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# Control charts for identifying systematic errors using control sera to detect antibody to *Salmonella* in an indirect ELISA

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**Summary**
This study evaluated the preparation of Shewhart's control charts using the concept of rational subgroups for monitoring the *Salmonella* antibody ELISA used for surveillance of Danish pig herds. Control charts were prepared for a buffer control sample, a negative serum sample and a positive serum sample. The quality control variables were the natural logarithm (ln) of the uncalibrated optical density (OD) for the buffer control sample and the negative serum sample, and the calibrated OD (OD%) for the positive serum sample. Testing round (run) within laboratory robot was chosen as the subgroup, and separate control charts were prepared for five robots. The control limits were set at six times the standard deviation for ln(OD) and three times the standard deviation for OD%. Evaluation based on a number of sensitising rules for control charts produced from historical data showed that use of the control charts could reveal systematic analytical errors.

**Keywords**
Control chart – ELISA – Quality control – Rational subgroup – Salmonella – Surveillance.

## Introduction

Quality control procedures are essential to diagnostic laboratories, because they provide confidence in the results from control samples and give information on unacceptable trends in performance (15). In the statistical component of quality control (QC), Shewhart's control charts are important tools (7, 9, 11), because these charts can differentiate systematic variation from random variation (7, 9). For continuous variables, Shewhart introduced x- and s-charts, in which the arithmetic mean ($\bar{x}$) of a sample is used as an estimate of the true mean of the distribution ($\mu$) and the sample standard deviation s is an estimate of the true standard deviation ($\sigma$). The control charts consist of a central line, which is the average of the sample averages, and upper and lower control limits (UCL and LCL) indicate the expected variation when the process is in statistical control (7, 8, 17). A key element in the Shewhart procedure is to sample data in rational subgroups according to known sources of variance (17). In the evaluation of the control charts, a number of sensitising rules can be applied. In general, at least one rule should be selected to reveal systematic errors, and another rule should be able to detect an increase in random errors (19). Sampling in rational subgroups maximises the chance for shifts in the process to occur between samples, thereby increasing the possibility of detection of systematic errors on the charts (11).

Despite the fact that the enzyme-linked immunosorbent assay (ELISA) has been widely used since the 1970s, relatively little has been published on methods for QC (20). Preparation of control charts has been described for some ELISAs (4, 5, 8, 14). None of these papers used the concept of rational subgroups, probably because these

assays all processed relatively few test samples each day. The ELISA is readily adapted to automation and hence to large-scale testing programmes. Advances in ELISA technology, including miniaturisation and mechanisation, have resulted in its application to the detection of infection caused by several pathogens of humans, poultry, pigs and cattle. Recently, ELISA technology has been applied to detecting *Salmonella* infections in poultry, cattle and pigs on a large scale (3).

The objective of this study was to evaluate whether control charts using rational subgroups would be useful for QC of an ELISA in a large-scale testing programme. As an example, data from the *Salmonella* antibody ELISA (12) used for surveillance in Danish pig herds (1) were used. The *Salmonella* ELISA processes more than 600,000 samples a year (10) and, therefore, control charts for the *Salmonella* ELISA could be produced from subgroups of the control sample results, thereby applying the important concept of rational subgroups. A severe technical error (malfunction of an automatic ELISA washer) occurred in 2003. The fact that this error was undetected for more than a year (10) emphasised the need for improvement of the QC procedures. However, it also provided an opportunity to test the control chart strategy on historical data from a period where sufficient QC procedures should have been able to detect errors.

# Materials and methods

## Control samples

An indirect ELISA that detects antibodies to the *Salmonella* O-antigen factors 1, 4, 5, 6, 7 and 12 was used (12). Five control samples were analysed in duplicate at fixed positions on every microtitre plate. These comprised a buffer control sample, a negative serum sample from a pig not infected with *Salmonella*, two positive serum samples from pigs infected with *Salmonella* Infantis and a positive serum sample from a pig infected with *S.* Typhimurium. The last serum sample was introduced into the study in January 2004.

## Control of results

For calibration of sample results, four standard sera from pigs infected with *Salmonella* Infantis were included on every microtitre plate. Optical density (OD) readings for serum samples were corrected for inter-plate variation by converting into corrected optical densities (ODCs) using linear regression analysis of the OD values of the negative control and the four *S.* Typhimurium standard sera from the same plate (13). The normative OD values used in the regression analysis were obtained as the median OD values of these standards as results accumulated over a period of time. The ODCs were expressed as OD% on a fixed linear scale with the normative value of the negative control at –1.520 OD% and the normative value of the highest *S.* Typhimurium control at 80.102 OD% (Lars Ekeroth, personal communication). The QC programme included approval/rejection of individual microtitre plates based on acceptance limits for the OD% of the sera used for the regression analysis, the OD% of the control sera and the coefficient of variation between control sera analysed in duplicate.

## Data selection and data control

Data for the preparation of control charts were extracted from the laboratory database. The variables were: date, multi-probe robot, microtitre plate, well number on microtitre plate, OD and OD%. Data originated from the years 2003 and 2004, and data control was carried out for one year at a time. All variables were checked for missing values, and the data for the variables 'robot' and 'well number' were checked for illegal values. Intervals of dates and plate numbers were listed, and results from microtitre plates that had been discarded by the existing QC were excluded.

## Choice of quality control variables

Control charts were prepared for three of the control samples, the buffer control sample (buffer control), the negative serum sample (negative serum) and the *S.* Typhimurium positive serum sample (positive serum). For the buffer control and negative serum, the OD was used as the test result, whereas the OD% was used for the positive serum. The observations included in the subgroups for the control charts were the individual control sample results, thereby giving two observations per control sample per microtitre plate. The two positive control sera from pigs infected with *S.* Infantis could not be included in the control charts for these historical data, because the OD% had not been calculated for the individual samples but only for the duplicate.

It is preferable that QC variables are normally distributed (7, 17). Therefore, the normality of the OD and OD% was evaluated using histograms and the Kolmogorov–Smirnov test for normality. This test can be used to determine whether two sets of data could come from the same distribution, or whether a set of data could originate from a standard distribution (in this case the normal distribution). The raw data and the simple transformations natural logarithm (ln) and square root were considered, and normality was tested for individual observations and for the mean within each microtitre plate.

## Preparation of control charts

The charts were produced using PROC SHEWHART in the SAS system. In PROC SHEWHART, several options are available for preparation of control charts, each giving rise to a different chart. For this study, different combinations of options were used to prepare control charts with varying approaches regarding the subgroup, control limits and sensitising rules. The procedure has been described by the SAS Institute Inc. (16), and version 8 of the SAS program was used.

## Choice of subgroup

Subgroup candidates were chosen according to known sources of variance. These sources could be found in the hierarchical structure of the process (Fig. 1). Each day a number of multi-probe robots were started simultaneously in one or more testing rounds (runs), and on each robot three microtitre plates were analysed at a time. Other possible sources of variance (e.g. change of reagent batches or technician) appeared less systematically, and they could not be included in the subgroup considerations. From the hierarchy, the levels 'robot' and 'run' were chosen as subgroup candidates.

As it is important for the calculation of control limits to have many subgroups on a control chart with the same number of observations, the most frequent number of observations and the frequency of this number of observations were counted in the data set from 2004. Furthermore, data from 2004 were used to prepare control charts using the candidates as subgroups, and a visual inspection of the x-charts made with different subgroups was used to determine whether some of the subgroups revealed more variation than others. Control charts made with intermittent sampling from different robots, or from different runs, were prepared to examine whether such a sampling procedure would reveal differences between robots or runs.

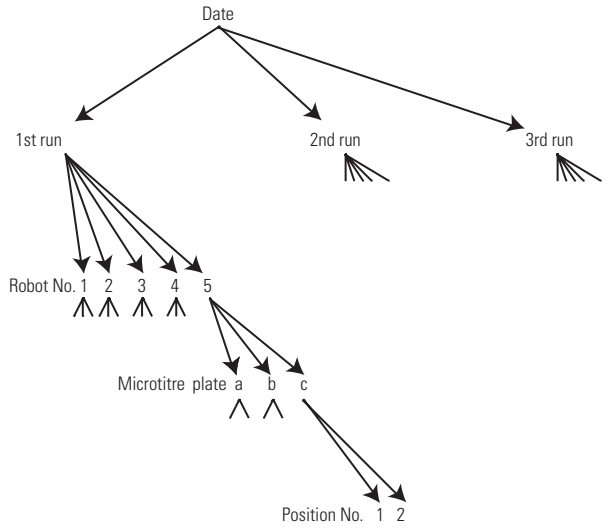



**Fig. 1**
**Hierarchical structure of sources of variance for the antibody enzyme-linked immunosorbent assay used in the surveillance programme for *Salmonella* in Danish slaughter pigs**

For comparison of parallel control charts for different robots an analysis of variance (ANOVA) was performed using the mean of the subgroups. The level of significance was set at $\alpha$=0.05, and in case of a significant difference, pairwise comparisons were made to identify the differing robot(s). For these comparisons the p-value was reduced to 0.05/10=0.005 according to the number of pairs (Bonferroni adjustment) (18).

## Choice of control limits

Upper and lower control limits were calculated as the arithmetic mean plus or minus a number (y) of standard deviations ($x \pm ys$), and the control limits were adjusted for each point on the charts. To avoid confusion, the limits will be referred to as y$\sigma$-limits even though the correct term is ys-limits, because $\sigma$ is the notation used by SAS. The control limits were calculated from the most common subgroup size, because the distance between the control limits will vary with varying subgroup size; subgroups smaller or larger than the common size were labelled differently on the control charts to point out that they had not been included in the calculations.

## Choice of sensitising rules

PROC SHEWHART in SAS can apply eight standardised sensitising rules to the data on Shewhart's control charts (16). An overview of the rules and their interpretation is given in Table I. The reaction that resulted from application of the rules on the charts to data from 2004 formed the basis for the final decision on which rules to apply.

## Evaluation of sampling strategies

To evaluate the approach chosen from the control charts made with data from 2004, additional charts were made with data from 2003. In 2003, the laboratory experienced a technical problem, which was corrected in September (10). The error was already present in January 2003, and retrospective analyses of test sample results (unpublished) showed an increase in the error rate in February 2003. Control charts using data from 2003 were expected to reveal the increase of the error in February when data from January to March 2003 were analysed, and to show the correction in September when data from January to

**Table I**
**Descriptions of the sensitising rules that SAS can apply to Shewhart's control charts for continuous variables to reveal systematic errors**
Based on 3σ control limits

| Rule No. | Pattern description | Interpretation |
|---|---|---|
| 1 | One point beyond the control limits | The original Shewhart rule, which shows points out of control |
| 2 | Nine points in a row on the same side of the central line | Early warning of a shift in the mean |
| 3 | Six points in a row steadily increasing or steadily decreasing | Indicates an upwards or downwards tendency |
| 4 | Fourteen points in a row alternating up and down | Positive when the subgroups shift between two different sources |
| 5 | Two out of three points in a row beyond the 2σ-line | Very sensitive to small changes in the process |
| 6 | Four out of five points in a row beyond the 1σ-line | Very sensitive to small changes in the process |
| 7 | Fifteen points in a row within the 1σ-line | Positive when observations within the subgroup have multiple sources |
| 8 | Eight points in a row on either or both sides of the central line with no points within the 1σ-line | Positive when subgroups are taken from one source at a time, i.e. not from the same population |

Based on information from SAS Institute Inc., 1999 (16)

September 2003 were used. Control charts for 2003 were only prepared for the negative serum sample, because the positive serum sample had not been used in 2003.

# Results

## Data control

The final data set from 2004 contained 13,570 observations per control sample, with two observations on each of 6,785 microtitre plates distributed on 217 dates. In this data set 1,104 observations (control sample results) per control sample had been excluded because the microtitre plates had been discarded by the QC. A missing value for 'robot' for all control samples was found on nine microtitre plates, which were also excluded. The final data from the 2003 set contained 14,908 observations per control sample distributed on 235 dates. In this data set 452 observations per control sample were excluded, because the microtitre plates had been discarded by the QC.

## Choice of quality control variables

It was not possible to find QC variables that were normally distributed according to the Kolmogorov–Smirnov test. Therefore, the shape of the histogram was used to compare the normality of the original data to that of the transformed data. Based on this evaluation the data with a distribution closest to a normal distribution were the ln-transformed data for the buffer control and the negative serum, but for the positive serum the histogram for the untransformed data was closest to a normal distribution. Figure 2 shows the histograms for the buffer control and the negative control for the OD and ln(OD).

## Choice of subgroup type

When control charts made with data from different robots in the same period were compared, the curves had no visible differences in shape. A control chart prepared with alternate sampling from the first, second, third, fourth and fifth robot throughout one month did not reveal any systematic differences between the robots, and the sensitising rule for subgroups from different populations (rule 8) was not positive at any time (results not shown). An ANOVA comparing the five robots revealed no differences between the mean value of the subgroups from the five robots for the buffer control ($p=0.9461$) or the negative control ($p=0.2093$), but for the positive control, a significant difference was identified ($p<0.0001$). Pairwise comparisons showed that robot No. 1 was significantly different from robots 2, 3 and 4 (p-values 0.0006, <0.0001 and <0.0001), and that robot No. 5 was different from robots 3 and 4 (p-values 0.0004 and 0.0039), even though this had not been visible on the charts with intermittent sampling.

Because the ANOVA had shown a difference between the robots, the level 'run' was only tested as a subgroup within a robot. A visual comparison of control charts made with subgroups, taking the control sample results from one robot in the first run, one robot in the second and third run and one robot across runs, revealed only minor differences in the shape of the curves on the x-charts, although the size of the peaks showed some variation. The differences that could be identified were not stable, because it was not consistently the same subgroup that was different from the other two subgroups. As for the different robots, a control chart prepared with alternate sampling from first, second and third robots, run throughout one month, failed to show any systematic differences between the three runs (Fig. 3). However, comparison of the mean and control limits for the chart revealed that the mean OD or OD% for

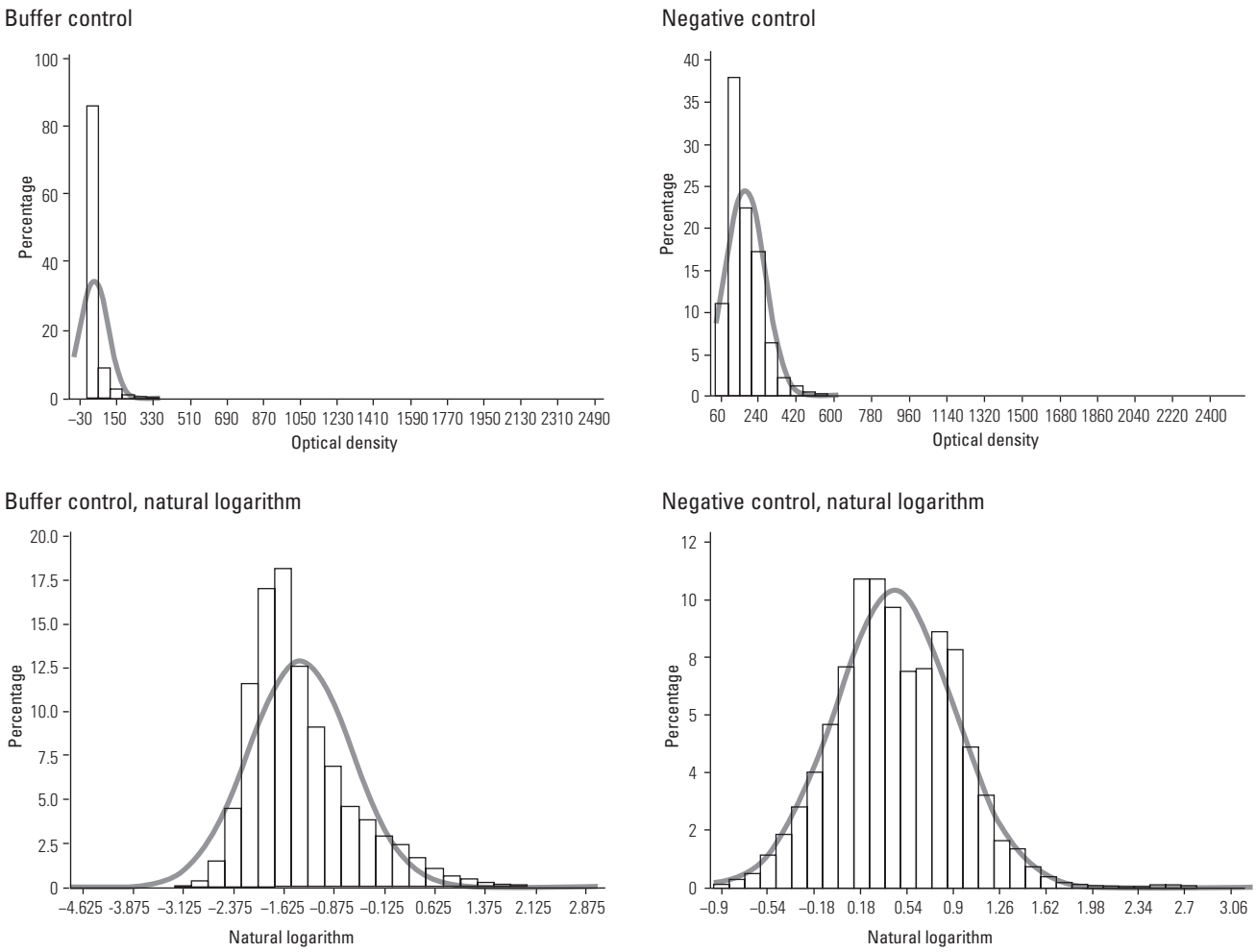



**Fig. 2**

**Histograms showing the distribution of data (control sample results) from the enzyme-linked immunosorbent assay for *Salmonella* antibody in 2004**

Comparison of non-transformed data (optical density; OD) and data transformed with the natural logarithm (ln[OD])

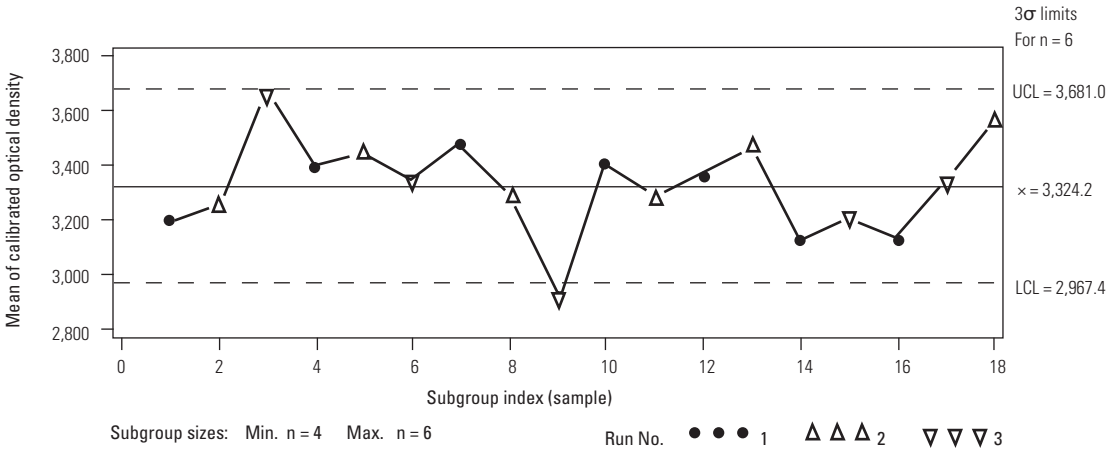



**Fig. 3**

**Control chart of control sample results prepared by intermittent sampling from first, second and third testing round in a day**

Data from the *Salmonella* Typhimurium positive control serum analysed in the *Salmonella* antibody enzyme-linked immunosorbent assay in 2004

the second and third run were approximately 1% higher than the values for the first run for all robots. Data for the positive serum are given in Table II.

Table III shows frequency counts for dates and robots. The most common number of observations per control sample per day for each robot was 12, as shown in the table, but acquisition of 18 observations was almost as common. Therefore, the use of robot as a subgroup would give many subgroups with deviating subgroup size. In contrast, the first run within each robot had a subgroup size of six observations on between 79% and 92% of the days. Because the control charts with intermittent sampling did not reveal any differences between runs or robots, despite the fact that such differences were probably present, and because of the stable subgroup size, run within robot was chosen as the subgroup.

## Choice of control limits

For the buffer control and the negative serum, for which the control charts were prepared using uncalibrated OD readings, almost none of the subgroups had a mean value

**Table II**
**Summary measures from control charts with different subgroups for the calibrated optical density of the positive control serum used in the *Salmonella* antibody enzyme-linked immunosorbent assay**

| Robot | Run | Mean x [a] | SD | UCL | LCL |
|---|---|---|---|---|---|
| 1 | 1st | 30.81 | 1.45 | 35.17 | 26.45 |
| | >1st | 31.94 | 1.43 | 36.22 | 27.66 |
| | All | 31.44 | 1.37 | 34.55 | 28.33 |
| 2 | 1st | 30.04 | 1.12 | 33.40 | 26.68 |
| | >1st | 31.26 | 1.17 | 34.76 | 27.75 |
| | All | 30.73 | 0.85 | 33.28 | 28.18 |
| 3 | 1st | 29.70 | 1.30 | 33.60 | 25.80 |
| | >1st | 30.50 | 1.33 | 34.50 | 26.50 |
| | All | 30.16 | 0.94 | 32.98 | 27.33 |
| 4 | 1st | 29.82 | 1.30 | 33.70 | 25.90 |
| | >1st | 30.96 | 1.26 | 34.75 | 27.17 |
| | All | 30.47 | 0.95 | 33.26 | 27.68 |
| 5 | 1st | 30.43 | 1.34 | 34.45 | 26.42 |
| | >1st | 31.90 | 1.26 | 35.67 | 28.14 |
| | All | 31.26 | 0.95 | 34.10 | 28.41 |
| All | 1st | 30.15 | 0.59 | 31.92 | 28.38 |
| | >1st | 31.28 | 0.59 | 33.06 | 29.50 |
| | All | 30.79 | 0.61 | 32.62 | 28.96 |

a) Mean x: The mean value of the subgroup means during 2004 given as calibrated optical density
SD: Standard deviation ($\sigma$) of mean x
UCL: Upper control limit (mean x+3$\sigma$)
LCL: Lower control limit (mean x–3$\sigma$)

**Table III**
**Number of observations per day for the control samples used in the *Salmonella* antibody enzyme-linked immunosorbent assay, given per day and per robot** (data from 2004)

| Robot | Most frequent number of observations per day | | | % of days that had the most frequent number of observations | | |
|---|---|---|---|---|---|---|
| | 1st run | >1st run | All runs | 1st run | >1st run | All runs |
| 1 | 6 | 6 | 12 | 79 | 33 | 29 |
| 2 | 6 | 6 | 12 | 91 | 35 | 32 |
| 3 | 6 | 6 | 12 | 90 | 34 | 32 |
| 4 | 6 | 6 | 12 | 84 | 31 | 29 |
| 5 | 6 | 6 | 12 | 92 | 32 | 31 |
| All | 30 | 30 | 60 | 48 | 13 | 11 |

within the 3σ-limits. Therefore control limits were re-calculated using 4, 5, 6 or 7σ. Evaluation of the control charts showed many points outside the control limits even with 7σ-limits. For both 6σ-limits and 7σ-limits the control charts had more than one third of the points within the control limits, and the points were evenly distributed in the full interval between the limits; therefore the 6σ-limits were chosen. For control charts for the positive serum, for which the control charts were prepared using the OD%, only one subgroup every second month resulted in a point outside the 3σ-limits (results not shown).

### Choice of sensitising rules

Initially, rules 1, 2, 3 and 4 were applied to all control charts as recommended in the SAS manual (16). Rules 5 and 6 were not used, because these tests increased the sensitivity of the control charts beyond a practical level for an ELISA. Rules 7 or 8 were only applied to control charts produced from subgroup data, which could give rise to unwanted stratification patterns. The use of the primary Shewhart rule (rule 1) on the control charts obtained from the uncalibrated OD readings gave rise to a large number of reactions. The use of rule 2, for a series of points on the same side of the central line, and rule 3, for increasing or decreasing mean values, gave only a few reactions on the control charts for the buffer control, the negative serum and the positive serum during the year 2004. Rule 4, for points alternating up and down, did not give any reactions on any of the control charts, and neither did rule 7, for large variation within subgroups. Rule 8, for subgroups originating from different populations, gave rise to multiple reactions. Many of these reactions seemed to be false, because they were accompanied by a reaction from rule 2, and therefore probably did not indicate subgroups from different populations.

Hence, the rule for a series of points on the same side of the central line (rule 2) and the rule for increasing or decreasing mean values (rule 3) were applied to the control charts for the buffer control, the negative serum and the positive serum. The rule for points alternating up and down (rule 4) was also applied to control charts for all variables, because unexpected factors such as change of technician could give rise to this pattern. The risk of false reactions was considered to be low, because no reactions were recorded during the year 2004. The rule for large variation within subgroups (rule 7) was applied to all charts, because the two wells on the same microtitre plate could have systematically different control sample results. The rule for sampling from different populations (rule 8) was not applied to any charts, because differences between runs or robots would not appear with the subgroup strategy chosen.

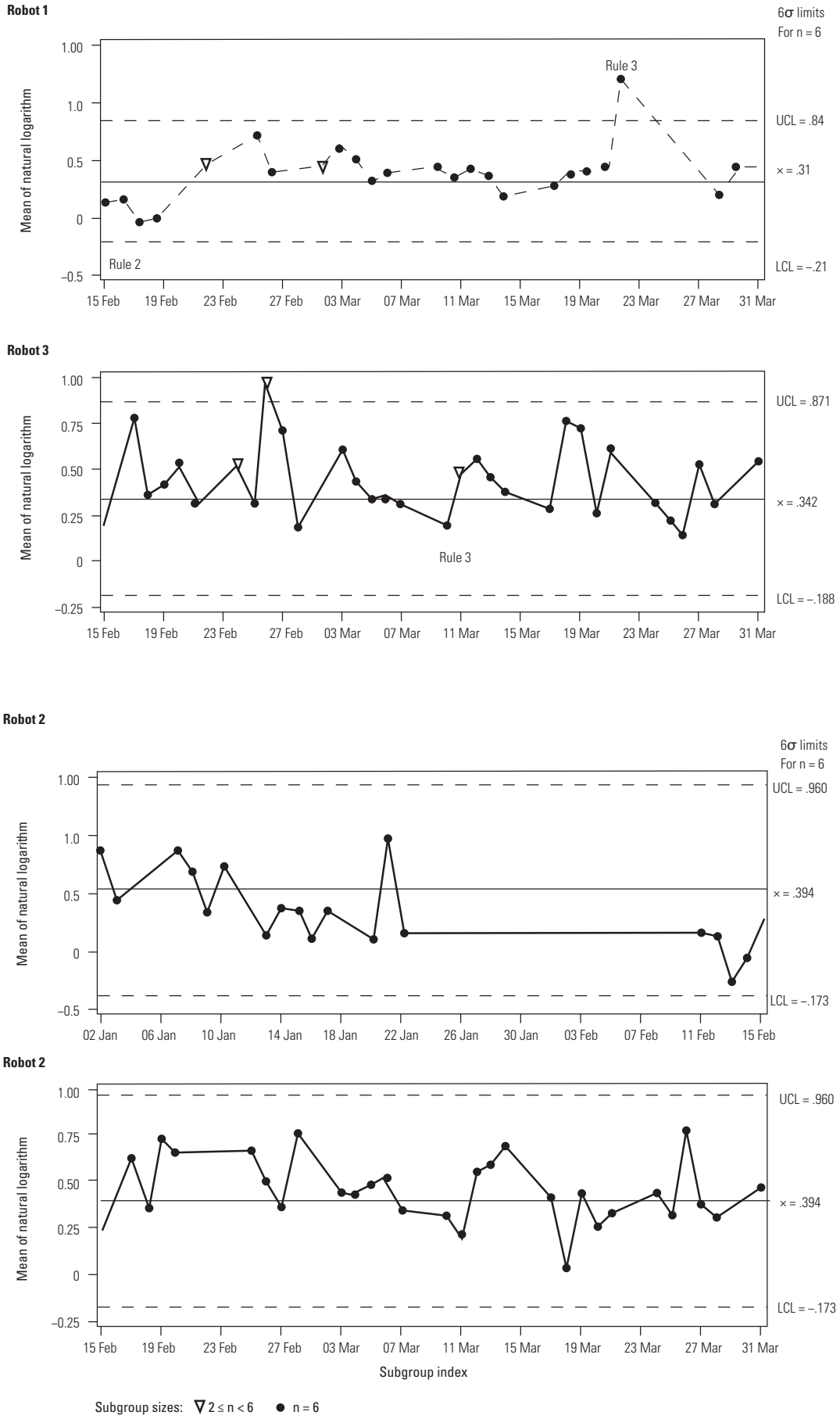
### Evaluation of sampling strategies

Control charts for the negative serum in 2003 were prepared using ln(OD) as the QC variable, and the subgroups were the first run for each robot with one control chart for each robot. The increase in the error in February 2003 was detected with reactions from rule 2 and/or rule 3 for three of the five robots (robots 1, 3 and 4). However, the non-reacting robots were not used two weeks before (robot No. 2) or two weeks after (robot No. 5) the increased error occurred, and this may have interfered with the reactions. The ANOVA performed as described above did not reveal any difference between the robots ($p=0.2055$). The charts for the five robots are shown in Figure 4.

In September 2003, the correction of the mean value was visible on the control charts for all five robots, but for robot No. 4 the switch was unclear and gave no reaction from the sensitising rules. The switch did not appear on exactly the same dates. The charts for the five robots are shown in Figure 5, and it can be seen that there were reactions from rule 2 for robot No. 4 in the weeks before the switch. The differences between the charts for different robots indicated a difference between the control sample results from the five robots, and this was confirmed by ANOVA ($p=0.0004$). Pairwise comparisons pointed out that robot No. 5 was significantly different from robots No. 1 ($p=0.0005$) and No. 4 ($p=0.0001$).

## Discussion

Before control charts are introduced to monitor a process, the process should be described thoroughly to reveal possible sources of variation. If all points on initial control charts are within the control limits without any particular tendency, the process is regarded as being in statistical control (9). For the *Salmonella* ELISA, only control sample results from microtitre plates that met the specifications for approval were included in the data set, because a deviating test result for a control sample on a rejected microtitre plate may result from causes not relevant to this specific control sample, e.g. dilution errors for another control sample. When evaluated with the Kolmogorov–Smirnov test, the data were not normally distributed. Alternatively, the Shapiro–Wilk test or the Anderson–Darling test could have been used, but taking the shape of the histograms into consideration, there was no reason to believe that these tests would have given a different result. When the possible outcome of an analysis is bounded to one side, normality cannot be expected. For the buffer control and the negative control, the control sample results were expected to be very low. Hence, the distributions were expected to be skewed to the left compared with the normal distribution.


Robot 1
6σ limits
For n = 6
Mean of natural logarithm
1.00
1.0
0.5
0
–0.5
Rule 3
Rule 2
UCL = .84
× = .31
LCL = –.21
15 Feb
19 Feb
23 Feb
27 Feb
03 Mar
07 Mar
11 Mar
15 Mar
19 Mar
23 Mar
27 Mar
31 Mar
Robot 3
1.00
0.75
0.50
0.25
0
–0.25
UCL = .871
× = .342
Rule 3
LCL = –.188
Robot 2
6σ limits
For n = 6
UCL = .960
× = .394
LCL = –.173
02 Jan
06 Jan
10 Jan
14 Jan
18 Jan
22 Jan
26 Jan
30 Jan
03 Feb
07 Feb
11 Feb
15 Feb
Robot 2
UCL = .960
× = .394
LCL = –.173
Subgroup index
Subgroup sizes: ∇ 2 ≤ n < 6 ● n = 6

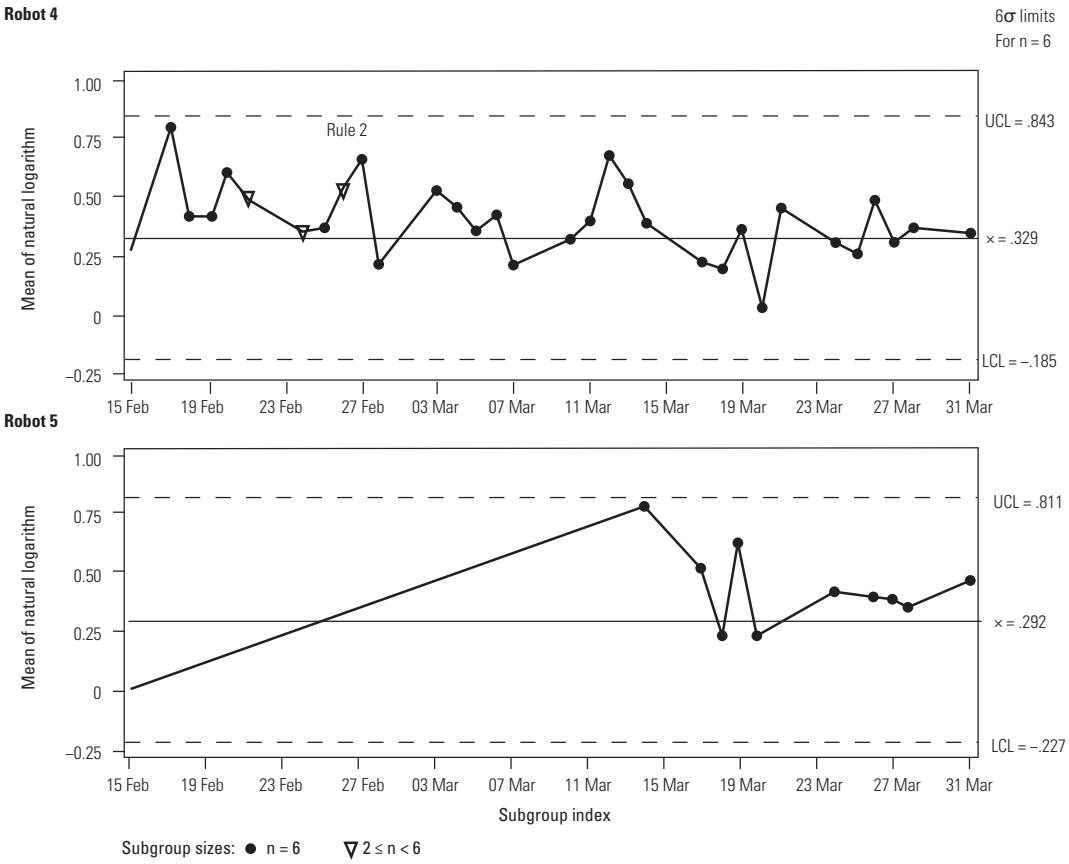
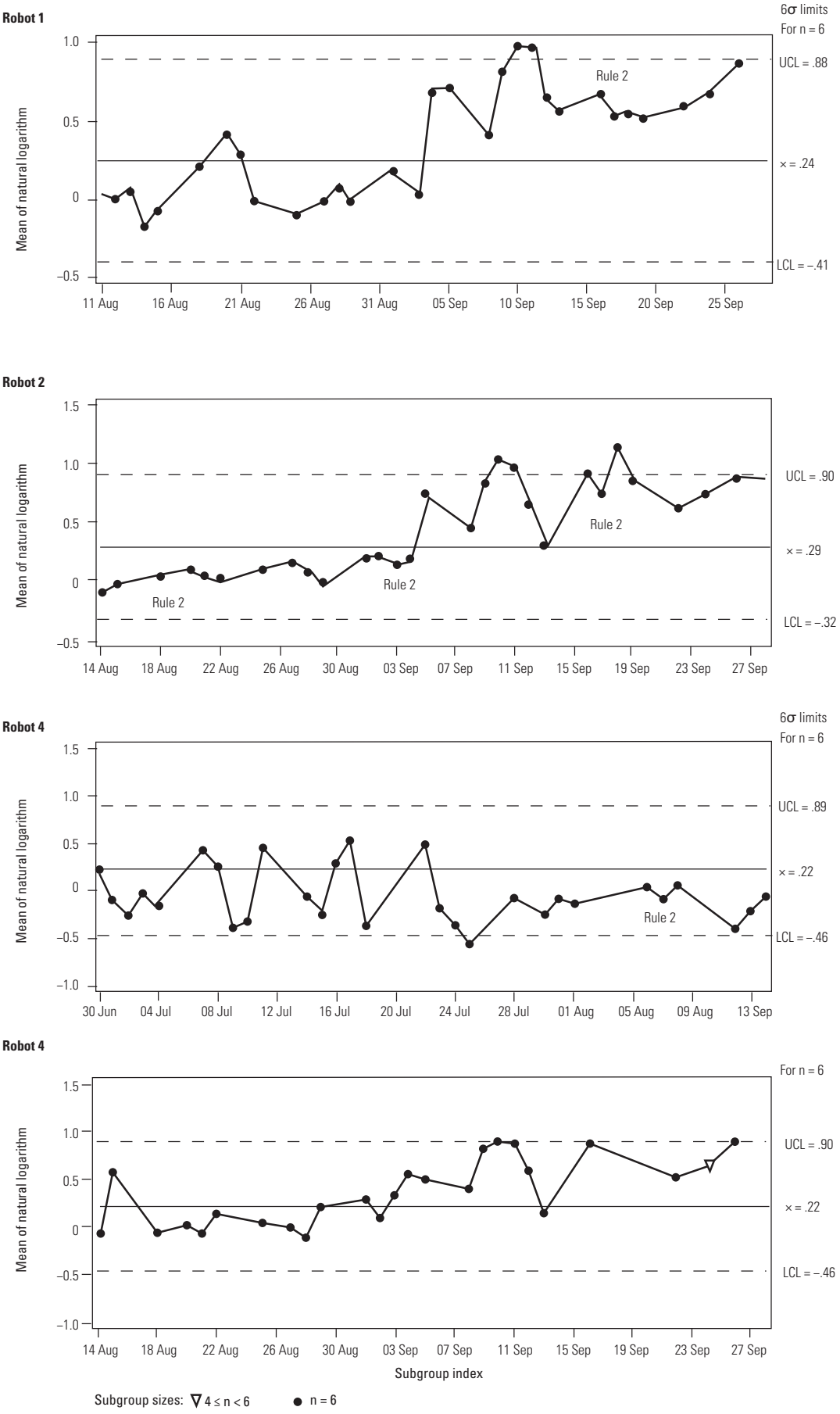



**Fig. 4**
**Control chart for the negative control sample in the *Salmonella* antibody enzyme-linked immunosorbent assay in the middle of February 2003**
Reaction from sensitising rule 2: the series of points on the same side of the central line indicate a change in the mean value, and from sensitising rule 3: upwards trend. The extra chart for robot No. 2 shows that this robot was not used for the two weeks before the expected reactions

Statistical control could only be obtained for the positive serum, whereas the control charts for the buffer control and the negative serum showed large day-to-day variation with many points out of control. This also occurred in 2004, when the ELISA was believed to perform normally. However, even for calibrated results, the day-to-day coefficient of variation for this ELISA has previously been found to be 18.9% (12). Therefore, it was expected that sampling of control sample results from uncalibrated ODs would show a process out of control. After all, the test results are calibrated to overcome this variation (13), and therefore the large variation for the buffer control and the negative serum was unavoidable, even though it was disturbing for the interpretation of the control charts.

Sampling of data for the preparation of control charts can be carried out using one of two strategies. Strategy one is a snapshot within a time period, including consecutive units of production in a subgroup. It minimises the chance of variability due to causes within the sample, but maximises the chance of variability between samples, if systematic errors are present (7). Strategy two is to take a random sample of all output during the sampling interval and to use the sample to approve the output of a process between two samples (7, 11). In general, the snapshot strategy will be the most useful (7). For the *Salmonella* ELISA, results from the individual microtitre plates were approved or rejected according to specification limits calculated for the buffer control, the negative serum and the positive serum. It would not be recommended to replace this approval/rejection procedure for individual microtitre plates by control charts prepared from a sample of the data and, therefore, the snapshot strategy was chosen.

In the choice of QC variables, the two observations for each control sample on the microtitre plates were treated as independent observations, even though there will be a correlation between samples analysed on the same microtitre plate. However, this violation of the general rules for statistics was carried through to maximise the subgroup size to six observations. The fact that the duplicate control samples were added to the microtitre plates by different probes on the multi-probe robots allowed for variations between the two control samples on


Robot 1
6σ limits
For n = 6
UCL = .88
× = .24
LCL = –.41
Rule 2
Mean of natural logarithm
1.0
0.5
0
–0.5
11 Aug
16 Aug
21 Aug
26 Aug
31 Aug
05 Sep
10 Sep
15 Sep
20 Sep
25 Sep
Robot 2
UCL = .90
× = .29
LCL = –.32
Rule 2
Rule 2
Rule 2
1.5
1.0
0.5
0
–0.5
14 Aug
18 Aug
22 Aug
26 Aug
30 Aug
03 Sep
07 Sep
11 Sep
15 Sep
19 Sep
23 Sep
27 Sep
Robot 4
6σ limits
For n = 6
UCL = .89
× = .22
LCL = –.46
Rule 2
1.5
1.0
0.5
0
–0.5
–1.0
30 Jun
04 Jul
08 Jul
12 Jul
16 Jul
20 Jul
24 Jul
28 Jul
01 Aug
05 Aug
09 Aug
13 Sep
Robot 4
For n = 6
UCL = .90
× = .22
LCL = –.46
1.5
1.0
0.5
0
–0.5
–1.0
14 Aug
18 Aug
22 Aug
26 Aug
30 Aug
03 Sep
07 Sep
11 Sep
15 Sep
19 Sep
23 Sep
27 Sep
Subgroup index
Subgroup sizes: ∇ 4 ≤ n < 6
● n = 6

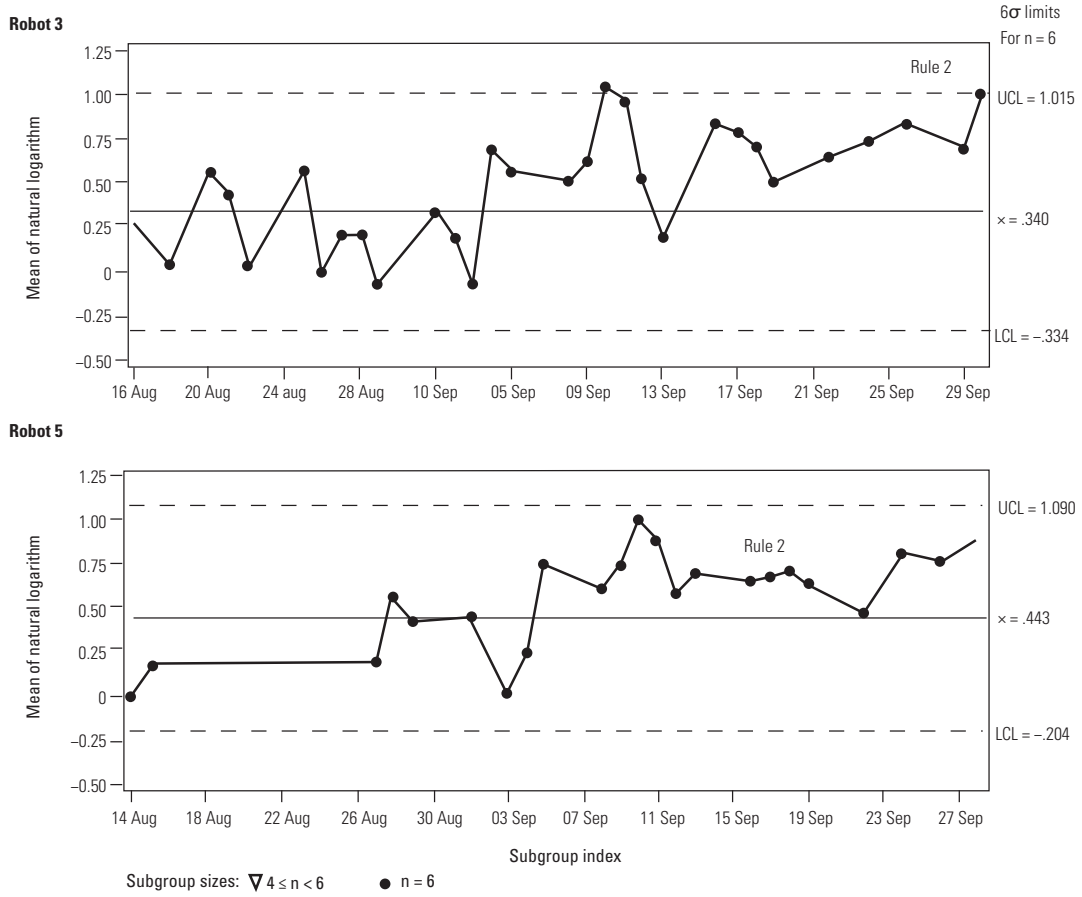



**Fig. 5**
**Control chart for the negative control sample in the *Salmonella* antibody enzyme-linked immunosorbent assay in September 2003**
Reaction from sensitising rule 2: the series of points on the same side of the central line indicates a change in the mean value

the same microtitre plate. Control charts were prepared for three different variables, because these related to different aspects of the assay. Control charts for the positive serum were produced from the calibrated OD-value, i.e. the OD%, because this was recommended for weakly positive controls (8). This meant that the control charts for the positive serum were correlated to the control charts for the buffer control and the negative serum, because the ODs of these variables were used for the calibration. Still, both charts were useful, as could be seen for the robot comparisons, where the ANOVA showed a difference between robots for the positive control, but not for the buffer control or the negative control.

When data are divided into subgroups in which the batch of reagents, machine and other factors are common, the variation within the subgroups can be regarded as similar to the variation due to chance (9). For the *Salmonella* ELISA minor differences originated from run number, but the day-to-day variation was identified as the largest contributor to the total variation. The subgroup chosen was run within robot, because this gave only a few subgroups with deviating sizes, thereby including most of the observations in the calculation of the control limits. Because differences were found between the robots both in 2003 and in 2004, it was decided to prepare one control chart for each robot in order to point out systematic errors more exactly. Summary data from each robot were used for an ANOVA against data from the other robots to ensure that deviating control sample results from one robot would be identified even if the control chart for the robot in question did not show lack of control. The use of ANOVA for comparison of performances in different subgroups has been described before (8), and for the *Salmonella* ELISA it was successful in pointing out differences that were not visible on inspection of the charts.

A common approach is to set the UCL as the arithmetic mean plus three times the standard deviation ($x+3\sigma$) and the LCL as the mean minus three times the standard deviation ($x–3\sigma$); the so-called $3\sigma$-limits (7, 11). The control limits for the positive serum were set as the standard $3\sigma$-limits, but because of the large variation in the uncalibrated QC variables, the control limits for the buffer control and the negative serum had to be widened to $6\sigma$, if the control charts were to reflect a process in statistical control. Even with these wide control limits the primary Shewhart rule (rule 1) was only applied to the control

charts for the positive serum, because the many reactions from rule 1 would have complicated the interpretation of the control charts for the buffer control and the negative control. The specification limits for approval of microtitre plates would assure that control sample results from the buffer control and the negative serum were detected if they were deviating severely from the normative values.

To determine whether the control charts were useful for QC, control sample results from 2003 were used for preparation of control charts. In September 2003, the number of seropositive test samples from slaughter pigs in the surveillance programme suddenly increased from 5.6% to 10.1%. The increase turned out to have been caused by a technical problem in the laboratory that had caused some of the samples to be analysed incorrectly, resulting in an artificially low level of test samples with a positive test result (10). A logistic analysis of the test sample results from 2003 revealed that the probability of a test sample result being categorised as 'positive' had decreased from 8% in the top corner of the microtitre plate to 2% in the bottom corner (2). An investigation in the laboratory identified the automatic ELISA washer as the cause of the problem. Retrospective analyses of data from the test sample analyses revealed that the washer had been washing the microtitre plates slightly unevenly since August 2002, and that the problem increased in February 2003 and persisted until September 2003. Apparently, the ELISA washer had returned to optimal function in September 2003, and this was the reason for the detection of the error.

The evaluation of the control charts with data from 2003 showed that both the increasing systematic error in February 2003 and the correction of the systematic error in September 2003 would have been detected immediately, if these control charts had been used at that time. Therefore, the use of control charts would have prevented this systematic error from remaining undetected for more than a year. After the introduction of the positive control on each microtitre plate, it is likely that systematic errors will appear even more clearly on control charts.

To improve the QC even more, a fourth control chart could be prepared for one of the sera from pigs infected with *S.* Infantis, provided the calculations of OD% can be performed on the individual observations as well as on the duplicate. Other sources of variance, such as a change in the batch of reagents, should also be marked on the charts in order to increase the attention paid to the results in critical periods but, unfortunately, this was not possible in this retrospective analysis. A multivariate control chart for the buffer control, the negative control and the positive serum was considered, e.g. as a joint control ellipse, which could reveal mutual out-of-control points undiscovered by the use of individual control charts. The disadvantage of the joint ellipse is that the time sequence of the plotted points is lost. Another approach is to create a new variable such as a $\chi^2$. The disadvantage is that such a value will be far from the original data and thus more difficult to interpret (11). The individual control charts simplify the readings and make the charts understandable for the people in the laboratory, because it is easily seen if a control sample result increases or decreases.

On the other hand, mutual control points could be out-of-control even if none of the individual charts show lack of control, and vice versa, and a multivariable control chart could improve the QC even further (6). However, this would have demanded the installation of new computer software on the laboratory computers, and that was not permitted, in order to secure the data system. Therefore, the Shewhart control charts were chosen to allow a rapid improvement of the QC procedures. Hopefully, the fact that even these simple charts proved to be useful can stimulate a more thorough revision of the QC system, e.g. the implementation of new computer software with broader possibilities in the choice of QC variables.

# Conclusion

Shewhart's control charts prepared with the concept of rational subgroups proved to be a valuable tool in the QC of the *Salmonella* ELISA. The control charts were able to reveal systematic errors that appeared in the year 2003, even though the data did not fulfil the standard criteria for a process in statistical control in the period before this error occurred.

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# Cartes de contrôle utilisant des sérums de contrôle pour déceler les erreurs systématiques avec l'ELISA indirect appliquée à la détection d'anticorps vis-à-vis de *Salmonella*

H. Bak & K. Barfod

**Résumé**
Les auteurs ont évalué le recours aux cartes de contrôle de Shewhart, basées sur le concept de sous-groupes rationnels, pour contrôler les performances de l'épreuve immuno-enzymatique (ELISA) utilisée pour détecter les anticorps dirigés contre *Salmonella* dans le cadre de la surveillance du cheptel porcin au Danemark. Des cartes de contrôle ont été préparées, respectivement pour une suspension de contrôle, un sérum négatif et un sérum positif. Les variables du contrôle de qualité sont le logarithme naturel (ln) de la densité optique (DO) non calibrée de la suspension de contrôle et du sérum négatif, et la DO calibrée (pourcentage de DO) pour le sérum positif. Un cycle de tests automatisés a été choisi comme sous-groupe, et des cartes de contrôle distinctes ont été préparées pour cinq automates. Les seuils de contrôle ont été fixés à six fois l'écart-type pour le ln(DO) et à trois fois l'écart-type pour le pourcentage de DO. Il ressort de cette évaluation, conduite en suivant un certain nombre de règles de sensibilité applicables aux cartes de contrôle telles qu'établies lors de travaux antérieurs, que le recours à ces cartes de contrôle permet de mettre en évidence les erreurs analytiques systématiques.

**Mots-clés**
Carte de contrôle – Contrôle de la qualité – Épreuve immuno-enzymatique – Salmonella – Sous-groupe rationnel – Surveillance.

# Diagramas de control para determinar errores sistemáticos en el uso de sueros testigo en un ELISA indirecto para detectar anticuerpos contra *Salmonella*

H. Bak & K. Barfod

**Resumen**
Los autores describen un estudio destinado a evaluar la preparación de diagramas de control de Shewhart partiendo del concepto de subgrupos racionales con el objetivo de controlar la prueba ELISA de detección de anticuerpos contra *Salmonella* utilizada para la vigilancia de rebaños de cerdos en Dinamarca. Se confeccionaron diagramas de control para las respectivas muestras testigo de solución tampón, suero negativo y suero positivo. Las variables de control de calidad eran el logaritmo natural (ln) de la densidad óptica (DO) no calibrada, en el caso de las muestras testigo de tampón y suero negativo, y la DO calibrada (DO%) en el caso del suero positivo. Como subgrupo se definió una serie de prueba realizada en robot, y se prepararon diagramas de

control para cada uno de los cinco robots del laboratorio. Como límites de control se fijaron el séxtuplo de la desviación típica para el ln(DO) y el triple de la desviación típica para la DO%. La evaluación, basada en una serie de reglas de sensibilidad de los diagramas de control definidas a partir de datos históricos, demostró que el uso de diagramas de control podía servir para descubrir errores de análisis sistemáticos.

**Palabras clave**
Control de calidad – Diagrama de control – ELISA – Salmonella – Subgrupo racional – Vigilancia.

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