Development and validation of an ATP method for rapid estimation of viable units in lyophilised BCG Danish 1331 vaccine

Sten E. Jensen a,*, Peter Hubrechts a, Bjarke M. Klein b, Kaare R. Hasløv a

a Quality Control Department, Statens Serum Institut, 5 Artillerivej, DK-2300 Copenhagen S, Denmark
b Biostatistics Unit, Statens Serum Institut, 5 Artillerivej, DK-2300 Copenhagen S, Denmark

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Abstract

An assay for quantifying viability in BCG vaccine by determining intracellular ATP content was developed and validated. ATP content was determined by measuring bioluminescence in the presence of luciferin/luciferase. During development and validation the ATP method was compared to the conventional viable count method. A key step to obtain correlation between ATP content and CFU was found to be a period of pre-incubation in a growth medium before ATP determination. During the validation, the robustness, linearity, accuracy, precision, and range were studied. The method validation study showed that the method applied was robust and applicable to determine ATP content in lyophilised BCG for estimating viability in the BCG samples. By comparison with a conventional viable count method, a high correlation between ATP content and the viable count was found; this relationship can be applied in routine quality control to estimate viable count from the ATP content determined in a sample.

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Keywords: ATP assay; Validation; BCG viability; Rapid alternative microbiological method; Colony forming units

1. Introduction

The current vaccine for control of the tuberculosis disease is the Mycobacterium bovis bacillus Calmette Guerin (BCG) vaccine. It is a vaccine consisting of live bacteria derived from an attenuated strain of M. bovis [1].

One of the key tests on BCG vaccine is to determine the viability of the BCG in the product. The conventional method for estimating viability in vaccines containing lyophilised BCG is to perform a test for colony forming units (CFU). In this test, a suspension of the BCG is inoculated onto a solid medium and after a period of incubation the number of colonies is counted. One of the main disadvantages of the viable count test is that it is very slow; depending on the growth medium, it may require an incubation period of up till five weeks before the number of colonies can be counted.

Another method for determining viability in bacterial samples is to measure the content of intracellular ATP [2]. The ATP can be quantified by measuring bioluminescence in the presence of luciferin/luciferase [3], a method recently introduced in the European Pharmacopoeia 5.5 as an alternative method for control of microbiological quality [4]. The method has been applied to BCG and the first studies were reported in the 1970s [5,6]. In the following years several studies on measuring ATP content in BCG were reported [7–12], and more recently an improved method for extraction of ATP from BCG has been described [13]. In some of these studies the ATP content measured was compared to viability determined by count of viable units. However, none of the studies reports on a validated ATP method that can replace the viable count method for use in quality control in a GxP (i.e. good laboratory practice, good manufacturing practice or good clinical practice) regulated environment.

Alternative methods must be validated and compared to the conventional method before they can be implemented for use in quality control. Here we report on the development and
validation of a method for determining intracellular ATP in lyophilised BCG, and describe the correlation between the proposed ATP method and a conventional CFU method. The present study is, to our best knowledge, the first to demonstrate a highly significant quantitative correlation between the CFU test and a validated rapid biochemical test.

2. Materials and methods

2.1. BCG samples

BCG Vaccine SSI, Danish strain 1331 (Statens Serum Institut, Copenhagen, Denmark) in vials was used. Each vial contained 0.75 mg lyophilised BCG and 3.75 mg sodium glutamate. Samples from production scale batches were used in the study. Samples of different BCG vaccine batches were stored for different length of time and at different temperatures before analysis to have BCG samples with different viability. Low viability samples: BCG treated at 37 °C in the study. Samples from production scale batches were used to test and a validated rapid biochemical test.

2.2. Analytical reagents

Dubos medium with 0.045% Tween 80 and 0.5% bovine albumin (SSI Diagnostika, Hillerød, Denmark). Tris-acetate buffer (0.1 M) with 2 mM EDTA, pH 7.75 (SSI Diagnostika, Hillerød, Denmark). ATP Reagent SL (BioThema, Haninge, Sweden); the reagent was reconstituted with 10 ml water for injection (Statens Serum Institut, Copenhagen, Denmark). ATP Standard, 1 × 10⁻³ M (BioThema, Haninge, Sweden); dilutions of ATP Standard were made in Tris-acetate buffer with EDTA; working standard solutions were stored at ≤−18 °C. Diluted sauton SSI for reconstitution of lyophilised BCG (Statens Serum Institut, Copenhagen, Denmark). Apyrase (Sigma–Aldrich Co., St. Louis, MO, USA); working solutions of 5 units/ml of apyrase were prepared in Diluted Sauton SSI containing 8 mM CaSO₄ (Merck KGaA, Darmstadt, Germany) and stored at ≤−18 °C. Löwenstein–Jensen medium in glass tubes (SSI Diagnostika, Hillerød, Denmark).

2.3. Determination of ATP in BCG

2.3.1. ATP extraction, samples without pre-incubation

Each vial containing lyophilised BCG was reconstituted with 400 µl diluted sauton SSI (the solution used for reconstitution of BCG Vaccine SSI before vaccination) and 100 µl apyrase (to degrade extracellular ATP present in the lyophilised product) working solution; thus the total volume for reconstitution was 500 µl and it gave a suitable concentration of BCG for ATP extraction. Ten minutes after reconstitution, the ATP was extracted from the BCG bacteria. Extraction was performed in Tris-acetate buffer with EDTA; the buffer, 500 µl, was preheated to 98–99 °C. From each vial 3 × 100 µl BCG suspension was withdrawn and added directly to 3 × 500 µl preheated buffer. The BCG-buffer mixture was kept at 98–99 °C for 6 min. After heat treatment, the resulting extracts were allowed to cool to room temperature.

2.3.2. ATP extraction, samples with pre-incubation at 37 °C

Each vial containing lyophilised BCG was reconstituted in a full mycobacterial growth medium, the Dubos medium with Tween and bovine albumin; 1.0 ml medium was added per vial and it could support sufficient growth of the BCG bacteria. In a laminar flow hood, medium was added through the rubber stopper of the vial with a needle and syringe. The content was mixed and the rubber stopper removed to allow airing of the vial for 10–15 min before reseating the vial with the rubber stopper. The object of airing is to vent N₂ filled in the vials during lyophilisation. After reconstitution, the vials were, unless otherwise specified, incubated 24 h at 37 °C before ATP was extracted from the BCG bacteria. From each vial 100 µl BCG suspension was withdrawn for ATP extraction. Otherwise, extraction was performed as described in Section 2.3.1.

2.3.3. Measurement of ATP

To the cooled ATP extract (100 µl BCG suspension + 500 µl buffer), 100 µl ATP reagent was added and the luminescence of the sample was measured in a 1251 Luminometer (Bio-Orbit Oy, Turku, Finland) at 25 °C. The luminescence signal was recorded as integrated signal over 10 s (mV × 10 s). As blanks, 100 µl Sauton/apyrase or Dubos medium was heat treated in buffer as described for BCG; after addition of 100 µl ATP reagent the background luminescence was measured. The amount of ATP extracted from the BCG samples was calculated from a standard curve of ATP. The ATP standard concentrations were prepared by adding 10 or 20 µl of the ATP working standards to blanks containing ATP reagent. After mixing the ATP standard solutions, the luminescence was measured. For each BCG sample, the ATP extracted was measured in four or eight vials and the average amount of ATP extracted determined.

2.4. Determination of CFU

Eight BCG vials were reconstituted in diluted sauton SSI to a concentration of 0.75 mg BCG/ml. The BCG suspensions from the eight vials were pooled and the suspension diluted in Diluted Sauton SSI. Three serial dilutions of the suspension were inoculated onto Löwenstein–Jensen medium and incubated at 37 °C for 5 weeks before counting and calculating the number of CFU [14]. The Löwenstein–Jensen medium was used for the CFU determinations as this medium is a well-established solid medium for viability tests on BCG Vaccine SSI. Other solid media may also be applicable [14].

2.5. Establishment of correlation ATP–CFU

BCG samples with different viability were assayed with both the ATP method (two studies, ±pre-incubation in growth medium) and the CFU method. The viability of the samples...
covered a range from low viability in samples heat-treated 28 days at 37 °C to high viability, samples of newly prepared lyophilised BCG stored refrigerated for a short period. In the study of the ATP method with pre-incubation, samples with intermediate viability (stored 12–18 months at 4 °C) were also included.

2.6. Validation of the method with pre-incubation step

2.6.1. Robustness

The assay robustness to changes in eight different factors on the ATP content measured was assessed. The investigation was carried out in a fractional factorial design. The eight factors were studied in two designs and each design contained four factors with two levels. JMP® 6.0 statistical software was used to make the design. The designs were balanced and each design had two blocks (assay runs) and in each block the ATP content in 16 BCG vaccine vials was determined. Vials of a BCG vaccine sample with intermediate viability were used in the study. In the first design four factors relating to the pre-incubation of samples in growth medium before ATP extraction were studied: (1) time span for airing of vials prior to incubation, (2) temperature during incubation, (3) incubation time—time span for incubation, and (4) the pH in the incubation medium. In the second design four factors relating to ATP extraction before measurement of luminescence were studied: (1) time to preheat extraction buffer, (2) temperature of extraction buffer, (3) time span of heat treatment during ATP extraction, and (4) covering of heated buffer-sample in cuvettes during extraction—cuvettes non-covered or covered with aluminium foil to minimise evaporation.

2.6.2. Linearity of ATP standard and of ATP extracted from BCG

Linearity of the ATP standard was studied by adding working standard solutions of different concentrations so the final range covered was 1.0–25 × 10⁻¹² mol ATP per cuvette. Linearity was also assessed for ATP extracted from BCG. In this investigation the total BCG concentration (live and dead bacteria) in the sample was held constant by addition of heat-killed BCG and only the concentration of extracted ATP was varied. In this way, the samples prepared for testing linearity of the ATP response from BCG, resemble samples for testing viability in a BCG vaccine. To cuvettes containing 500 μl preheated Tris-EDTA buffer, 100 μl BCG suspension was added and the intracellular ATP extracted from BCG by heat treatment. From each vial with BCG preincubated in Dubos medium, a volume of 20, 40, 60, 80 and 100 μl BCG suspension was assayed; for the volumes 20–80 μl, the BCG sample volume was adjusted to 100 μl by addition of heat-killed BCG in Dubos medium. Four vials with different levels of viability were assayed to obtain dose-response curves with responses overlapping to cover a larger response range; the vials were incubated in Dubos growth medium for more than 24 h to obtain samples with higher ATP response. An ATP standard curve covering the range 1.0–15 × 10⁻¹² mol ATP/cuvette was included in the assay of each vial to convert the BCG-ATP response to mole ATP.

Double determinations were made at each concentration of ATP standard and ATP extracted from BCG.

2.6.3. Accuracy

During development of the ATP method, a correlation between ATP content using pre-incubation and numbers of CFU was established (cf. Section 2.5). For this study of accuracy, new samples were assayed with these two methods, and the results were compared to the previously found relationship between ATP content and numbers of CFU. From the data (n = 35) obtained during development, a figure with the regression line and 95% prediction limits was made. Data for the new samples were plotted in this figure.

2.6.4. Precision

The study of precision was carried out by three analysts on three days. A factorial design was employed to assess repeatability and intermediate precision for samples with low viability (a BCG vaccine heat-treated 28 days at 37 °C) and high viability (a newly prepared BCG vaccine stored refrigerated for a short period). Repeatability: in each assay run two samples (2 × 8 vials) of the same sample type were assayed and the coefficient of variation (CV) for repeatability was calculated. Intermediate precision: data from the design were analysed, and the variation between the reportable values and the overall CV for intermediate precision calculated.

2.7. Data analysis

Calculation of the ATP content of a vial was done in an Excel spreadsheet (Microsoft® Office Excel 2003 SP1); linear regression analysis were done on the standard curve data and the ATP content of a vial calculated and expressed as ng ATP per vial. Calculation of the number of CFU was done in an Excel spreadsheet using the formulae in the World Health Organisation (WHO) technical guide 77.9 [14].

The data of the robustness study were analysed with the JMP® 6.0 statistical software to determine if the studied levels of a factor had a significant effect on the ATP content measured in the sample.

Otherwise, analyses of correlation (Pearson product-moment), calculation of CV, simple linear regression, predictions limits for regression line, plot of residuals and QQ-plot were done in SAS® 9.1.3 SP4 for windows.

3. Results

3.1. Establishment of the ATP–CFU correlation

To obtain data for assessing the correlation between viability determined by the ATP method and the CFU method, samples of lyophilised BCG with different levels of viability were assayed. On each sample the ATP content and the number of CFU were determined. The ATP content in the first study of correlation was determined after reconstitution of the
lyophilised BCG in diluted sauton medium and no incubation step before ATP determination (Fig. 1). The data show a significant positive correlation \( (P < 0.01) \) between ATP content and CFU number with a coefficient of correlation, \( r = 0.53 \). However, for the ATP method, the levels of ATP content of the low and high viability samples were overlapping.

To improve the discrimination between ATP content determined in samples with low and high viability, the introduction of an incubation step in growth medium before ATP determination was studied (Fig. 2). The data show a significant positive correlation \( (P < 0.01) \) between ATP content and CFU number with a coefficient of correlation, \( r = 0.93 \). Thus, pre-incubation of lyophilised BCG in growth medium significantly improved the ATP–CFU correlation.

3.2. Method validation

A method validation study to examine robustness, linearity, range, accuracy and precision was conducted on the ATP method that included a 24 h pre-incubation step in growth medium before ATP extraction.

3.2.1. Robustness

The robustness of the method was studied in factor designed experiments. The factors, the levels studied and the results are shown in Table 1. For each factor, the method was shown to be robust to variations in the levels studied; at \( \alpha = 0.05 \) no significant effect on the ATP content measured could be demonstrated.

3.2.2. Linearity

Linearity of the ATP standard was studied in the range \( 1.0 - 25 \times 10^{-12} \) mol ATP in four independent experiments. Simple linear correlation analysis on the dose-response data gives correlation coefficients, \( r \geq 0.99 \) for all four experiments (data not shown).

Curve fit was also assessed for ATP extracted from BCG. In four independent experiments (one vial per experiment), vials with different levels of viability were assayed to examine an extended range of the dose–response curves. Analysis of the linear relationship for dose-response for ATP extracted from BCG assayed at constant concentration of BCG in the sample shows correlation coefficients, \( r \geq 0.99 \) for all four curves. Data for one of the curves in the medium range are shown in Fig. 3.

3.2.3. Range

An ATP standard curve in the range \( 1.0 - 15 \times 10^{-12} \) mol ATP was included in the assay of each vial in the linearity study of ATP extracted from BCG. The responses from the BCG samples extended beyond the lower and upper responses from the standard curve, i.e. the vials with lower viability had

![Fig. 1. Correlation between ATP content and number of CFU. The BCG samples were not pre-incubated in growth medium before ATP determination. Samples \( (n = 43) \) with low viability (●) and high viability (▲) were assayed for ATP content (ng ATP per vial) and for viable units (10^6 CFU per vial).](image1)

![Fig. 2. Correlation between ATP content and number of CFU. The BCG samples were pre-incubated 24 h in growth medium before ATP determination. Samples \( (n = 35) \) with low viability (●), intermediate viability (▲) and high viability (■) were assayed for ATP content (ng ATP per vial) and for viable units (10^6 CFU per vial).](image2)

![Table 1

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<th>Factor</th>
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<td>B. Factors relating to extraction of ATP</td>
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responses starting below the ATP standard curve for the smallest volumes and the vials with higher viability had responses that were above the ATP standard curve for the largest volumes. Thus the study of linearity indicates that the range of the assay is at least \(1.0 \times 10^{15}/C_2\) mol ATP for 100 \(m\)l BCG suspension assayed. This range corresponds to \(10^{150}/C_2\) mol ATP per BCG vaccine vial.

3.2.4. Accuracy

Based on the data from development of the method with 24 h pre-incubation (Fig. 2), a figure with a line that shows the expected relationship between logarithmic transformed (log) ATP content and log CFU number was made (Fig. 4). The dotted lines show the 95% prediction limits based on the 35 observations; if the relationship between ATP and CFU found during development is reasonable, then at least 95% of the observations from the accuracy study should be within the prediction limits. Fig. 4 shows that all of the 15 new observations were within the prediction limits.

The relationship between log ATP content and log CFU number for all the 50 observations obtained during development and validation of the method with pre-incubation is shown in Fig. 5. The quantile-quantile plot of residuals (Fig. 6A) and the plot of residuals versus fitted log(CFU) observations (Fig. 6B) indicate that for logarithmic transformed data the assumption of a normal distribution and homogeneity of variance is reasonable. When analysis of correlation is performed on all of the data from development and validation, a significant positive correlation \((P < 0.0001)\) is found between ATP content and number of CFU with correlation coefficients of \(r = 0.928\) and 0.934 for untransformed and logarithmic transformed data, respectively. From these data \((n = 50)\) an equation for the relationship between ATP content and CFU can be derived as: \(\log(\text{CFU}) = -1.426 + 1.398 \log(\text{ATP})\) or \(\text{CFU} = 0.037 \times \text{ATP}^{1.398}\).

3.2.5. Precision

Results from the study of precision are shown in Table 2. From these data CV for repeatability and intermediate precision were calculated. For samples with low viability CVs of

![Fig. 3. Relationship between dose and response for ATP extracted from BCG. The total BCG concentration (live and dead bacteria) was held constant by addition of heat-killed BCG and only the concentration of extracted ATP from live BCG (dose) was varied. The linear regression line is included. Data from one experiment in the medium response range.](image1)

![Fig. 4. The linear relationship (straight solid line) between log(ng ATP per vial) and log(10^6 CFU per vial) with 95% prediction limits (dotted lines) illustrated for the 35 observations obtained during method development. New samples \((n = 15)\) were assayed for ATP content and viable units and data plotted in the diagram: five samples with low (○), intermediate (+) and high (■) viability, respectively. All BCG samples were pre-incubated 24 h in growth medium before ATP determination.](image2)

![Fig. 5. Correlation between logarithmic transformed data for ATP content and number of CFU for all the observations \((n = 50)\) obtained during development and validation of the ATP method. Samples with low (○), intermediate (▲) and high (■) viability were assayed for ATP content (ng ATP per vial) and for viable units (10^6 CFU per vial). For ATP determination, all BCG samples were pre-incubated 24 h in growth medium.](image3)
5.5% and 15% were found for repeatability and intermediate precision, respectively. For samples with high viability CVs of 5.1% and 9.0% were found for repeatability and intermediate precision, respectively.

4. Discussion

The estimation of viability in BCG vaccines is a pivotal test which is used to document quality and stability of the vaccine. The current test to estimate viability, count of viable units, is slow (especially with Löwenstein–Jensen medium), labour-intensive and highly dependent on the choice of substrate and other technical parameters. Thus introduction of a rapid biochemical test to replace it would be preferable both in routine quality control of BCG and in development of new live TB vaccines of mycobacterial origin. To make such methods work for quality control one must look at the concepts behind the current assay.

The test for count of viable units reflects the properties on a solid growth medium of the freeze-dried BCG vaccine in its final container. In this state there is potentially a lot of metabolic activity preserved by freeze-drying that will be lost upon seeding on a solid medium. Only those bacteria that have sufficient metabolic activity to support growth on the chosen solid medium will form colonies. Hence, a biochemical method performed directly on the freeze-dried BCG vaccine do not stand a good chance to be successfully validated against the gold standard of BCG vaccine quality control, the count of viable units method. This becomes clear when biochemical methods are evaluated on accelerated stability samples made by exposure to 37 °C. Samples exposed to this temperature for 4 weeks may encounter a reduction in viability up to 80% in terms of CFU, which is the limit indicated in the European Pharmacopoeia and the WHO requirements. Biochemical methods based, e.g., on enzyme activity alone may, as shown in the present study, for such samples show only a small and some times indistinguishable decrease in activity. Therefore, a very important marker in evaluation of the applicability of any rapid assay for viability is the ability to detect a significant reduction in viability in accelerated stability samples.

In the present study, an assay of the content of intracellular ATP was developed as an alternative biochemical method to estimate viability in BCG. During development of the ATP method, it was compared with a conventional CFU method for count of viable units. For BCG samples with different viability, a high correlation must be demonstrated between ATP content and number of CFU. An essential step to obtain high correlation between ATP content and CFU was found to be a period of pre-incubation in a growth medium before ATP determination. By the introduction of this step, the BCG bacteria will be in an active growth phase at the point of extraction and measurement of intracellular ATP. Compared with an ATP assay without pre-incubation in growth medium, the correlation between ATP content and number of CFU was markedly improved by the introduction of the pre-incubation step. Moreover, a clear discrimination in viability was found between low viability samples treated at 37 °C for 4 weeks and high viability (untreated) samples of the same vaccine batch; this heat treatment of samples is a compulsory quality control test for stability of the vaccine. Assay of intracellular ATP

![Fig. 6. For the logarithmic transformed correlation data (n = 50), A: quantile-quantile plot of residuals with normal line, and B: plot of residuals versus fitted log(CFU) observations for samples with low (●), intermediate (+) and high (■) viability.](image)

Table 2

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A. Low viability samples

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Assay runs by three different analysts on three different days; two reportable values (ng ATP/vial) per assay run. A: samples with low viability (a BCG vaccine heat-treated 28 days at 37 °C); B: samples with high viability (a newly prepared BCG vaccine).

a Assay runs on separate days. The samples with low or high viability were assayed on different days.
without pre-incubation in growth medium gave overlapping ATP levels between heat-treated and untreated samples and could therefore not be used for routine quality control purposes. In contrast, the method variation with pre-incubation of samples in growth medium worked well in this respect.

As an alternative method, the ATP method must be validated and compared to the conventional method for count of viable units [16]. The ATP method was subjected to a formal method validation. During validation the robustness, linearity, accuracy, precision, and range were studied. No separate experiments were done to study specificity of this ATP method. The luciferase enzyme used in the method is specific for ATP [18], but not for BCG. Because only samples containing BCG will be assayed, there is no point in demonstrating specificity using a range of appropriate micro-organisms as suggested in the European Pharmacopoeia [4]. Instead, specificity of the ATP method for estimating viability in BCG was demonstrated by comparison with viability estimated by an independently established method, the count of viable units method. Such a comparison will also show if interfering factors affect the correlation between ATP content and number of CFU. Indeed, the data and results obtained from the study of accuracy on pre-incubated BCG samples with different levels of viability showed a high correlation between viability estimated by the ATP method and the count of viable units method, clearly demonstrating that the ATP method gives a signal that specifically can express viability in BCG. This analysis of correlation was based on observations of ATP content and CFU number determined in 50 different BCG samples. From the data obtained, a model for the relationship between ATP content and number of CFU was established. In this model, the correlation between ATP content and number of CFU shows a linear relationship between logarithmic transformed ATP content and logarithmic transformed CFU number. The assumptions of the model were tested and plots of residuals showed no indications of bias in the model.

Linearity of the ATP response for ATP extracted from BCG was demonstrated in the range 10–150 x 10^{-12} mol ATP per BCG vaccine vial, corresponding to 5–76 ng ATP per vial; the samples assayed in the studies of precision and accuracy were all within this range. Sample types of both low and high viability showed acceptable repeatability and intermediary precision, which was better than that found for the colony count method (data not shown). ATP content and viable units showed high correlation in the range 8–45 ng ATP per vial. This range corresponds to a range for viable units of 0.7–7.6 x 10^6 CFU per vial when ATP content is converted to number of CFU with the equation CFU = 0.037 x ATP^{1.398} (cf. Section 3.2.4). The documented range covers for practical purposes the specification for BCG Danish 1331, which is 2–8 x 10^6 CFU per vial. It is, however, reasonable to expect that the correlation is likewise good in the full linear range of 5–76 ng ATP per vial.

In conclusion, we have developed a rapid, robust and precise ATP method for estimation of BCG viability. Using this method, we have established a model resulting in a close correlation with the count of viable units method. This model also allows precise calculation of the viability reduction in samples heat-treated at 37 °C for 4 weeks. The ATP method is therefore suitable for routine BCG quality control purposes, although its continued relationship to the count of viable units method should be demonstrated at suitable intervals.

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