuEPO products and other cytokines including recombinant human growth hormone (rhGH), recombinant human epidermal growth factor(rhEGF), recombinant human basic fibroblast growth factor(rhBFGF), interferon α-2a(IFNα-2a), interleukin-11(IL-11), interleukin-2(IL-2), recombinant human granulocyte colony-stimulating factor(rhG-CSF) and recombinant human granulocyte-macrophage colony-stimulating factor(rhGM-CSF) were provided by different manufacturers. All rHuEPO products used in this study were produced in Chinese hamster ovary cells.

2.3. Construction of the reporter gene vector pGL4-SG-Luc

PGL4.26 vector (Promega) contains a minimal promoter followed by a luciferase gene. Three tandem repeats of consensus SIE (5′-GTCGACATTTCCCGTAAATC-3′) and GAS (5′-GTATTTCCCAGAAAAGGAAC-3′) were sequentially connected and inserted into the multiple cloning site of pGL4.26 vector and the positive clone was verified by DNA sequencing.

2.4. Development of UT-7-SG-Luc cells stably transfected with pGL4-SG-Luc

The plasmid pGL4-SG-Luc was introduced into UT-7/epo cells (ATCC) by electroporation. Cells were selected beginning at 48 h after transfection in selective media (IMDM containing 10% fetal bovine serum, 1 IU/mL EPO and 500 μg/mL hygromycin B). After about 4 weeks' selection, hygromycin-resistant cells were then cloned by limited dilutions to obtain single cell clones and screened for the induction of luciferase by treating cells with gradient concentrations of EPO. After that the positive clones were routinely maintained in selective media.

2.5. RGA procedure

UT-7-SG-Luc cells were washed three times by phosphate buffer solution and incubated at 37 °C with 5% CO_2 in assay medium (RPMI 1640 containing 5% fetal bovine serum and 10 mM HEPES) for 17–20 h. Cells were then collected and seeded into 96 well Costar plates, with 6×10^4 cells in the presence of rHuEPO standards or test samples in a total volume of 100 μL per well, followed by incubation at 37 °C with 5% CO_2 for 4 h. Then 100 μL of Promega Bright Glo Luciferase Assay reagent was added, and the plate was subsequently shaken for 5 min on a titre-plate shaker. Luciferase activity was finally determined by a Luminoscan Ascent plate reader.

2.6. Reticulocyte assay for EPO potency determination

Eight-week-old BALB/c mice were allocated to standard and sample groups in fully randomized order, with 5 mice per treatment group. The standard and test samples were diluted to appropriate concentrations with saline containing 0.1% bovine serum albumin. A single dose of 7.5, 15 or 30 IU EPO/0.2 mL per mouse was injected subcutaneously into the respective animal on day 1. On day 4, blood was taken from the orbital venous sinus of each mouse and reticulocytes were counted by the Sysmex R-500 Hematology Analyzer. The study was approved by the Ethic Committee of National Institute for Food and Drug Control.

2.7. Specificity

The specificity of the RGA was evaluated by determining whether the luciferase output could be affected by the presence of aggregated rHuEPO and other therapeutic cytokines including rhGH, rhEGF, rhBFGF, IFNα-2a, IL-11, IL-2, rhG-CSF and rhGM-CSF. The aggregated rHuEPOs were prepared by incubating rHuEPO samples at 90 °C for 1 h. All samples were diluted to gradient concentrations of 10, 2.5, 0.63, 0.16, 0.039, 0.0098, 0.0024 and 0.00061 μg/mL in assay medium, and then used to treat the cells under the same condition as described above for the analyses of rHuEPO. The levels of luciferase activity were compared to those obtained with intact rHuEPO treatment.

2.8. Bland–Altman analysis

The agreement between the results of RGA and reticulocyte assay was evaluated by a Bland-Altman plot. Thirty-four batches of qualified rHuEPO injections or powder injections that ranged from 2500 to 45,000 IU per vial were obtained from different manufacturers. Both assays were performed on each sample, and the relative potency was calculated respectively, resulting in 68 data points. Each of the 34 samples was then represented on the graph by assigning the log-transformed average of two measurements as the abscissa (x-axis) value, and the ratio of RGA vs. reticulocyte assay as the ordinate (y-axis) value.

2.9. Statistical analyses

Data analyses of reticulocyte assays were carried out according to the protocol of Chinese Pharmacopeias by parallel line methods. A four-parameter logistic model (Section 5.3 of European Pharmacopoeia) was used to calculate the relative potency, dose response and linear range for RGA. Statistical techniques for method validation involved the coefficient of variation (CV), recovery rate, ANOVA and Bland–Altman plot. Analyses were carried out using Sigmaplot 12.0 for dose response and linear range determination, SoftMax Pro software (Molecular Devices, USA) for relative potency estimation and SPSS 19.0 for method validation.

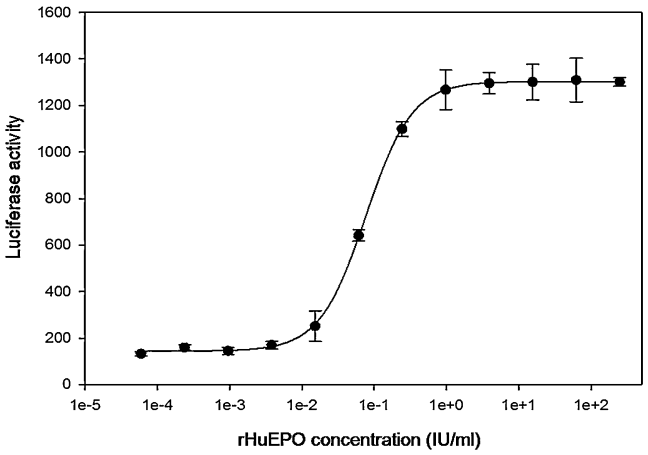

Fig. 1. Responsiveness of UT-7/G7 cells to rHuEPO. UT-7/G7 cells were treated with gradient concentrations of rHuEPO. Each point and error bar represents the mean and standard deviation of three replicates, respectively.

3. Results

3.1. Response of UT-7/G7 cells to the treatments of rHuEPO

The reporter gene vector pGL4-SG-Luc was successfully constructed and transfected to UT-7/epo cells. Resistant clones were selected in the presence of hygromycin B, and then subcloned by limited dilution. As shown in Fig. 1, one of the positive clones was found to produce high levels of luciferase in response to rHuEPO treatment and showed high sensitivity and a dose–response curve (a four-parameter logistic curve) with excellent curve-fitting (*R*-squared was 1.0). This clone was then designated as UT-7/G7 cell line and employed to establish a reporter gene assay for rHuEPO products.

3.2. Optimization of RGA procedure

To obtain stronger reactivity and better sensitivity, crucial experiment parameters of RGA were subsequently optimized. UT-7/G7 cells were routinely maintained in the presence of EPO, which should be removed before tests to reduce the expression of background luciferase. Our tests found that being washed three times by phosphate buffer solution and incubated in the absence of EPO for 17–20 h (starvation time) was sufficient to maintain the survival of UT-7/G7 cells and low-level expression of background luciferase. Besides, other parameters including UT-7/G7 cell number, rHuEPO working concentration and stimulation time were also optimized. Table 1 summarized the optimal conditions used in all subsequent experiments. With the optimal condition, EC_{50} (50% effective concentration) was 0.077 IU/mL and the linear range was 0.03–1.0 IU/mL.

Table 1
Optimized parameters for the RGA.

Experiment parameters	Optimal values
Cell starvation time	17–20 h
Cell number (per well)	6.0×10^4
Initial concentration of rHuEPO	2 IU/mL
rHuEPO dilution multiple	2
rHuEPO stimulation time	4 h
Sensitivity (EC_{50})	0.077 IU/mL
Linear range	0.03–1.0 IU/mL

Crucial experiment parameters of the new RGA were optimized, and the optimal conditions used in all subsequent experiments were listed in the table as well as the sensitivity and linear range of the assay.

3.3. Method validation of RGA

To validate the new RGA, two rHuEPO pharmaceutical products, injection for sample 1 and powder injection for sample 2, were tested in triplicates for each dose and their respective potency was estimated using the National Standard for rHuEPO as a reference for activity. All tests were conducted according to ICH Guidelines, including precision, accuracy, specificity and robustness.

3.3.1. Precision

In order to determine the precision of the new RGA, we ran the assay on five different days, with three repeated analyses of rHuEPO products each day. Such design made it possible to better understand the plate-to-plate variability as well as inter-assay variation. As shown in Table 2, our analyses resulted in intra-assay (within-day) CV of 3.1% to 14.0% and inter-assay (between-day) CV of 4.8% to 7.4%. The linear range (logarithmic phase) of all dose–response curves ran almost parallel to each other, indicating that each group behaved nearly indistinguishably (data not shown). Besides, ANOVA suggested no statistically significant day effect (*P* values: 0.255 and 0.615 for sample 1 and sample 2, resp.).

3.3.2. Accuracy

The accuracy was evaluated applying the new method to the analyses of the in-house mixture of the rHuEPO formulations with known amounts of rHuEPO standard preparation. The accuracy was calculated as the percentage of rHuEPO standard recovered from rHuEPO formulations according to Chinese Pharmacopeias. The experiment consisted of six repeated assays on different days. As shown in Table 3, the CV of six potency estimates was less than 5.0% for both rHuEPO formulations (3.5% and 4.7%, respectively) and the recovery rates were between 81.7% and 102.4%,

Table 2
Precision of the new RGA.

	Sample 1				Sample 2			
	Plates			Intra-assay CV (%)	Plates			Intra-assay CV (%)
	1	2	3		1	2	3	
Day 1	3520	3768	4069	7.3	3215	3437	4177	14.0
Day 2	4016	4142	4274	3.1	3675	3879	4085	5.3
Day 3	4008	4335	4693	7.9	3871	3832	4471	8.8
Day 4	3699	3812	4061	4.8	3782	3506	4094	7.8
Day 5	3740	4057	3888	12.1	3847	3761	3931	6.1
Mean	3797	4023	4197	–	3678	3683	4152	–
Inter-assay CV (%)	5.6	5.9	7.4	–	7.3	5.4	4.8	–

Two rHuEPO pharmaceutical products were tested by RGA and their respective potencies were estimated using the National Standard for rHuEPO as a reference for activity.

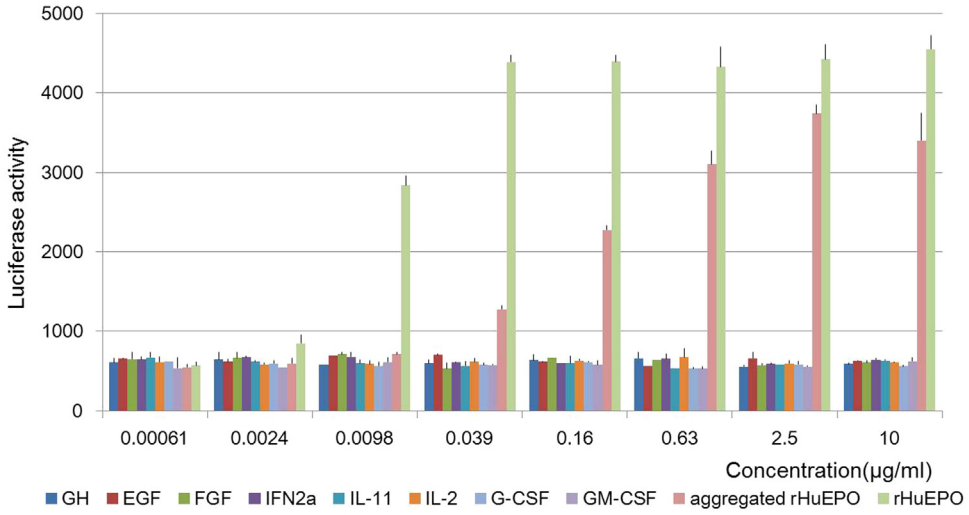

Fig. 2. Specificity of the assay. Responsiveness of UT-7/G7 cells to a variety of different cytokines including rhGH, rhEGF, rhBFGF, IFNα-2a, IL-11, IL-2, rhG-CSF and rhGM-CSF as well as aggregated rHuEPO were detected. Each column illustrates the mean of three replicates.

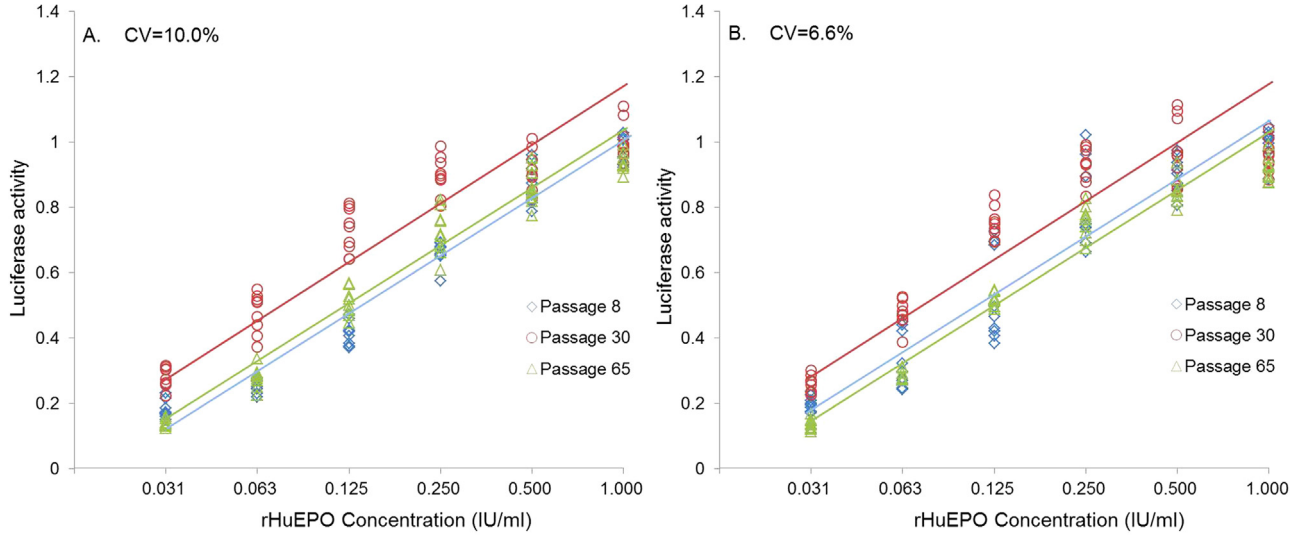

Fig. 3. Stability of UT-7/G7 cell lines. Sample 1(A) and sample 2(B) were rHuEPO pharmaceutical products. The analyses of both samples were performed on cells at three different stages (passage # 8, 30, 65) respectively. The graphs depict the linear range of dose–response curves. The luciferase activity is represented by the ratio of individual luciferase output vs. maximal luciferase intensity for each group.

demonstrating that no significant interference of matrix formulation was found in this assay.

3.3.3. *Specificity of the assay*

Since JAK2-SATAT5 signal pathway is not exclusive for EPO, experiments were conducted to assess whether the presence of other cytokines could affect the expression of luciferase in UT-7/G7 cells. As shown in Fig. 2, none of the eight cytokines except for rHuEPO could influence the luciferase activities in a wide range of drug concentrations (0.0006–10 μg/mL), and the aggregated rHuEPO showed remarkably reduced activity, suggesting UT-7/G7 cells were specifically responsive to EPO in this assay.

3.3.4. *Stability of UT-7/G7 cell line*

The stability of UT-7/G7 cell line is crucial to the robustness of the new RGA, which was evaluated by comparing the responsiveness of cells at three different stages (passage # 8, 30, 65) to two rHuEPO samples respectively. No significant difference was found in potency estimates between three different passages (one-way analysis of variance, *P* values: 0.772 and 0.943 for sample 1 and sample 2, respectively) and the CV of three potency estimates from cells at different stages was 10.0% for sample 1 and 6.6% for sample 2, suggesting no effect of the passage number on responsiveness of UT-7/G7 cells to EPO. Fig. 3 showed that the cells at different stages behaved nearly indistinguishably.

3.4. *Agreement between RGA test and reticulocyte assay*

The agreement between RGA and reticulocyte assay was assessed using a Bland–Altman plot, which is widely accepted as a powerful tool to quantify the difference between two methods, particularly for the analyses of a new method vs. a gold-standard [27,28]. Given the wide range of drug concentrations, all averages of the two measurements were log-transformed in Fig. 4. The average ratio of RGA vs. reticulocyte assay was 0.99 while 95% agreement limits were between 0.82 and 1.16. As shown in Fig. 4, most of the points in terms of ratios of RGA to reticulocyte assay were between 0.82 and 1.16, with only one point out of the range. The

Table 3
Recovery test.

Round	Sample 1		Sample 2	
	Estimated potency (IU)[a]	Recovery rate[b] (%)	Estimated potency (IU)	Recovery rate (%)
1	3874	85.4	3776	89.0
2	3805	95.1	3746	102.4
3	3772	86.6	3833	81.7
4	3675	85.4	3603	82.9
5	3985	96.3	3801	87.8
6	4035	85.4	4142	91.5
Mean	3857.67	–	3564.5	–
CV%	3.5	–	4.7	–

[a] Estimated potency was calculated using the National Standard for rHuEPO as a reference for activity.
[b] Recovery rate was calculated as the percentage of rHuEPO standard recovered from rHuEPO formulations according to Chinese Pharmacopeias.

pattern remained generally symmetric around the average ratio, which was close to one, revealing statistical consistency between the two methods across a wide range of drug concentrations.

4. Discussion

The pharmacological use of rHuEPO has been well established and has had a dramatic impact on the quality of life of patients with renal disease [5,6]. EPO is a glycoprotein, with approximately 40% of the molecular mass of the mature molecule made up of four carbohydrate chains (three N-linked and one O-linked). The carbohydrate moieties of EPO contain at least 10 molecules of sialic acid, which is an important determinant of the pharmacokinetic behavior of EPO in vivo by preventing degradation and delaying clearance of EPO from circulation [10,11]. The protein structure of EPO molecules is associated with the receptor-activating ability of EPO, which is always estimated by in vitro bioassays. Only when in vitro bioactivities are consistent with in vivo bioactivities can the rHuEPO products be used clinically [8]. Since rHuEPOs produced in mammalian cells contain multiple isoforms with heterogeneous carbohydrate moieties, potency determination of sialic acid and isoelectric focusing electrophoresis are also essential test items in routine quality control of rHuEPO pharmacological products (Chinese Pharmacopoeia (2010)).

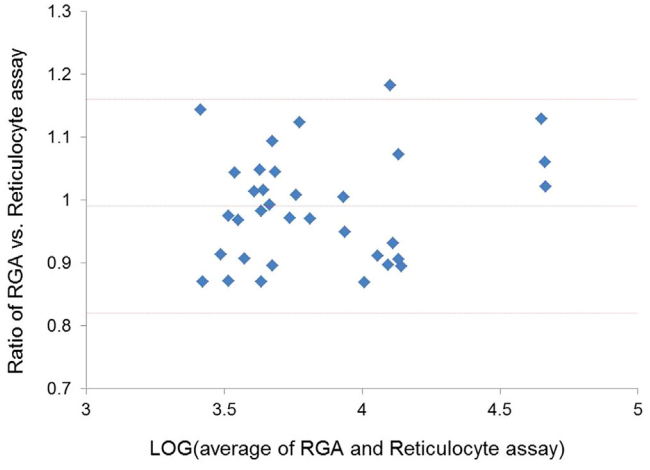

Fig. 4. Bland–Altman plot. Each plot represents the ratio of RGA vs. reticulocyte assay against the log-transformed average of the two assays. The upper and lower dotted lines define the agreement limits within which 95% of differences between the two methods are expected to lie, and the middle dotted line represents the average ratio of RGA vs. reticulocyte assay.

On binding to EpoR, EPO could activate several signal pathways including JAK2-STAT5, Ras-MAPK, JAK2-NF-KappaB, PI3K-ATK and DAG-PKC. JAK2-STAT5 pathway has been clearly elaborated and considered to be directly correlated with the bioactivity of EPO [29,30]. Based on these findings, two specific DNA response elements of STAT5, SIE and GAS and an EPO-dependent cell line UT-7/epo were chosen to explore a novel bioassay for rHuEPO products.

In this study, a novel RGA was developed employing a UT-7/epo cell line stably transfected with luciferase gene under the control of SIE and GAS promoter and subsequently validated for its precision, specificity, robustness and agreement with reticulocyte assay. The constructed UT-7/G7 cell line showed strong reactivity to rHuEPO and excellent curve-fitting (Fig. 1). The optimized assay could be completed within 24 h and proved to be more sensitive with an EC_{50} of 0.077 IU/mL. Our analyses resulted in both intra- and inter-assay variation less than 15.0%, and recovery rates ranged from 81.7% to 102.4%, demonstrating good precision and accuracy, which were even better than most of currently-used methods [8,13]. None of the eight cytokines involved in our study except for rHuEPO could affect the expression of luciferase in UT-7/G7 cells, suggesting good specificity. Data obtained from cells at three different stages (passage # 8, 30, 65) showed no statistically significant difference in potency estimates and inter-passage CV values were found to be below 10%, suggesting no effect of the passage number on responsiveness to rHuEPO in this assay. Finally, Bland–Altman analysis suggested that for rHuEPO products with proper carbohydrate structures, the results of RGA were highly consistent with in vivo bioactivities of rHuEPO [18].

In conclusion, here for the first time we reported a new RGA for rHuEPO with good specificity, precision, accuracy and robustness. The new assay also showed a high consistency with reticulocyte assay in terms of results. However, it has many advantages over reticulocyte assay, such as simpler operation, higher efficiency and no need of animal test. This RGA could be used as a viable supplement to reticulocyte assay and employed in potency determination of rHuEPO pharmaceutical products. Besides, it could also be used in potency determination of other erythropoiesis-stimulating agents sharing the same receptor with EPO and in detection of neutralizing antibodies against therapeutic EPO in clinical serum samples.

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