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EVALUATION OF SUITABILITY OF METHODS FOR TESTING BLOOD PROTEIN HYDROLYSATES FOR THEIR BIOLOGICAL ACTIVITY AND TOXICITY

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SUMMARY

The use of high quality nutrient media comprising protein hydrolysates as the main components for maintaining their nutrient value is a critical prerequisite for appropriate final product preparation in FMD vaccine biotechnology. The incoming control of raw materials, in particular testing blood protein hydrolysates of different batches for their quality, is of considerable importance. Currently, there are different methods for the determination of hydrolysate efficacy using cell cultures and unicellular organisms as test systems. We used suspended baby hamster kidney (BHK-21) cell line as a model for testing blood protein hydrolysates in the raw materials intended for FMD vaccine production for their biological activity and toxicity. The validation of the proposed method was carried out to confirm the reliability of the test results obtained. The following main validation characteristics were determined through testing 560 blood protein hydrolysate samples: trueness, specificity, sensitivity, as well as precision under repeatability and reproducibility conditions. The validation results for the proposed method demonstrated high reliability (Fisher's criterion – 0.021; ≤0.050) and full compliance with acceptance criteria.

Key words: blood protein hydrolysate, biological activity, toxicity, specificity, test-system sensitivity, trueness of methods, precision.

INTRODUCTION

Currently, there is an apparent and intensively growing trend in cell biotechnology for preparation of nutrient media containing raw protein hydrolysates as growth amino acid and peptide component [3, 7, 9].

Even subtle deviations from the technology can result in blood protein hydrolysate (BPH) toxicity; reducing growth-promoting properties of the BPH-containing nutrient media up to cell death [7, 11]. Therefore, incoming control of raw materials, in particular testing blood protein hydrolysates (BPHs) of different batches for their quality, is of considerable importance for biotechnological production.

Currently there are various methods for identifying hydrolysate effectiveness with cell cultures and unicellular organisms as test-systems [10]. However, the analysis of publications revealed no evidence of using suspension baby hamster kidney (BHK-21) cell line as a model for testing BPHs for their biological activity and toxicity.

System for raw material incoming control requires ensuring reliability of analysis results. Procedure for validation of analytical methods is established for this purpose that is one of the in-process medicine control elements [13].

Validation is a procedure for verification of appropriate compliance with all requirements for the said specific use by providing objective evidence (ISO 9000:2005) [5]. Validation procedure implies establishment of objective test system parameters that include trueness, repeatability, reproducibility under different test conditions, specificity and sensitivity [1, 2, 12, 13].

Test result reliability can be ensured only when accurate method is used. In metrology accuracy is a closeness of measured value to the reference one [4]. Terms "trueness" and "precision" are used for thorough explanation of quantitative method accuracy. Trueness refers to the proximity of experiment results to true value in the whole field of measuring. Systematic error is a main factor determining trueness. Precision refers to the closeness of independent measurements obtained under certain established experiment conditions to each other. Intralaboratory (intermediate) precision of the method under validation is assessed

under the working conditions of the same laboratory (different days, different operators, different equipment, etc.). In order to assess the extent of achievement of the required result quality the analysis is carried out under repeatability and reproducibility conditions including calculation of absolute and relative variance values [1, 8, 13].

The study was aimed at evaluation of suitability of the method for testing blood protein hydrolysates of different batches for their biological activity and toxicity designed for testing raw materials used for anti-FMD vaccine production.

MATERIALS AND MEHODS

Cell line. Suspension baby hamster kidney cell line (BHK-21) at 1st and 2nd passage and concentration of at least 3.0×10^6 cell/ml was used.

Medium for cell cultivation. Culture medium based on Hank's solution containing salts, amino acids and vitamins required for the cell growth and supplemented by 5% of fetal bovine serum (SERANA) inactivated at 56 °C for 30 min as well as tested blood protein hydrolysate was used for BHK-21 cell line cultivation. The blood protein hydrolysates were sterilized by autoclaving at temperature of 132 ± 2 °C and pressure of 0.2 MPa for 1 hour.

Tests of BPHs for their biological activity. BHK-21 cell line was grown in culture flasks containing prepared nutrient medium solution at cell seeding density of $0.4–0.7 \times 10^6$ cell/ml. BPHs at two concentrations, 0.25 and 0.50% dry weight, were tested, for their biological activity. The cells were incubated in glass propagators placed in temperature-controlled shaker (Incubation Schüttelschrank BS 4) with rotation rate of 150 ± 5 RPM and at temperature 37.0 ± 0.5 °C. pH level was maintained at 7.0–7.2 with sterile 7.5% sodium bicarbonate solution. Cells were successively passaged five times every 48 hours.

Determination of cell multiplication index. BHK-21 cell concentration in suspension was determined using Goryaev's chamber for hemocyte counting. Number of cells in 1 ml of the suspension was calculated according to the following formula [11]:

$$X = \frac{A \times B \times 4000}{3600} \times 1000,$$

where X – number of cells in 1 ml;
A – total number in the chamber;
B – suspension dilution.

Increase in cell concentration was estimated based on multiplication index (MI) according to the following formula:

$$\mathrm{MI} = \frac{a}{b},$$

where a – cell density 48 hours after inoculation;
b – cell inoculation density.

Interpretation of tests of BPHs for their biological activity and toxicity. BPH quality was assessed based on MI mean as follows:

if MI_{mean} value (MI_{mean}) after 5 passages was 4.5–5.0, BPH was considered biologically active;

if MI_{mean} after 5 passages was 3.0–4.5, BPH was considered acceptable;

if MI_{mean} after 5 passages was less than 3.0, BPH was rejected.

BPH toxicity was assessed as follows: if BHK-21 cells had remained viable for five passages (at least 60% of viable cells at 4th and 5th passage) the BPH was considered non-toxic, if not the hydrolysate was rejected.

The results were interpreted in such a way since BPHs were tested for their biological activity and toxicity in cells grown in the absence of 5% CO_2 atmosphere. The cell viability severely declined up to passage 4–5 under such conditions.

Method trueness assessment. To assess the method for its trueness medium supplemented with BPH at concentration of 0.25 and 0.50%, as well as medium supplemented with warmed 5% calf fetal serum only were tested in triplicate. Term "percentile" was used for obtained data characterization. Percentile indicated the value below which a given percentage of samples in a group of samples fell in such a way that 0% percentile equaled to the smallest value, 100% percentile equaled to the largest value and 50% percentile equaled to the median value, etc. Intermediate values had percentiles ranking $100/(n – 1)$. Fisher's variance ratio (F) was estimated for comparative analysis of variance dispersion (when BPHs at concentration of 0.25 and 0.50% were used) [4].

Method specificity assessment. Method specificity is ability of the method to detect all known negative samples as negative. In the said study BPHs were considered negative (were rejected) when they were toxic for suspension continuous BHK-21 cell line and induced growth at multiplicity index less than 3.0. The said BPH samples were tested in triplicate and test results were used for mean cell multiplicity index calculation and for proving that tested BPH batches were unsuitable for production.

Method sensitivity assessment. To assess the method for its sensitivity known positive samples were tested and suspension BHK-21 cell culture multiplicity index was calculated.

Assessment of intermediate precision under reproducibility and repeatability conditions. To assess precision of the quantitative method of testing BPHs for their biological activity were performed under the same measuring conditions (equipment, laboratory time period (10 days), the same operator) except for difference in cell suspension (different passages). Analysis was also carried out under the same measuring conditions (equipment, cell suspension, laboratory except for subjectivity factor (the analysis was performed by two operators)) and time period (within 24 days). Analyses were performed in triplicate. Absolute and relative variance values were calculated for assessment of the quantitative method precision under repeatability and reproducibility conditions.

Calculation of absolute variance values. Variation range (R) was calculated as difference between the greatest and least threshold cycle values: $R = Ct_{max} – Ct_{min}$. Individual linear deviation was calculated according to the following formula: $d_i = |Ct_i – Ct_{mean}|$. Mean linear deviation (d_{mean}) was calculated as arithmetic mean of individual linear deviation according to the following formula:

$$d_{mean} = \Sigma\,|d_i|\,/N,$$

where d_i – individual linear deviation;
N – number of observations.

Dispersion (δ^2) of values was calculated according to the following formula: $\delta^2 = (\Sigma d_i^2)/N$. Mean squared variation (δ) was calculated to measure variance according to the following formula: $\delta = \sqrt{(\delta^2)}$ [1, 8].

Calculation of relevant variance values. Relative standard deviation (RSD) was calculated according to the formula: $RSD = \delta/Ct_{mean}$. Repetitive variation (V_R) was calculated according to the formula: $V_R = (R/Ct_{mean}) \times 100$. Linear co-

efficient of deviation (C_d) was calculated according to the formula: $C_d = (d_{mean}/Ct_{mean}) \times 100$. To assess variability of individual threshold cycle values coefficient of variation (C_δ) was calculated as follows: $C_\delta = (\delta/Ct_{mean}) \times 100$ [1, 8].

RESULTS AND DISCUSSION

At the first stage of our study, the method of testing BPHs for their biological activity was assessed for its trueness. The main determining factor here was a systematic error resulting from comparison with the results of a previously described method. However, analysis of available scientific data have not found any methods used for specific testing BPH for its biological activity. Therefore, for comparative analysis BHK-21 cells were cultivated in growth medium supplemented with 5% of warmed calf fetal serum and in growth medium supplemented with BPHs. Results of BPH tests for 5 successive passages and in triplicate are given in Table 1 and in the Figure.

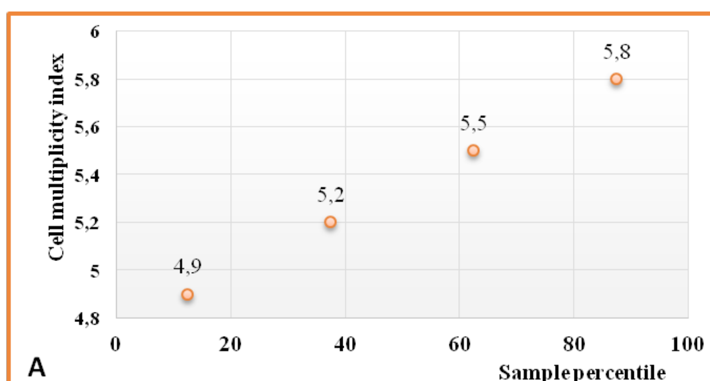
Data given in Table 1 show that multiplicity index was 5.2–5.3, when BPHs at the concentration of 0.25 and 0.50% were used. When the medium supplemented only with calf fetal serum was used, the cell population decreased more than twice.

Analysis results are shown in the Figure in the form of equations: $y_x = 5.989 + 0.3158x$ (A), $y_x = -3.653 - 4.421x$ (B) and prove that BHK-21 cell line multiplicity index meets the required level >3.0 in accordance with the developed method when BPHs at concentrations of 0.25 и 0.50% were used [11].

Testing of 560 BPH samples at 0.25% and 0.50% concentrations using the developed methods resulted in data, whose statistical analysis allowed for conclusion that at 0.25% BHP concentration Fisher's variance ratio (F) amounted to 0.021 that is significantly lower than 0.05. Otherwise stated, the demonstrated model was significant. Upon the increase of the hydrolysate content up to 0.50% the tested criterion amounted to 2.08, which is significantly higher than 0.05. Therefore, the model was statistically insignificant [4].

The analysis of the obtained data thus indicated that use of the validated methodology at BHP concentration 0.25% demonstrated error free results. With the increase of the hydrolysate concentration up to 50% the demonstrated method can be used for the evaluation of the drug toxicity through the determination of the percentage of live cells in the suspension.

During the next stage, specificity of the method of testing BPHs for their biological activity was assessed during testing of 14 toxic samples on the suspension continuous BHK-21 cell line during three subsequent passages in triplicates. The results of the analysis are shown in Table 2.



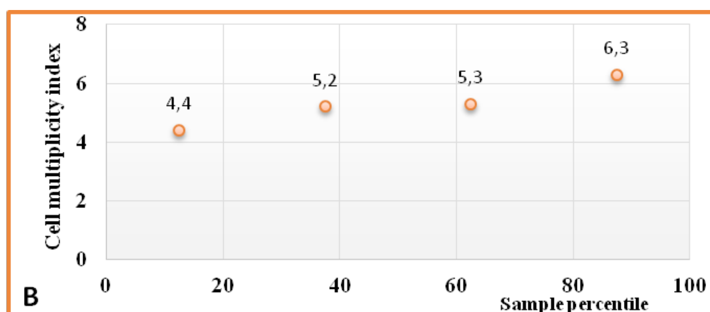


Fig. Accuracy assessment of the validated method during the analysis of biologically active samples containing BHP at concentration 0.25% (A) and 0.50% (B)

With hydrolysate concentration 0.25% the multiplicity index declined to 1.2–1.8 and the percentage of live cells amounted to 40–60%. With BPH concentration 0.50% the multiplicity index amounted to 0.7–1.5 and the percentage of live cells was between 25–42%. Thus, the analysis results demonstrated that all BPH samples were toxic for suspension BHK-21 cell line resulting in the cell deaths (percentage of live cells <60%) and decline of the multiplicity index (MI < 3.0) [10]. In view of the determination that all fourteen negative samples were toxic the conclusion was made that specificity of the validated method amounted to 100%.

During the next stage of the study, sensitivity of the method proposed for testing BPHs for their biological activity at concentration 0.25% was assessed while testing of 546 known proper hydrolysate samples. The results are shown in Table 3.

The data in Table 3 demonstrate that 76% of tested samples were defined as biologically active (4.5 < MI < 5.0) and 24% were considered as satisfactory (3.0 < MI < 4.5) for

Table 1
Trueness of the method for testing blood protein hydrolysates for their biological activity

No.	Sample characteristics		Multiplicity index within 5 passages /percentage of viable cells				
			Expected results	Obtained results			$M \pm m$
				in singlicate	in duplicate	in triplicate	
1	BPH 0.25% (batch 6)	Calf fetal serum, 5%	≥4.5/98–100	5.8/98	4.9/98	5.5/98	5.2 ± 0.3
2	BPH 0.50% (batch 6)	Calf fetal serum, 5%	≥4.5/98–100	5.2/99	4.4/99	6.3/98	5.3 ± 0.9
3	Calf fetal serum, 5%		<3.0/98–100	1.9/99	2.0/98	2.2/99	2.0 ± 0.1

Table 2
Specificity assessment of the method of testing BPHs for their biological activity and toxicity

No.	Multiplicity index during three passages/percent of live cells													
	Expected result		Obtained result											
			0.25% BPH						0.50% BPH					
			Passage 1		Passage 2		Passage 3		Passage 1		Passage 2		Passage 3	
1	<3.0	<60%	1.8 ± 0.1	60	1.7 ± 0.1	55–56	1.4 ± 0.2	42–44	1.5 ± 0.2	42–43	1.3 ± 0.1	40	1.0 ± 0.2	35–38
2			1.5 ± 0.1	58–60	1.5 ± 0.2	52–55	1.2 ± 0.1	45–46	1.3 ± 0.1	42–44	1.3 ± 0.2	40–42	1.1 ± 0.1	29–30
3			1.4 ± 0.1	55–58	1.3 ± 0.2	50–52	1.2 ± 0.3	45–48	1.2 ± 0.2	40	1.1 ± 0.2	40–43	0.8 ± 0.1	30
4			1.5 ± 0.2	58–60	1.3 ± 0.1	51–53	1.3 ± 0.1	40–45	1.4 ± 0.2	41–43	1.2 ± 0.2	38–39	1.0 ± 0.2	35
5			1.6 ± 0.1	59–60	1.5 ± 0.1	50–55	1.3 ± 0.1	42–44	1.5 ± 0.1	35–38	1.3 ± 0.2	31–34	0.9 ± 0.1	28–31
6			1.7 ± 0.1	60	1.5 ± 0.1	49–55	1.2 ± 0.2	40–42	1.5 ± 0.1	39–40	1.4 ± 0.1	33–39	1.0 ± 0.1	25–38
7			1.7 ± 0.2	56–57	1.5 ± 0.1	50–55	1.3 ± 0.1	40–45	1.5 ± 0.1	40	1.3 ± 0.3	35–41	0.9 ± 0.1	26–29
8			1.5 ± 0.1	54–58	1.3 ± 0.2	50–54	1.3 ± 0.1	41–43	1.3 ± 0.1	42–45	1.2 ± 0.1	35–38	0.9 ± 0.2	25–26
9			1.4 ± 0.1	55–60	1.3 ± 0.1	45–51	1.2 ± 0.1	40	1.3 ± 0.1	41	1.2 ± 0.1	35–36	0.8 ± 0.1	25–30
10			1.7 ± 0.1	5458	1.6 ± 0.1	46–50	1.4 ± 0.2	42–45	1.4 ± 0.1	40–44	1.0 ± 0.1	30–35	0.9 ± 0.1	29–31
11			1.7 ± 0.1	52–55	1.5 ± 0.2	44–48	1.3 ± 0.1	40–44	1.5 ± 0.1	42–44	1.2 ± 0.1	34–39	1.0 ± 0.2	28–30
12			1.6 ± 0.2	50–55	1.5 ± 0.2	45–50	1.2 ± 0.2	40–45	1.3 ± 0.1	36–37	1.1 ± 0.1	30–33	0.8 ± 0.1	29–34
13			1.6 ± 0.2	49–54	1.4 ± 0.2	45–50	1.2 ± 0.1	40–42	1.2 ± 0.2	35–40	1.0 ± 0.2	33–40	0.8 ± 0.1	25–28
14			1.6 ± 0.1	54–60	1.3 ± 0.1	47–48	1.2 ± 0.1	40	1.2 ± 0.1	36–42	1.0 ± 0.2	35–38	0.7 ± 0.1	28–32

production purposes. Thus, the analysis of known proper BPH samples all samples were defined as suitable for operation. This fact confirms that the proposed BPH biological activity test-system demonstrates high sensitivity.

During the next stage of the study, precision of the method under repeatability conditions was assessed by testing the samples in triplicates under the same measuring conditions (equipment, laboratory, 10-day interval, the same operator) except for different cell suspension (different passages).

Statistical analysis results are shown in Table 4 demonstrating that during testing of two serial BPH at 0.25% concentration in three subsequent passages the mean deviation (d_{mean}) amounted to 0.044–0.067. Therefore, in all samples MI_i variability degree is low against the mean value of the variable. Variation coefficient ($C_δ$) varied from 0.878 to 1.899 (≤2%) [1, 8] thus confirming high reliability of the results in each group of samples. Analysis of two 0.50% BPH replicates in three passages demonstrated d_{mean} = 0.044–0.089. In other words, the MI_{mean} degree of difference from the mean value is low, herewith the $C_δ$ value ranged from 0.918 to 1.768 (≤2%) [1]. Therefore, the test results of each sample group are reliable.

Precision of the method was also assessed under the reproducibility conditions. For this purpose two BPH batches were tested in triplicates under the same measuring conditions (equipment, cell suspension, laboratory), excluding human factor (the test was performed by two different operators at different time) and time interval (within 24 days). The results are demonstrated in Table 5.

Statistical analysis of two BPH batches at concentration 0.25% during three subsequent passages demonstrated mean linear deviation 0.030–0.060, and at concentration 0.50% – 0.027–0.043. Otherwise stated, MI_i degree of variation in the demonstrated observations is low. During the assessment of BPH at concentration 0.25% coefficient of variation ($C_δ$) varied from 1.185 to 2.234, during the assessment of BPH at 0.50% concentration – from 1.375 to

Table 3
Sensitivity of the test-system for BPH biological activity assessment (546 samples, *n* = 3)

Multiplicity index			Percentage of live cells in the suspension, %					
Expected result	Actual result		Expected result		Actual result			
	3.0–4.5	4.5–5.0			95–100	90–95	60–90	<60
>3.0	131	415	Passages 1–3	98–100	302	125	119	0
			Passages 4–5	>60	235	177	134	0

Table 4
Precision of the test-system for BPH biological activity assessment under repeatability and reproducibility conditions, 3 passages ($n = 3$)

No.	Properties of the tested sample	Statistic values								
		Highest value (MI_{max})	Lowest value (MI_{min})	Variation range (R)	Mean linear deviation (d_{mean})	Dispersion (δ^2)	Mean squared variation (δ)	Repetitive variation (V_R'), %	Linear coefficient of deviation (C_d)	Coefficient of variation (C_δ), %
1	BPH, batch 6, concentration 0.25%									
	Passage 1	5.1	5.0	0.1	0.044	0.002	0.047	1.974	0.877	0.930
	Passage 2	5.4	5.3	0.1	0.044	0.002	0.047	1.863	0.828	0.878
	Passage 3	5.1	5.0	0.1	0.044	0.002	0.047	1.987	0.883	0.937
2	BPH, batch 6, concentration 0.50%									
	Passage 1	5.2	5.1	0.1	0.044	0.002	0.047	1.948	0.866	0.918
	Passage 2	5.4	5.2	0.2	0.089	0.009	0.094	3.750	1.667	1.768
	Passage 3	4.9	4.8	0.1	0.044	0.003	0.047	2.055	0.913	0.969
3	BPH, batch 53, concentration 0.25%									
	Passage 1	4.1	4.0	0.1	0.044	0.002	0.047	2.459	1.093	1.159
	Passage 2	4.4	4.3	0.1	0.044	0.002	0.047	2.308	1.026	1.088
	Passage 3	4.4	4.2	0.2	0.067	0.007	0.082	4.651	1.550	1.899
4	BPH, batch 53, concentration 0.50%									
	Passage 1	4.2	4.1	0.1	0.044	0.002	0.047	2.400	1.067	1.131
	Passage 2	4.4	4.3	0.1	0.044	0.002	0.047	2.290	1.018	1.080
	Passage 3	4.3	4.2	0.1	0.044	0.002	0.047	2.362	1.050	1.114

1.913 (≤3%) [1, 8], that indicated insignificant degree of deviation from arithmetic mean and high reliability level of the results in each group of samples.

Comparative analysis of the data obtained during the precision assessment demonstrated that under repeatability conditions the degree of reliability of the BPH activity assessment result was higher as compared to the assessment performed under the reproducibility conditions that was consistent with the well-known statistical expectations [1]. Herewith, the method was compliant with the acceptance criteria as for all absolute and relative variations.

CONCLUSION

Basic validation properties of the method for blood protein hydrolysate biological activity and toxicity presence/absence were assessed for its further use in the analysis of raw materials for FMD vaccine manufacture.

During the use of non-toxic BPH at concentrations 0.25% and 0.50% the BHK-21 multiplicity intensity amounted to >3.0 that was consistent with the requirements of the developed method. Testing of 560 samples of BPH concentrations 0.25 and 0.50% using the developed method demonstrated that use of hydrolysate at concentration 0.25% resulted in Fisher's criterion value 0.021 (<0.05). Otherwise stated, the demonstrated model was significant and confirmed the validity of the method being validated. After the increase of the hydrolysate amount to 0.50% the demonstrated test can be used for assessment of the preparation toxicity by means of determination of the live sell percentage in the suspension.

Testing of 546 biologically active and satisfactory samples as well as 14 toxic BPH samples using the method being validated confirmed high sensitivity and specificity of the demonstrated test-system.

Analysis of the precision under repeatability and reproducibility conditions demonstrated that the obtained values of the tested variation parameters were consistent with the statistical expectations as for the method trueness and meet the acceptance criteria.

The proposed method is valid and can be used for biological activity determination and assessment of BPH toxicity during testing of raw materials for FMD vaccine manufacture.

Conflict of interests. The authors declare no conflict of interest.

Table 5
Precision of the test-system for BPH biological activity assessment under reproducibility conditions und performance by different operators ($n = 3$)

No.	Properties of the tested sample	Statistic values								
		Highest value (MI_{max})	Lowest value (MI_{min})	Variation range (R)	Mean linear deviation (d_{mean})	Dispersion (δ^2)	Mean squared variation (δ)	Repetitive variation (V_R), %	Linear coefficient of deviation (C_d)	Coefficient of variation (C_δ), %
1	BPH, batch 6, concentration 0.25%									
	Passage 1	6.0	5.5	0.5	0.037	0.007	0.086	8.646	0.634	1.489
	Passage 2	6.0	5.5	0.4	0.030	0.005	0.071	6.897	0.517	1.219
	Passage 3	6.0	5.5	0.4	0.030	0.005	0.068	6.936	0.520	1.185
2	BPH, batch 6, concentration 0.50%									
	Passage 1	4.7	5.1	0.4	0.035	0.005	0.074	8.136	0.712	1.497
	Passage 2	4.7	5.0	0.4	0.027	0.005	0.068	8.054	0.537	1.375
	Passage 3	4.6	5.0	0.5	0.043	0.009	0.093	10.247	0.890	1.913
3	BPH, batch 53, concentration 0.25%									
	Passage 1	4.9	4.9	0.3	0.030	0.004	0.061	6.338	0.634	1.279
	Passage 2	4.5	5.0	0.5	0.060	0.011	0.107	10.453	1.150	2.234
	Passage 3	4.6	5.0	0.4	0.040	0.008	0.089	8.333	0.833	1.863
4	BPH, batch 53, concentration 0.50%									
	Passage 1	4.4	4.7	0.3	0.033	0.004	0.066	6.498	0.722	1.440
	Passage 2	4.3	4.7	0.4	0.040	0.007	0.082	8.824	0.882	1.801
	Passage 3	4.5	4.8	0.3	0.027	0.003	0.058	6.498	0.578	1.266

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