2-3. **Robustness**. The robustness of an analytical procedure is a measure of its capacity to remain unaffected by small but deliberate variations in method parameters, and provides an indication of its reliability during normal usage.

The evaluation of robustness should be considered during the development phase. It should show the reliability of the analytical procedure with respect to deliberate variations in method parameters. For NAT, small variations in the method parameters can be crucial. However, the robustness of the method can be demonstrated during its development when small variations in the concentrations of reagents (e.g. $MgCl_2$, primers or deoxyribonucleotides) are tested. Modifications of extraction kits or extraction procedures as well as different thermal cycler types may also be evaluated.

Finally, robustness of the method can be evaluated through collaborative studies.

3. *GUIDELINE FOR COMPARABILITY STUDY*

NAT may be used instead of official methods (indicator cell culture method and/or culture method). In this case a comparability study should be carried out. This comparability study should include mainly a comparison of the respective detection limits of the alternative method and official methods. However, specificity (mycoplasma panel detected, putative false positive results) should also be considered.

For the detection limit, acceptability criteria are defined as follows:

– if the alternative method is proposed to replace the culture method, the NAT system must be shown to detect 10 CFU/ml for each mycoplasma test species described in paragraph 2-2;
– if the alternative method is proposed to replace the indicator cell culture method, the NAT system must be shown to detect 100 CFU/ml for each mycoplasma test species described in paragraph 2-2.

For both cases, suitable standards calibrated for the number of nucleic acid copies and the number of CFUs may be used for establishing that these acceptability criteria are reached. The relation between CFUs and nucleic acid copies for the reference preparations should be previously established to compare the performance of the alternative NAT method with the performance of the official methods.

1 of the following 2 strategies can be used to perform this comparability study:

– perform the NAT alternative method in parallel with the official method(s) to evaluate simultaneously the detection limit of both methods using the same samples of calibrated strains;
– compare the performance of the NAT alternative method using previously obtained data from official method validation. In this case, calibration of standards used for both validations as well as their stabilities should be documented carefully.

Comparability study reports should describe all the validation elements described in section 2 (specificity, limit of detection and variability, as well as robustness) in order to assess all the advantages and/or disadvantages of the alternative NAT method compared to official methods.

01/2008:20608

2.6.8. PYROGENS

The test consists of measuring the rise in body temperature evoked in rabbits by the intravenous injection of a sterile solution of the substance to be examined.

Selection of animals. Use healthy, adult rabbits of either sex weighing not less than 1.5 kg, fed a complete and balanced diet not containing antibiotics, and not showing loss of body mass during the week preceding the test. A rabbit is not be used in a pyrogen test:

a) if it has been used in a negative pyrogen test in the preceding 3 days, or

b) if it has been used in the preceding 3 weeks in a pyrogen test in which the substance under examination failed to pass the test.

Animals' quarters. Keep the rabbits individually in a quiet area with a uniform appropriate temperature. Withhold food from the rabbits overnight and until the test is completed; withhold water during the test. Carry out the test in a quiet room where there is no risk of disturbance exciting the animals and in which the room temperature is within 3 °C of that of the rabbits' living quarters, or in which the rabbits have been kept for at least 18 h before the test.

Materials. Glassware, syringes and needles. Thoroughly wash all glassware, syringes and needles with water for injections and heat in a hot-air oven at 250 °C for 30 min or at 200 °C for 1 h.

Retaining boxes. The retaining boxes for rabbits whose temperature is being measured by an electrical device are made in such a way that the animals are retained only by loosely fitting neck-stocks; the rest of the body remains relatively free so that the rabbits may sit in a normal position. They are not restrained by straps or other similar methods which may harm the animal. The animals are put into the boxes not less than 1 h before the first record of the temperature and remain in them throughout the test.

Thermometers. Use a thermometer or electrical device which indicates the temperature with a precision of 0.1 °C and insert into the rectum of the rabbit to a depth of about 5 cm. The depth of insertion is constant for any one rabbit in any one test. When an electrical device is used it may be left in position throughout the test.

Preliminary test. After selection of the animals, one to three days before testing the product to be examined, treat those animals that have not been used during the previous 2 weeks by intravenous injection of 10 ml per kilogram of body mass of a pyrogen-free 9 g/l solution of *sodium chloride R* warmed to about 38.5 °C. Record the temperatures of the animals, beginning at least 90 min before injection and continuing for 3 h after the injection of the solution. Any animal showing a temperature variation greater than 0.6 °C is not used in the main test.

Main test. Carry out the test using a group of three rabbits.

Preparation and injection of the product. Warm the liquid to be examined to approximately 38.5 °C before the injection. The product to be examined may be dissolved in, or diluted with, a pyrogen-free 9 g/l solution of *sodium chloride R* or another prescribed liquid. Inject the solution slowly into the marginal vein of the ear of each rabbit over a period not exceeding 4 min, unless otherwise prescribed in the monograph. The amount of the product to be injected varies according to the product to be examined and is prescribed in the monograph. The volume injected is not less than 0.5 ml per kilogram and not more than 10 ml per kilogram of body mass.

Determination of the initial and maximum temperatures. The "initial temperature" of each rabbit is the mean of two temperature readings recorded for that rabbit at an interval of 30 min in the 40 min immediately preceding the injection of the product to be examined. The "maximum temperature" of each rabbit is the highest temperature recorded for that rabbit in the 3 h after the injection. Record

the temperature of each rabbit at intervals of not more than 30 min, beginning at least 90 min before the injection of the product to be examined and continuing 3 h after the injection. The difference between the maximum temperature and the initial temperature of each rabbit is taken to be its response. When this difference is negative, the result is counted as a zero response.

Rabbits showing a temperature variation greater than 0.2 °C between two successive readings in the determination of the initial temperature are withdrawn from the test. In any one test, only rabbits having initial temperatures which do not differ from one another by more than 1 °C are used. All rabbits having an initial temperature higher than 39.8 °C or less than 38.0 °C are withdrawn from the test.

Interpretation of results. Having carried out the test first on a group of three rabbits, repeat if necessary on further groups of three rabbits to a total of four groups, depending on the results obtained. If the summed response of the first group does not exceed the figure given in the second column of the Table 2.6.8.-1, the substance passes the test. If the summed response exceeds the figure given in the second column of the table but does not exceed the figure given in the third column of the table, repeat the test as indicated above. If the summed response exceeds the figure given in the third column of the table, the product fails the test.

Table 2.6.8.-1

Number of rabbits	Product passes if summed response does not exceed	Product fails if summed response exceeds
3	1.15 °C	2.65 °C
6	2.80 °C	4.30 °C
9	4.45 °C	5.95 °C
12	6.60 °C	6.60 °C

Rabbits used in a test for pyrogens where the mean rise in the rabbits' temperature has exceeded 1.2 °C are permanently excluded.

01/2008:20609

2.6.9. ABNORMAL TOXICITY

GENERAL TEST

Inject intravenously into each of 5 healthy mice, weighing 17 g to 24 g, the quantity of the substance to be examined prescribed in the monograph, dissolved in 0.5 ml of *water for injections R* or of a 9 g/l sterile solution of *sodium chloride R*. Inject the solution over a period of 15 s to 30 s, unless otherwise prescribed.

The substance passes the test if none of the mice die within 24 h or within such time as is specified in the individual monograph. If more than one animal dies the preparation fails the test. If one of the animals dies, repeat the test. The substance passes the test if none of the animals in the 2nd group die within the time interval specified.

IMMUNOSERA AND VACCINES FOR HUMAN USE

Unless otherwise prescribed, inject intraperitoneally 1 human dose but not more than 1.0 ml into each of 5 healthy mice, weighing 17 g to 24 g. The human dose is that stated on the label of the preparation to be examined or on the accompanying leaflet. Observe the animals for 7 days.

The preparation passes the test if none of the animals shows signs of ill health. If more than one animal dies, the preparation fails the test. If one of the animals dies or shows signs of ill health, repeat the test. The preparation passes the test if none of the animals in the 2nd group die or shows signs of ill health in the time interval specified.

The test must also be carried out on 2 healthy guinea-pigs weighing 250 g to 400 g. Inject intraperitoneally into each animal 1 human dose but not more than 5.0 ml. The human dose is that stated on the label of the preparation to be examined or on the accompanying leaflet. Observe the animals for 7 days.

The preparation passes the test if none of the animals shows signs of ill health. If more than one animal dies the preparation fails the test. If one of the animals dies or shows signs of ill health, repeat the test. The preparation passes the test if none of the animals in the 2nd group die or shows signs of ill health in the time interval specified.

01/2008:20610

2.6.10. HISTAMINE

Euthanise a guinea-pig weighing 250 g to 350 g that has been deprived of food for the preceding 24 h. Remove a portion of the distal small intestine 2 cm in length and empty the isolated part by rinsing carefully with solution B described below using a syringe. Attach a fine thread to each end and make a small transverse incision in the middle of the piece of intestine. Place it in an organ bath with a capacity of 10 ml to 20 ml, containing solution B maintained at a constant temperature (34 °C to 36 °C) and pass through the solution a current of a mixture of 95 parts of oxygen and 5 parts of carbon dioxide. Attach one of the threads near to the bottom of the organ bath. Attach the other thread to an isotonic myograph and record the contractions of the organ on a kymograph or other suitable means of giving a permanent record. If a lever is used, its length is such that the movements of the organ are amplified about 20 times. The tension on the intestine should be about 9.8 mN (1 g) and it should be adjusted to the sensitivity of the organ. Flush out the organ bath with solution B. Allow it to stand for 10 min. Flush 2 or 3 times more with solution B. Stimulate a series of contractions by the addition of measured volumes between 0.2 ml and 0.5 ml of a solution of *histamine dihydrochloride R* having a strength which produces reproducible submaximal responses. This dose is termed the "high dose". Flush the organ bath (preferably by overflow without emptying the bath) 3 times with solution B before each addition of histamine. The successive additions should be made at regular intervals allowing a complete relaxation between additions (about 2 min). Add equal volumes of a weaker dilution of *histamine dihydrochloride R* which produces reproducible responses approximately half as great as the "high dose". This dose is termed the "low dose". Continue the regular additions of "high" and "low" doses of histamine solution as indicated above, and alternate each addition with an equal volume of a dilution of the solution to be examined, adjusting the dilution so that the contraction of the intestine, if any, is smaller than that due to the "high dose" of histamine. Determine whether the contraction, if any, is reproducible and that the responses to the "high" and "low" doses of histamine are unchanged. Calculate the activity of the substance to be examined in terms of its equivalent in micrograms of histamine base from the dilution determined as above.

The quantity so determined does not exceed the quantity prescribed in the monograph.

If the solution to be examined does not produce a contraction, prepare a fresh solution adding a quantity of histamine corresponding to the maximum tolerated in the