Guidelines for the single-laboratory validation of qualitative real-time PCR methods
Foreword

Guidelines for conducting future validation studies of qualitative real-time PCR methods, both in single laboratories and in collaborative studies, have been developed by a subgroup of the § 64 LFGB working group "Development of methods for the identification of foodstuffs produced by means of genetic engineering techniques".

These guidelines are intended to ensure that real-time PCR methods developed by the § 64 LFGB working group are able to meet the performance criteria defined at the European (ENGL, CEN) and international level (ISO, Codex) [1] [2].

A further aim is to acquaint other relevant bodies (ENGL, CEN / ISO) with the newly developed method validation approaches in order to actively contribute to the process of establishing minimum required performance criteria in this area.

The procedure described in this document constitutes a recommendation that has proven itself in practice. Alternative approaches are possible as long as it can be shown that the performance criteria regarding limit of detection, specificity and robustness mentioned in the present document are satisfied.

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Inhaltsverzeichnis

1 Scope .................................................................................................................. 4
2 Performance characteristics .................................................................................. 4
  2.1 Limit of detection (LOD) .............................................................................. 4
  2.2 Specificity ..................................................................................................... 5
  2.3 Robustness .................................................................................................... 6

Annex 1 – Determination of copy numbers from genomic DNA ................................. 8
  A. Determination of the DNA concentration ...................................................... 8
     1. Determination by means of optical density at 260 nm ............................. 8
     2. Determination by means of the PicoGreen method ............................... 8
     3. Determination by means of digital PCR .................................................. 8
  B. Estimation of the number of copies of the target sequence ............................ 9

Annex 2 - Determination of limit of detection, precision data and PCR efficiency ....... 10
  A. Dilution series of the target DNA ................................................................ 10
  B. Preparation of PCR replicates ..................................................................... 10
  C. Determination of the limit of detection (LOD_{95\%}) .................................... 10
  D. Assessment of the observed variability of the measured number of copies around the
     limit of detection (optional) ......................................................................... 11
  E. Determination of the PCR efficiency (optional) ............................................. 12

Annex 3 - Example for conducting a robustness test .............................................. 13

References ............................................................................................................. 14

Impressum ............................................................................................................. 15
1 Scope

These guidelines describe the performance characteristics and the minimum performance criteria which should be taken into account when conducting a single-laboratory validation study for qualitative real-time PCR methods.

Validation data determined by the single laboratory will also help to decide whether the method in question should be validated in the framework of a collaborative study and be included into the BVL’s Official Collection of Methods.

2 Performance characteristics

In a single-laboratory validation study (e.g. by the developer of the method) for qualitative real-time PCR methods, data for the following performance characteristics should be determined:

- Limit of detection
- Specificity
- Robustness

On the basis of these data, it can be verified whether the minimum required performance criteria are fulfilled. A decision whether to conduct a validation of the method in the framework of a collaborative study can then be taken.

Annex 1 provides instructions for the determination of the DNA concentration as well as for the computation of the number of copies of the target sequence.

Annex 2 presents a work plan for the experimental determination of the limit of detection by a single laboratory. Optionally, data can be collected for the assessment of the variability of the measured number of copies around the limit of detection, and the efficiency of the method can be determined.

Annex 3 shows an example for conducting robustness tests.

2.1 Limit of detection (LOD)

Definition:

In qualitative PCR analysis (especially for the detection of GMO), the limit of detection of a single laboratory is usually understood as the concentration of the target DNA at which an amplification product is detected with a probability of at least 0.95 (LOD_{95%}). It is expressed in the number of copies of the target sequence.

Procedure:

The limit of detection should be determined by means of a dilution series of the target DNA, using a uniform concentration of non-target DNA (“background DNA”, cf. Annex 2) for each dilution level.

For each dilution level, 12 PCR replicate measurements are performed. The lowest dilution level (i.e. the lowest number of copies) for which all 12 replicates are positive is considered to be an approximate value for LOD_{95%}. Detailed instructions can be found in Annex 2 C.
In addition, Annex 2D describes a procedure for the assessment of the variability of the measured number of copies around LOD95%. To this end, the repeatability standard deviations are compared to the theoretical values resulting from the Poisson model.

The experimental data also allow the computation of the PCR efficiency (Annex 2E), the slope and the coefficient of determination.

**Performance criterion:**

The LOD95% of the qualitative real-time PCR method should not exceed 20 copies of the target sequence.

**Note:** For this value the mean amplification probability (\(\lambda\)) of the entire PCR is about 15% based on the parameter of the Poisson distribution (\(\lambda \times LOD_{95\%} = 2.996\)) [3].

### 2.2 Specificity

**Definition:**

The specificity is the property of a method to respond exclusively to the analyte.

**Procedure:**

**Theoretical test for specificity**

Carry out a computer-aided (“in-silico”) test, examining the oligonucleotide sequences (primer, probe) as well as the amplicon sequence for similarities to other sequences by searching suitable data bases (e.g. BLAST [4] in GenBank, or by means of the web service ‘JRC GMO-Amplicons’ [5]).

**Practical test for specificity**

a) Test for unexpected cross-reactions with non-target DNA

Check the PCR detection system for cross-reactivities with DNA from GMO containing similar genetic elements or constructs. Also check for plant species which are often present in samples (corn, soya, rape, rice, potato, wheat).

If non-target DNA is tested and a negative result is expected, at least 2500 copies should be added to the PCR reaction, if possible. If no reference material with sufficiently high concentrations of the non-target DNA is available, lower concentrations can be used and the number of copies added should be indicated. The amplificability of the non-target DNA should be verified by means of an independent test.

b) Test with target DNA

Add target DNA for which a positive result is expected in copy numbers in the range of the LOQ (here: the copy number for LOD95%, multiplied by a factor of 3, i.e. in general 20 to 60 copies per PCR). Add non-target DNA in a concentration of 100-200 ng / 25 µl of PCR mix to the target DNA, in order to simulate conditions which are relevant in practice and could influence the outcome.

It is sufficient to carry out each of the PCR tests according to a) and b) in duplicate determination.

**Performance criteria:**

The *in-silico* analysis should not show any unwanted similarities between sequences.

In the experimental test, all the PCR results should fulfill the theoretical expectations.
It there is cross-reactivity which is considered to be acceptable, it should be indicated and taken into account in the scope of the method.

2.3 Robustness

Definition:
The robustness of a method is a measure of its capacity to remain unaffected by small, accidental changes in the environment or in the conditions in which the measurement was performed. In order to determine robustness, measurement results corresponding to small, deliberate changes in the measurement conditions are collected.

Procedure:
A multifactorial experimental design is implemented [6]. For every combination of the factor levels listed in Table 1, target DNA is added in a concentration around the LOQ (e.g. the number of copies corresponding to the LOD\textsubscript{95\%}, multiplied by a factor of 3, in general 20 to 60 copies per PCR). Dilute the target DNA in non-target DNA (“background” DNA, e.g. 20 ng/µl, cf. Annex 2A). For each factor-level combination, PCR tests should be performed in triplicate. An example of the procedure can be found in Annex 3.

To check the robustness of the real-time PCR method, vary the following conditions for the factors listed in Table 1:

- Real-time PCR equipment (2 different types or manufacturers)
- PCR reagent kit (2 different manufacturers)
- Annealing temperature (e.g. ±1 °C)
- Master mix volume (±5 %, e.g. 19 µl of master mix + 5 µl of DNA versus 21 µl of master mix + 5 µl DNA)
- Primer concentration (minus 30 %)
- Probe concentration (minus 30 %)

Performance criteria:
The method should yield positive results for all mixes despite the modified conditions.

Note (1): In the case of negative results, the PCR test for the corresponding mixes should be repeated. In the case of repeated negative results, the method is not sufficiently robust and needs to be optimized.

Note (2): Considerable deviations between Ct values could be an indication that the robustness of the method is insufficient.

Table 1 Factors to be checked / modifications in the procedure conditions

<table>
<thead>
<tr>
<th>Factor</th>
<th>1</th>
<th>0</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCR equipment</td>
<td>A</td>
<td>B</td>
</tr>
<tr>
<td>PCR kit</td>
<td>X</td>
<td>Y</td>
</tr>
<tr>
<td>Primer concentration</td>
<td>unchanged</td>
<td>-30 %</td>
</tr>
<tr>
<td>Probe concentration</td>
<td>unchanged</td>
<td>-30 %</td>
</tr>
<tr>
<td>Volume of PCR mix</td>
<td>19 µl of master mix + 5 µl of DNA</td>
<td>21 µl of master mix + 5 µl of DNA</td>
</tr>
<tr>
<td>(total volume: 25 µl)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Annealing temperature</td>
<td>+1 °C</td>
<td>-1 °C</td>
</tr>
</tbody>
</table>
Table 2  Robustness test by means of an orthogonal experimental design [7]

<table>
<thead>
<tr>
<th>Factor</th>
<th>Combination</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
</tr>
<tr>
<td>PCR equipment</td>
<td>1</td>
</tr>
<tr>
<td>PCR kit</td>
<td>1</td>
</tr>
<tr>
<td>Primer concentration</td>
<td>1</td>
</tr>
<tr>
<td>Probe concentration</td>
<td>1</td>
</tr>
<tr>
<td>Volume of PCR mix</td>
<td>1</td>
</tr>
<tr>
<td>Annealing temperature</td>
<td>1</td>
</tr>
</tbody>
</table>

The orthogonal experimental design shown in Table 2 is characterized by the fact that, for any pair of factors (e.g. PCR kit/primer concentration), all four factor-level combinations (11, 10, 01, 00) occur with the same frequency (twice each). This structure guarantees that possible factorial interaction effects on the PCR test results can also be detected.
Annex 1 – Determination of copy numbers from genomic DNA

A. Determination of the DNA concentration

1. Determination by means of optical density at 260 nm

The optical density (OD 260) of the DNA treated with RNAse can be determined by means of spectrophotometry according to official method No L 00.00-119, Annex B.1 [8].

Criteria for acceptance:
\[
\text{OD}_{260}/\text{OD}_{280} > 1.8 \\
\text{OD}_{260}/\text{OD}_{230} > 2.0
\]

For \( \text{OD}_{260} = 1 \), the estimated DNA concentration is 50 µg/ml in case of double-strand DNA and 37 µg/ml in case of single-strand DNA.

2. Determination by means of the PicoGreen method

Alternatively, the DNA concentration can be determined fluorometrically by means of the PicoGreen method [9] (e.g. using the Quant-iT PicoGreen dsDNA quantitation kit, Molecular Probes).

3. Determination by means of digital PCR

The use of digital PCR equipment (e.g. digital droplet PCR) is a new approach which allows an accurate determination of the number of copies of a target sequence or the concentration of a DNA solution [10].
B. Estimation of the number of copies of the target sequence

The theoretical number of copies of the target sequence can be computed on the basis of haploid genome equivalents using genome weights, e.g. according to Arumuganathan et al. [11] or current databases [12]. The reference values and the reference used must be indicated.

In order to ensure the comparability of the computations carried out within the § 64 working group “Development of methods to identify foodstuffs produced by means of genetic engineering”, the genome weights listed in Table 3 should be applied in the validation of methods for the Official Collection.

Table 3: Mass of the haploid genome of different species [11], [12], [13]

<table>
<thead>
<tr>
<th>Species</th>
<th>Mass of haploid genome (in pg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cotton</td>
<td>2.33 [11]</td>
</tr>
<tr>
<td>Barley</td>
<td>5.55 [12]</td>
</tr>
<tr>
<td>Potato</td>
<td>1.8 [11]</td>
</tr>
<tr>
<td>Salmon</td>
<td>3.27 [13]</td>
</tr>
<tr>
<td>Linseed</td>
<td>0.70 [12]</td>
</tr>
<tr>
<td>Alfalfa</td>
<td>1.57 [11]</td>
</tr>
<tr>
<td>Corn</td>
<td>2.6 [11]</td>
</tr>
<tr>
<td>Papaya</td>
<td>0.39 [11]</td>
</tr>
<tr>
<td>Rape</td>
<td>1.15 [12]</td>
</tr>
<tr>
<td>Rice</td>
<td>0.5 [12]</td>
</tr>
<tr>
<td>Soya</td>
<td>1.13 [12]</td>
</tr>
<tr>
<td>Common wheat</td>
<td>17.33 [12]</td>
</tr>
<tr>
<td>Sugar beet</td>
<td>1.25 [12]</td>
</tr>
</tbody>
</table>

When using genomic DNA, first of all compute the number of genome equivalents on the basis of the mass of the respective haploid genome of the species according to the following equation:

\[
\text{Number of genome equivalents per } \mu\text{l} = \frac{\text{DNA concentration [ng/}\mu\text{l]} \times 1000}{\text{haploid genome mass [pg]}}
\]

The corresponding number of copies of the target sequence can be estimated on the basis of the number of genome equivalents. In this context, the number of copies per haploid genome as well as possible differences in the ploidy level and the zygosity of the employed reference material have to be taken into account.

When using plasmid DNA or amplicon DNA, the number of copies can be computed on the basis of the length of the sequence (in base pairs, bp) and the DNA concentration determined spectrophotometrically or fluorometrically (in ng/µl) according to the following equation:

\[
\text{Number of copies per } \mu\text{l} = \frac{\text{DNA concentration [ng/}\mu\text{l]} \times 6.022 \times 10^{14}}{\text{sequence length [bp]} \times 660}
\]

Plasmid DNA should be linearized by means of restriction digest before being used in the PCR.
Annex 2 - Determination of limit of detection, precision data and PCR efficiency

In the following, an exemplary work schedule for the experimental determination of LOD_{95%} (in copies of the target sequence) is described.

A. Dilution series of the target DNA

Prepare a dilution series of the target DNA, with a uniform concentration of non-target DNA for each dilution level. Supplement the DNA dilutions of the target DNA (genomic DNA, plasmid DNA or amplicon DNA) with sufficient background DNA (e.g. plant species DNA or salmon sperm DNA in concentrations of up to 200 ng per 25 µl of PCR mix), thus stabilizing them for the PCR.

Note: Before use, the background DNA must be tested for inhibition with regard to the target sequence (e.g. according to [14]).

To determine the LOD_{95%}, PCR tests should be performed for the six concentration levels given as examples in Table A2-1, with 12 replicates each.

By including an additional calibration series, it is possible to obtain data regarding both the variability of the measured number of copies around LOD_{95%} and the PCR efficiency (cf. paragraphs C to E).

For this purpose, prepare a dilution series with at least 4 levels (with background DNA, if possible) from a standard DNA solution (target DNA consisting of genomic DNA, plasmid DNA or amplicon DNA) (e.g. 2500, 500, 100, 50 copies per setup). Perform PCR tests for the different DNA dilutions, with at least 3 replicates each.

The calibration series is included in every PCR run in which target DNA dilutions are around LOD_{95%}.

B. Preparation of PCR replicates

In order to minimize possible errors caused by pipetting, prepare reaction mixtures from the PCR master mix (containing all components except for DNA) and from the DNA solution for every dilution level. These reactions mixtures are then distributed to the individual reactions (wells). The same applies for the preparation of the calibration series.

C. Determination of the limit of detection (LOD_{95%})

The LOD_{95%} is determined by means of the complementary log-log model. For this purpose, different software packages can be employed (e.g. “R” or “SAS”). Alternatively, the LOD_{95%}, the 95% confidence interval and the mean POD curve along with the corresponding 95% confidence range can be computed via a web service which is provided by the BVL [15] (enter the nominal copies in the PCR, the number of replicates, the number of positive results).

The LOD_{95%} is checked for plausibility. A value significantly smaller than 2.996 suggests that the numbers of copies of the target sequence that were actually added to the PCR reaction did not correspond to the (nominal) numbers of copies estimated for the DNA solutions.

For the verification of the DNA dilutions, a maximum of two results may be positive at the level with 0.1 copies per PCR of the target sequence; if there are more positive results than that, the estimation of the number of copies must be repeated.
Table A2-1: Example of a dilution series for the determination of the limit of detection

<table>
<thead>
<tr>
<th>Level</th>
<th>Preparation</th>
<th>Nominal copies/µl</th>
<th>Volume for PCR [µl]</th>
<th>Number of replicates</th>
<th>Copies in 5 µl</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>40 µl of DNA solution (20 copies/µl) plus 160 µl of dilution buffer*</td>
<td>4</td>
<td>100</td>
<td>12</td>
<td>20</td>
</tr>
<tr>
<td>2</td>
<td>100 µl of DNA solution (4 copies/µl) plus 100 µl of dilution buffer*</td>
<td>2</td>
<td>100</td>
<td>12</td>
<td>10</td>
</tr>
<tr>
<td>3</td>
<td>100 µl of DNA solution (2 copies/µl) plus 100 µl of dilution buffer*</td>
<td>1</td>
<td>120</td>
<td>12</td>
<td>5</td>
</tr>
<tr>
<td>4</td>
<td>80 µl of DNA solution (1 copies/µl) plus 120 µl of dilution buffer*</td>
<td>0.4</td>
<td>100</td>
<td>12</td>
<td>2</td>
</tr>
<tr>
<td>5</td>
<td>100 µl of DNA solution (0.4 copies/µl) plus 100 µl of dilution buffer*</td>
<td>0.2</td>
<td>180</td>
<td>12</td>
<td>1</td>
</tr>
<tr>
<td>6</td>
<td>20 µl of DNA solution (20 copies/µl) plus 180 µl of dilution buffer*</td>
<td>0.02</td>
<td>200</td>
<td>12</td>
<td>0.1</td>
</tr>
</tbody>
</table>

*contains background DNA up to a concentration of 100 ng per 25 µl of PCR mix (e.g. 20 ng/µl of salmon sperm DNA in 0.2 x TE buffer)

It may make sense to prepare other dilution levels of the copy numbers (e.g. 10 / 5 / 3 / 2 / 1.5 / 0.1 copies in 5 µl).

D. Assessment of the observed variability of the measured number of copies around the limit of detection (optional)

For the optional determination of copy numbers around the limit of detection, the copy numbers are assigned to the respective Ct values on the basis of an additional calibration series of target DNA (preparation cf. Annex 2A). Subsequently, the mean and the standard deviation are computed for each dilution level (example cf. Table A 2-2). Only dilution levels for which all replicates are positive can be taken into consideration for this statistical evaluation regarding the computed number of copies.

If the repeatability standard deviations are in the range of the standard deviation of the Poisson model, it can be concluded that the observed differences under repeatability conditions are due to the inevitable heterogeneity of the samples in the Poisson model. With this in mind, it makes sense to compute a standard deviation adjusted for the standard deviation of the Poisson model.

\[
\text{adjusted standard deviation} = \sqrt{\text{repeatability standard deviation}^2 - \text{Poisson standard deviation}^2}
\]

An adjusted standard deviation of more than 30 % indicates that the LOD_{95%} which can actually be achieved in routine analysis may be subject to high variations.

Table A2-2: Example for the statistical evaluation of a DNA dilution series (n=12)

<table>
<thead>
<tr>
<th>Nominal copy number of target sequence</th>
<th>Positive tests / all tests</th>
<th>Mean value (copies)</th>
<th>Std. dev.*</th>
<th>Relative std. dev.*</th>
<th>Poisson std. dev.</th>
<th>Adjusted std. dev.*</th>
<th>Relative adjusted std. dev.*</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>12/12</td>
<td>9.1</td>
<td>3.1</td>
<td>30.8 %</td>
<td>3.0</td>
<td>0.8</td>
<td>8.6 %</td>
</tr>
<tr>
<td>5</td>
<td>12/12</td>
<td>4.0</td>
<td>2.0</td>
<td>50.0 %</td>
<td>2.0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td>9/12</td>
<td></td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>6/12</td>
<td></td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>1.5</td>
<td>2/12</td>
<td></td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>0.1</td>
<td>0/12</td>
<td></td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

* under repeatability conditions
E. Determination of the PCR efficiency (optional)

**Definition:**
Percentage of the measured amplification rate per PCR cycle in relation to the theoretically achievable value of 2.

**Procedure:**
The efficiency can be computed in a single real-time PCR from the fluorescence measurement values during the exponential growth. However, these values are not available from every instrument. Therefore, the efficiency is usually determined with the help of a dilution series.

For this purpose the calibration series prepared in accordance with paragraph A can be used, yielding the following parameters:
- Slope (s)
- Coefficient of determination

**Computation of the PCR efficiency by means of linear regression analysis:**

PCR is an exponential process. There is a direct relationship between the number of copies of target DNA added at the beginning of the reaction and the measured Ct values. If copy numbers are plotted on the x-axis and the obtained Ct values on the y-axis, an exponential curve should result. This curve can be used to determine efficiency by means of linear regression analysis.

Consider the following equation:

\[ Y = X (1 + E)^n \]

where \( Y \) denotes the amount of amplicons produced, \( X \) denotes the initial number of copies, \( E \) denotes the efficiency of the reaction and \( n \) denotes the number of PCR cycles.

In order to generate the calibration curve, take the logarithm of both sides of Equation (1):

\[ \log Y = \log X + n \log(1 + E) \]

An expression for the slope (s) of the regression line can be derived from Equation (2):

\[ s = \frac{-1}{\log(1 + E)} \]

In the case of the base 10 logarithm, the ideal value is \( s = -3.3219 \).

Solving Equation (3) for \( E \), one obtains the efficiency (in %) as follows:

\[ E \% = 10^{\left(\frac{-1}{s}\right) - 1} \times 100 \]

A good efficiency should not deviate by more than ±10 % from the theoretical value of 100 % (corresponding to a slope in the interval -3.1 to -3.6). The coefficient of determination should be at least 0.98.
Annex 3 - Example for conducting a robustness test

<table>
<thead>
<tr>
<th>Factor</th>
<th>Combination</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCR equipment</td>
<td></td>
<td>A</td>
<td>A</td>
<td>A</td>
<td>A</td>
<td>B</td>
<td>B</td>
<td>B</td>
<td>B</td>
</tr>
<tr>
<td>PCR kit</td>
<td></td>
<td>X</td>
<td>X</td>
<td>Y</td>
<td>Y</td>
<td>X</td>
<td>X</td>
<td>Y</td>
<td>Y</td>
</tr>
<tr>
<td>Primer concentration</td>
<td>unchanged</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>-30%</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Probe concentration</td>
<td>unchanged</td>
<td></td>
<td></td>
<td>-30%</td>
<td>-30%</td>
<td>unchanged</td>
<td>-30%</td>
<td>unchanged</td>
<td>-30%</td>
</tr>
<tr>
<td></td>
<td>-30%</td>
<td></td>
<td></td>
<td>-30%</td>
<td>-30%</td>
<td>unchanged</td>
<td>-30%</td>
<td>unchanged</td>
<td>-30%</td>
</tr>
<tr>
<td>Master mix volume</td>
<td>19 µl</td>
<td>19 µL</td>
<td>21 µL</td>
<td>21 µL</td>
<td>21 µL</td>
<td>21 µL</td>
<td>19 µL</td>
<td>19 µL</td>
<td></td>
</tr>
<tr>
<td>Annealing temperature</td>
<td>+1 °C</td>
<td>-1 °C</td>
<td>+1 °C</td>
<td>-1 °C</td>
<td>-1 °C</td>
<td>+1 °C</td>
<td>-1 °C</td>
<td>+1 °C</td>
<td></td>
</tr>
</tbody>
</table>
References


Impressum

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