Original Article

A Novel Reporter Gene Assay for Pyrogen Detection

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SUMMARY: Fever is a systemic inflammatory response of the body to pyrogens. Nuclear factor κ B (NF- κ B) is a central signaling molecule that causes the excessive secretion of various pyrogen-induced pro-inflammatory factors. This study explored the feasibility of a novel reporter gene assay (RGA) for pyrogen detection using RAW264.7 cells stably transfected with the NF- κ B reporter gene as a pyrogenic marker. The RGA could detect different types of pyrogens, including the lipopolysaccharide of gram-negative bacteria, the lipoteichoic acid of gram-positive bacteria, and the zymosan of fungi, and a good dose-effect relationship was observed in terms of NF- κ B activity. The limits of detection of the RGA to those pyrogens were 0.03 EU/ml, 0.001 μ g/ml, and 1 μ g/ml, respectively. The method had good precision and accuracy and could be applied to many molecules (e.g., nivolumab, rituximab, bevacizumab, etanercept, basiliximab, *Haemophilus influenzae* type b conjugate vaccine, 23-valent pneumococcal polysaccharide vaccine, group A and group C meningococcal conjugate vaccine, diphtheria, tetanus, pertussis [acellular, component], poliomyelitis [inactivated] vaccine, and imject alum adjuvant). The results of this study suggest that the novel RGA has a wide pyrogen detection spectrum and is sufficiently sensitive, stable, and accurate for various applications.

INTRODUCTION

Pyrogens are fever-inducing substances, including exogenous pyrogens (e.g., the lipopolysaccharide [LPS] of gram-negative bacteria, the lipoteichoic acid [LTA] of gram-positive bacteria, the peptidoglycan and lipoprotein of gram-negative/positive bacteria, and the zymosan of fungi) and endogenous pyrogens (e.g., steroids, prostaglandin E, and proinflammatory cytokines) (1,2). Pyrogen testing is mandatory and is a critical for ensuring the safety of parenteral products.

The Chinese Pharmacopoeia has adopted the rabbit pyrogen test (RPT) and the bacterial endotoxin test (BET) for detecting pyrogenic contamination in products (3). Nevertheless, the results of the RPT are often affected by many factors, such as environmental, animal, and operational factors, and the BET can only detect the LPS from gram-negative bacteria. To replace the traditional pyrogen tests, the European Pharmacopoeia has adopted the monocyte activation test (MAT), which is mainly based on the use of monocytes and macrophages involved in the development of fever and production of proinflammatory cytokines [e.g., interleukin (IL)-6, IL-1 β , and tumor necrosis factor (TNF)- α] as pyrogenic markers (4–6). However, the MAT often needs large amounts of human blood, and its convenience needs to be improved. The

representativeness of using a single pro-inflammatory cytokine as the pyrogenic marker is also limited in theory.

In essence, fever is a systemic inflammatory response of the body to pyrogens (7–9). The mechanism by which pyrogens stimulate the secretion of proinflammatory factors in the body often involve the activation of NF- κ B, which is the central signaling molecule mediating the inflammatory response (10–13). Therefore, it is reasonable to use NF- κ B as a representative pyrogenic marker. As a result, the main aim of the present study was to evaluate the feasibility of utilizing murine macrophage RAW264.7 cells transfected with the NF- κ B reporter gene to detect pyrogens.

MATERIALS AND METHODS

Reagents: The national standard for bacterial endotoxins is LPS, which was obtained from *Escherichia coli* O55:B5 [10,000 endotoxin units (EU)/vial, batch 150600-200707, identical to the 2nd international WHO standard for endotoxin 94/580 from *Escherichia coli* O113:H10] and was provided by the National Institutes for Food and Drug Control (NIFDC). The following materials were also used in this work: LTA (Sigma-Aldrich, Darmstadt, Germany, Cat#L3265), zymosan (Sigma-Aldrich, Cat#Z4250), LPS (Sigma-Aldrich, Cat#L7895, Cat#L6143, Cat#L8274, Cat#L4516, Cat#L4263), glucan (Sigma-Aldrich, Cat#G5011), lectin (Sigma-Aldrich, Cat#L8754), dextran (Sigma-Aldrich, Cat#31392), imject alum adjuvant (Thermo Scientific, Shanghai, China, Cat#77161), foetal bovine serum (FBS, GeminiBio, West Sacramento, CA, USA, Cat#900-108), penicillin-streptomycin (Gibco, Shanghai, China, Cat#15140-122), L-glutamine (Gibco, Cat# 25030-081), hygromycin B (Amresco, Shanghai, China,

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Cat#V900372), Bright-Glo Luciferase Assay reagent (Promega, Madison, WI, USA, Cat#E2650), phosphate-buffered saline (PBS, HyClone, Beijing, China, Cat#SH30256.01), trypsin-EDTA (Gibco, Cat#25200-056), DMEM (Gibco, Cat#11995-065), pyrogen-free water for the BET (Zhanjiang A&C Biological LTD, Zhanjiang, Guangxi, China), *Tachypleus* amebocyte lysate (TAL, Zhanjiang A&C Biological LTD), nivolumab injection reagent (Bristol-Myers Squibb Holdings Pharma LTD Liability Company, Shanghai, China), rituximab injection reagent (Roche Diagnostics GmbH, Shanghai, China), bevacizumab injection reagent (Roche Diagnostics GmbH), etanercept solution for injection (Pfizer Ireland Pharmaceuticals, Beijing, China), *Haemophilus influenza* type b conjugate vaccine (Yuxi Walvax Biotechnology Co. LTD, Yuxi, Yunnan, China), 23-valent pneumococcal polysaccharide vaccine (Yuxi Walvax Biotechnology Co. LTD), group A and group C meningococcal conjugate vaccine (Yuxi Walvax Biotechnology Co. LTD), diphtheria, tetanus, pertussis (acellular, component), poliomyelitis (inactivated) vaccine (Sanofi Pasteur S.A., Shenzhen, China), and basiliximab for injection (Novartis Pharma Stein AG, Beijing, China).

Consumables: Ninety-six-well plates were used for both the RGA (Corning Inc., Corning, NY, USA, Cat#3917, white, flat bottom, tissue culture treated, polystyrene) and the BET (Corning Inc., Cat#M9005, flat bottom, polystyrene, tissue culture-treated). Other reagents/materials were purchased as sterile and free of pyrogens, and glassware was baked at 250°C for 1 h.

Construction of the reporter gene vector: The pCM1.1_luc_hygro vector contains a minimal promoter followed by a luciferase gene. NF-κB response element (5'-TCCTCGGAAAGTCCCCTCTGAGATCCTCGGAAGTCCCCTCTGAGATCTCAGAGGGGACTTTCCGAGGA-3') was synthesized by overlap PCR and inserted into the multiple cloning site ahead of the mini-promoter region, and the positive clone was verified by DNA sequencing.

Development of RAW264.7 cells stably transfected with the reporter gene vector: The plasmid pCM1.1_NF-κB_luc_hygro was introduced into RAW264.7 cells (ATCC) by electroporation. The cells were selected at 48 h after transfection in selective medium (DMEM containing 10% FBS, 1% penicillin-streptomycin, 1% glutamine, and 150 µg/ml hygromycin B). After being selected for 3 weeks, hygromycin-resistant cells were then cloned by limited dilution to obtain a single cell clone and were then screened for the induction of luciferase activity by treatment with gradient concentrations of LPS (e.g., 1,000 ng/ml, 100 ng/ml, and then 1:3 dilutions, 10 series). The resulting positive clones were routinely maintained in the selective medium.

FACS analysis: RAW264.7 cells were centrifuged (300 × g for 5 min) at 4°C, washed twice with ice-cold PBS, and then blocked with 200 µg/ml mouse IgG (Jackson ImmunoResearch, West Grove, PA, USA, Cat#015-000-003) on ice for 10-20 min. The cells were resuspended in PBS containing 4% bovine calf serum (BCS) at a concentration of 2 × 10⁶ cells/ml and aliquoted into 96-well plates (50 µl/well), after which 50 µl of 2 µg/ml fluorescence-labelled antibodies was

added for detection of TLR2 (R&D, Shanghai, China, Cat#FAB1530G), TLR4 (R&D, Cat#FAB27591G), TLR6 (R&D, Cat#FAB1533G), CD40 (Invitrogen, Shanghai, China, Cat#14-1401-81), CD83 (Biolegend, San Diego, CA, USA, Cat#121518), and CD86 (Invitrogen, Cat# 14-0862-81); rat IgG2a Alexa Fluor 488-conjugated antibody was used as an isotype control (R&D, Cat#IC006G). The cell-antibody mixture was incubated on ice for 45 min in the dark, washed twice with PBS containing 4% BCS, and resuspended in 200 µl of 5 µg/ml propidium iodide (Sigma-Aldrich, Cat#P4170) in PBS to stain dead cells. Data were collected using a BD FACSCanto system and analysed with FlowJo software.

RGA: Selected cells in the logarithmic growth stage were washed with PBS and digested with 0.25% trypsin-EDTA. Confluent cell monolayers were suspended in the selective medium at the required concentration, seeded into 96-well plates (100 µl/well), and allowed to attach for 24 h. Then, the selective medium was discarded, and sample solutions prepared with assay medium (DMEM containing 10% FBS) were added to the 96-well plates (100 µl/well, *n* = 4). The plates were incubated at 37°C in an atmosphere of 5% CO₂ in air for the indicated time period. After incubation, Bright-Glo Luciferase Assay reagent was added to the 96-well plates (100 µl/well), which were subsequently shaken for 1 min. Finally, luciferase activity was determined using a Luminoskan Ascentreader.

BET: The kinetic chromogenic TAL assay was performed according to the manufacturer's instructions (Zhanjiang A&C Biological LTD). A total of 100 µl/well of the sample/standard solutions (*n* = 2) prepared with pyrogen-free water was mixed with an equal volume of an endotoxin-specific TAL reagent, also prepared with pyrogen-free water, in 96-well plates. The rate of color development was measured at 37°C using a specially equipped microplate reader (Synergy HT, BioTek Instruments Inc., Winooski, VT, USA). The endotoxin contents of the samples were calculated according to the parallel line assay method using the logarithmically transformed dose and the rate of colour development and are expressed as EU/ml, referring to the standard endotoxin.

Application of the RGA to drugs: Drugs within the maximum valid dilution (MVD) pass the interference assay when the spike recovery is within the range of 50-200%. Spike recovery = 100% (the mean concentration of endotoxin detected in the diluted solution containing the added endotoxin - that detected in the diluted solution)/the added endotoxin. The recovery concentration of LPS in the drugs was 4.0 EU/ml, and the limit of detection (LOD) of LPS used in the experiment was 0.125 EU/ml.

Comparison between the RGA and BET: Drugs were tested at their MVD, each of which was calculated as the endotoxin limit concentration in EU/ml divided by the LOD (in this case, 0.5 EU/ml).

Each drug presented five blinded spikes, two of which were defined as negative, meaning they were below 0.5 EU/ml (0 and 0.25 EU/ml), while three were positive (0.5, 1.0, and 2.0 EU/ml). These spikes were tested by both the RGA and BET.

After the interference test was passed, the samples

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Fig. 1. The NF- κ B activity levels of RAW264.7 cells at a density of 10×10^5 cells/ml after stimulation with LPS (A), LTA (B), and zymosan (C) for different times ($n = 4$). &: $p > 0.05$ vs. the negative control (e.g., 0 EU/ml, 0 µg/ml); #: $p < 0.05$ vs. the negative control; *: $p < 0.01$ vs. the negative control.

were classified by a so-called prediction model, which classified the samples as negative (N) when the mean concentration of endotoxin equivalents in each of the sample replicates calculated by the endotoxin standard curve was less than the contaminant limit concentration

specified for the samples. The samples were otherwise classified as positive (P).

Within-laboratory reproducibility was calculated as the proportion of samples classified identically in three independent runs. Reproducibility between methods

was calculated as the proportion of samples classified identically. Sensitivity was defined as the probability of correctly classifying positive samples, and specificity was defined as the probability of correctly classifying negative samples.

Statistical analysis: All experiments were repeated three times. The data are expressed as the mean and standard error of the mean. The data were compared between groups using Student's t-test.

RESULTS

The time-effect relationships of pyrogens (LPS, LTA, and zymosan) activating NF-κB: The results of this experiment are presented in Fig. 1. The data show that the different pyrogens, i.e., LPS, LTA, and zymosan, could activate NF-κB in a dose-dependent manner. Increasing the stimulation time up to 10–12 h, almost all concentrations of the three pyrogens could activate NF-κB to an extreme extent.

The dose-effect relationships of pyrogens (LPS, LTA, and zymosan) with respect to activation of NF-κB in RAW264.7 cells at different cell densities: The results of this experiment are presented in Fig. 2. The data of Fig. 2A show that at different cell densities, LPS had a dose-effect relationship with NF-κB activity to a certain extent. The dose-response curve was relatively flatter for low cell density (5×10^5 cells/ml) than for the medium and high cell densities (10×10^5 and 15×10^5 cells/ml, respectively). However, LPS had a relatively narrower concentration range (32–128 EU/ml) for maintaining maximum NF-κB activity at the high cell density (15×10^5 cells/ml) compared with that (32–512 EU/ml) at the medium cell density (10×10^5 cells/ml). Besides, the NF-κB activity at the high cell density (15×10^5 cells/ml) was much more susceptible to the “edge effect” of plates in the culture, which might lead to a decrease in NF-κB activity at that density stimulated by the 512 EU/ml of LPS. These results indicate that LPS

had the best dose-effect relationship with NF-κB activity at a cell density of 10×10^5 cells/ml. The LOD of the NF-κB response at that cell density to LPS was 0.03 EU/ml ($p = 0.005$ vs. the negative control).

The data in Figs. 2B and 2C show that at different cell densities, non-endotoxin pyrogens, including LTA and zymosan, had significant dose-effect relationships with NF-κB activity. However, the larger the cell density was, the higher the background value of NF-κB was. The highest concentration of LTA (1,000 µg/ml) might have cytotoxic effects, which can lead to a significant decrease in NF-κB activity at different cell densities. The LODs of the NF-κB response to LTA at the high, medium, and low densities were 0.01 ($p = 0.005$), 0.001 ($p = 0.006$), and 0.1 µg/ml ($p = 0.007$), respectively, which were all 1 µg/ml ($p = 0.008$, $p = 0.003$, $p = 0.005$) for zymosan.

The data in Fig. 2D show that there were also significant dose-effect relationships between LPS from different strains, including *Salmonella typhosa*, *Salmonella enterica* serotype typhimurium, *Escherichia coli* O26:B6, *Escherichia coli* O127:B8, *Klebsiella pneumoniae*, and other non-endotoxin pyrogens, including glucan, lectin, peptidoglycan, and the NF-κB response.

The dose-effect relationships of LPS in activating NF-κB in RAW264.7 cells and cell phenotypes at different cell passages: The results of this experiment are presented in Fig. 3. The data of Fig. 3A show that at different cell passages with a density of 10×10^5 cells/ml, LPS had a stable dose-effect relationship with NF-κB activity. Furthermore, the dose-response curves at different cell passages were relatively parallel. The data in Figs. 3B and 3C show no significant difference in expression of TLRs, including TLR2, TLR4, and TLR6, in RAW264.7 cells before and after transfection with NF-κB, which was also not significant at different passages of the transfected RAW264.7 cells. The data in Figs. 3D and 3E show that expression of CDs, including CD40, CD83, and CD86, on transfected RAW264.7 cells was significantly higher than that on wild-type RAW264.7 cells. However, no significant difference in CD expression on transfected RAW264.7 cells at different passages was observed. These results indicate good stability for both the activity and phenotype of the transfected RAW264.7 cells.

Precision and accuracy of the RGA for detecting LPS in the laboratory: Four samples of LPS, whose expected concentrations were 1.5 (sample 1), 3.0 (sample 2), 6.0 (sample 3), and 12.0 (sample 4) EU/ml, respectively, were detected by the RGA. The results are presented in Table 1. The data show that the measured concentrations of LPS in the samples were 1.761 ± 0.126 (sample 1), 3.241 ± 0.101 (sample 2), 5.906 ± 0.390 (sample 3), and 11.028 ± 1.760 (sample 4) EU/ml, and the intraassay and interassay coefficients of variation (CVs) were generally less than 13% and 16%, respectively. A good linear relationship was confirmed between the expected (x) and measured (y) concentrations, $y = 0.8772x + 0.5497$ ($R^2 = 0.9995$), in the tested LPS concentration range (1.5–12.0 EU/ml), suggesting that the method also had good accuracy.

Application of the RGA to drugs: The results are presented in Table 2, and they indicate that the method

Table 1. Precision of the RGA for detecting LPS

	Round 1	Round 2	Round 3	Interassay CV (%)
Sample 1 (EU/ml)	1.927	1.791	1.717	7
	1.879	1.653	1.765	
	1.725	1.871	1.522	
Intraassay CV (%)	6	6	8	/
Sample 2 (EU/ml)	3.319	3.216	3.103	3
	3.418	3.265	3.255	
	3.089	3.258	3.249	
Intraassay CV (%)	5	1	3	/
Sample 3 (EU/ml)	5.426	5.678	5.924	7
	6.065	5.683	6.755	
	5.719	5.726	6.175	
Intraassay CV (%)	6	0	7	/
Sample 4 (EU/ml)	11.91	8.752	9.632	16
	11.646	9.039	12.121	
	14.372	11.125	10.655	
Intraassay CV (%)	12	13	12	/

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Fig. 2. (Color online: D) The NF- κ B activity levels of RAW264.7 cells at 5×10^5 , 10×10^5 , and 15×10^5 cells/ml after stimulation with LPS (A), LTA (B), and zymosan (C) for 10 h; at 10×10^5 cells/ml after stimulation with LPS from different strains and non-endotoxin pyrogens including glucan, lectin, and peptidoglycan for 10 h ($n = 4$). LPS-1: from *Salmonella typhosa*; LPS-2: from *Salmonella enterica* serotype typhimurium; LPS-3: from *Escherichia coli* O26:B6; LPS-4: from *Escherichia coli* O127:B8; LPS-5: from *Klebsiella pneumonia*; Glucan: from baker's yeast (*Saccharomyces cerevisiae*); Lectin: from *Phaseolus vulgaris* (red kidney bean); Peptidoglycan: from *Bacillus subtilis*.

Fig. 3. (Color online: B and D) The NF-κB activity levels of RAW264.7 cells at passage 14, 21, 30, and 40 with a density of 10×10^5 cells/ml (A) after stimulation with LPS for 10 h ($n = 4$). Expression of TLR2, TLR4, and TLR6 (B and C) by wild-type RAW 264.7 cells and transfected RAW264.7 cells at passage 8, 19, 30, and 45 was analysed by FACS, and expression of CD40, CD83, and CD86 (D and E) was also assessed. Rat IgG2a AF488 was used as an isotype control. Δ : $p > 0.05$ vs. wild-type RAW264.7 cells; $\#$: $p < 0.05$ vs. wild-type RAW264.7 cells; $*$: $p < 0.01$ vs. wild-type RAW264.7 cells.

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Table 2. Recovery of the RGA for detecting LPS

Drug	Fold-dilution	NF-κB response	
		Spike recovery (%)	Interference
Nivolumab injection	16	121	no
Rituximab injection	8	125	no
Bevacizumab injection	16	161	no
Etanercept solution for injection	168	105	no
<i>Haemophilus influenzae</i> type b conjugate vaccine	400	74	no
23-Valent pneumococcal polysaccharide vaccine	400	75	no
Group A and group C meningococcal conjugate vaccine	8,000	85	no
Basiliximab for injection	64	70	no
Diphtheria, tetanus, pertussis (acellular, component), poliomyelitis (inactivated) vaccine	1,600	96	no
Imject alum adjuvant	1,000	129	no

Table 3. Classification of samples by the RGA and BET

Drug	Spike (EU/ml)	Truth	RGA			BET		
			Round 1	Round 2	Round 3	Round 1	Round 2	Round 3
<i>Haemophilus influenzae</i> type b conjugate vaccine	0	N	N	N	N	N	N	N
	0.25	N	N	N	N	N	N	N
	0.5	P	N	P	P	N	N	N
	1	P	P	P	P	P	P	P
	2	P	P	P	P	P	P	P
23-Valent pneumococcal polysaccharide vaccine	0	N	N	N	N	N	N	N
	0.25	N	N	N	N	N	N	N
	0.5	P	P	P	P	N	N	N
	1	P	P	P	P	P	P	P
	2	P	P	P	P	P	P	P
Sensitivity (%)			94 (17/18)			67 (12/18)		
Specificity (%)			100 (12/12)			100 (12/12)		
Within-laboratory reproducibility (%)			90 (27/30)			100 (30/30)		
Reproducibility between assays (%)			87 (52/60)					

Bold types, false classifications.

has potential for various applications. Specifically, the aluminum adjuvant can adsorb or mask the endotoxin, which can strongly inhibit the combination of endotoxin and limulus lysate in BET, and the interference cannot be eliminated by diluting the solution, even to 10,000-fold (data not shown). However, aluminum adjuvant is often used in the preparation of vaccines, which may be why a strong interference effect is often observed with BET for vaccines. The data show that RGA can overcome the problem of adsorbed or masked endotoxin-induced by the aluminum adjuvant in BET, and can be applied for pyrogen detection in adjuvant and related products (e.g., diphtheria, tetanus, pertussis [acellular, component], poliomyelitis [inactivated] vaccine).

Comparison between the RGA and BET: The results are presented in Table 3. The BET assay, in principle, is a physicochemical binding assay. Protein components in products often interfere with the binding of LPS to the *Limulus* agents, leading to the fact that the interference of the product is mostly manifested as inhibition. Even if the interference of the product at a large dilution is eliminated, false-negative results

are more likely to occur in the BET, as shown by the sensitivities of the BET (67%) and RGA (94%). However, the accuracy of the BET will be affected when the interference of the product at a large dilution is eliminated. The RGA can overcome the above problems of the BET, and the results of the RGA should be more accurate.

DISCUSSION

Currently, three pyrogen tests, including the RPT, BET, and MAT, have been adopted by pharmacopeias (3,4). The RPT involves the use of animals in vivo, does not correspond with the 3Rs principle, has variations in response depending on many factors, and is expensive. The BET is a physicochemical test for detecting the LPS of gram-negative bacteria and is not a functional activity test (14). The MAT is mainly based on the use of monocytes and macrophages involved in the febrile response and can overcome those problems. However, it often needs large amounts of human blood, and its convenience needs to be improved. The

representativeness of using a single pro-inflammatory cytokine as the pyrogenic marker is also limited in theory.

Compared with other sources of monocytes and macrophages, such as whole blood (15–17) and peripheral blood monocytes (18–20), monocytic and macrophage cell lines are more stable and easier to use (21–24). RAW264.7 cells are mouse-derived macrophages and express the main receptors that can bind pyrogens, including TLR2, TLR4, and TLR6. The results of this study also confirm that pyrogens from different sources can activate an NF- κ B reporter gene when it is transfected into RAW264.7 cells.

Most existing MATs often use various proinflammatory cytokines (e.g., IL-6, IL-1 β , and TNF- α) as pyrogenic markers (6,25). Although this approach has a certain rationality, pyrogens stimulate the body via different mechanisms to induce immune cells to secrete different proinflammatory factors. Our previous study found differences between the secretion of IL-6 and IL-1 β , even from the same cryopreserved or fresh pooled human whole blood (26,27). However, the synthesis and secretion of various pro-inflammatory factors mostly involve the activation of NF- κ B. Therefore, it is more reasonable to use NF- κ B as a representative pyrogenic marker, which might be verified by the fact that LPS from different strains had similar dose-effect relationships in the RGA.

Additionally, the time-effect relationships of the NF- κ B activation were similar among the pyrogens, which might also verify that NF- κ B is a central signaling molecule that mediates the fever reaction induced by pyrogens. We confirmed that the activation of NF- κ B by stimulation with different pyrogens, including LPS, LTA, and zymosan, showed a good correlation with the secretion of proinflammatory factors (e.g., IL-1 β , IL-6, and TNF- α) induced by those pyrogens (data not shown). We also found that the fresh pooled human whole blood (27) and RGA had similar reactivities to LTA and zymosan.

In conclusion, this study establishes a novel bioassay for pyrogen detection using RAW264.7 cells transfected with an NF- κ B reporter gene as a pyrogenic marker. This method can be used to detect multiple pyrogens; is sensitive, stable, and accurate; and can be applied widely.

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Conflict of interest None to declare.

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