International Rules for Seed Testing 2027

Validated Seed Health Testing Methods

7-030: Detection of *Acidovorax valerianellae* in *Valerianella locusta* (corn salad) seed

Including changes and editorial corrections adopted at the Ordinary General Meeting 2026 in Calgary, Canada

Effective from 1 January 2027

Validation reports

See References. Copies are available by e-mail from the ISTA Secretariat at ista.office@ista.ch.

Please send comments, suggestions or reports of problems relating to this method to the ISTA Seed Health Committee, c/o ISTA Secretariat.

Disclaimer

Whilst ISTA has taken care to ensure the accuracy of the methods and information described in this method description, ISTA shall not be liable for any loss or damage, etc. resulting from the use of this method.

Safety precautions

Ensure you are familiar with hazard data and take appropriate safety precautions, especially during weighing out of ingredients. It is assumed that persons carrying out this test are in a laboratory suitable for carrying out microbiological procedures and familiar with the principles of Good Laboratory Practice, Good Microbiological Practice, and aseptic techniques. Dispose of all waste materials in an appropriate way (e.g. autoclaving, disinfection) and in accordance with local health, environmental and safety regulations.

Note on the use of the translations

The electronic version of the International Rules for Seed Testing includes the English, French, German and Spanish versions. If there are any questions on interpretation of the ISTA Rules, the English version is the definitive version.

Published by
The International Seed Testing Association (ISTA)
Richtiarkade 18, CH-8304 Wallisellen, Switzerland

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Online ISSN 2310-3655

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7-030: Detection of *Acidovorax valerianellae* in *Valerianella locusta* (corn salad) seed

Host: *Valerianella locusta* (L.) Laterr.
Pathogen(s): *Acidovorax valerianellae* sp. nov.

Prepared by: International Seed Health Initiative for Vegetable Crops, ISF (ISHI-Veg)

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Revision history

Version 1.0, 2015-01-01
Version 1.1, 2017-01-01: Reporting results revised
Version 1.2, 2021-01-01: Safety precautions, Sample size and Methods revised
Version 1.3, 2024-01-01: Sample size revised
Version 1.4, 2027-01-01: Flowchart added in Methods

Background

Acidovorax valerianellae (Av) is a soil transmitted (Grondeau *et al.*, 2003) and seed transmitted (Grimault *et al.*, 2006) pathogen of corn salad (*Valerianellae locusta*). It causes circular water-soaked spots that turn black on cotyledons and leaves of corn salad plants (Gardan *et al.*, 2003).

This method for the detection of *Acidovorax valerianellae* on corn salad seeds is developed by the International Seed Health Initiative for Vegetable Crops, ISF (ISHI-Veg), and validated in a comparative test with results of six laboratories. It includes a grow-out test performed in a sweatbox followed by PCR test confirmation of symptomatic corn salad cotyledons at 14 or 21 days after sowing.

Seeds can be sown on vermiculite or potting soil that in case of high (suspected) saprophytic load can be treated with the fungicide Thiram at a ratio of 4 g per kg of seeds (Serandat, 2008) against the saprophytic development in the sweatbox. Two options of incubation conditions (Grimault *et al.*, 2008) are provided to give greater flexibility to users who operate in different laboratory facilities. Seed samples known to be infected with *Acidovorax valerianellae* and a non-positive seed sample were used as positive and negative controls, respectively.

The primers in the PCR test for the confirmation of corn salad *Acidovorax valerianellae*-symptomatic cotyledons were developed by J. Heldens (Enza Zaden BV, 2004, pers. comm.) and validated on collections of *Acidovorax valerianellae* and saprophyte isolates (Mazouni 2007; Portier, 2010) and through comparison to grow-out results (Mazouni, 2007).

Safety precautions

Refer to special instructions/safety data sheets (http://ecb.jrc.ec.europa.eu/esis/) to handle the Thiram fungicide.

Dispose of all waste materials in an appropriate way (e.g. autoclaving, disinfection) and in accordance with local health, environmental and safety regulations.

Ethidium bromide

Ethidium bromide is carcinogenic. If possible, use an alternative chemical, e.g. Gel Red™ (Biotium). Use ethidium bromide according to safety instructions. It is recommended to work with solution instead of powder. Some considerations are mentioned below.

- Consult the Material Safety Data Sheet on ethidium bromide before using the chemical.
- Always wear personal protective equipment when handling ethidium bromide. This includes wearing a lab coat, nitrile gloves and closed toe shoes.
- Leave lab coats, gloves, and other personal protective equipment in the lab once work is complete to prevent the spread of ethidium bromide or other chemicals outside the lab.
- All work with ethidium bromide is to be done in an 'ethidium bromide' designated area in order to keep ethidium bromide contamination to a minimum.

Ultraviolet light

Ultraviolet (UV) light must not be used without appropriate precautions. Ensure that UV protective eyewear is utilised when working with ethidium bromide.

Treated seed

This method can be applied on untreated seed, on seed treated with fungicides or on seed treated with hot water or hypochlorite.

Sample size

The sample size (total number of seeds to be tested) and subsample size depend on intended use, the maximum acceptable infection level and the analytical sensitivity of the method. The minimum recommended working sample size is 10 000 seeds and the maximum subsample size must be 5000 seeds.

Materials

Reference material: a known positive seed sample and a known negative seed sample or standardised reference material of at least 1000 seeds each

Sweatbox: e.g. Rotho, ref. 4045, 10 l (32.5 cm × 38.5 cm × 16.5 cm) or equivalent

Substrate: medium-size vermiculite or potting soil vermiculite

Thiram fungicide (optional): 80 % WP (bis(dimethyl-thiocarbamoyl) disulphide, $C_6H_{12}N_2S_4$

Balance: capable of weighing to the nearest 0.001 g

Module/growth chamber: capable of operating/maintaining temperature at 18–25 ±2 °C

Containers for grinding of cotyledons: e.g. extraction bags Universal, Art. No. 430100, BioReba, Switzerland

0.85 % sterile saline

Grinder: e.g. press grinder, homogeniser hand model (Bioreba, Art. No. 400010) or equivalent

Pipettes and sterile pipette tips

70 % ethanol or chlorine disinfecting product

Forceps

PCR primers (J. Heldens, Enza Zaden BV, 2004, pers. comm.):
ZTO57-F: GAT CGT GGG GGA TAA CGG A
ZTO58-R: GTC ATG AGC TCT CTT TAT TAG AAA CAG

PCR positive control: DNA of an *Acidovorax valerianellae* known strain or standardised reference material

Microliter pipettes: e.g. Gilson, Finn

Sterile filtered tips: 1–1000 µl

Sterile Eppendorf tubes: 1.5 ml and 0.2 ml

Thermal cycler

Agarose electrophoresis equipment

DNA visualising system: e.g. BET or analogue reagent, UV imaging apparatus

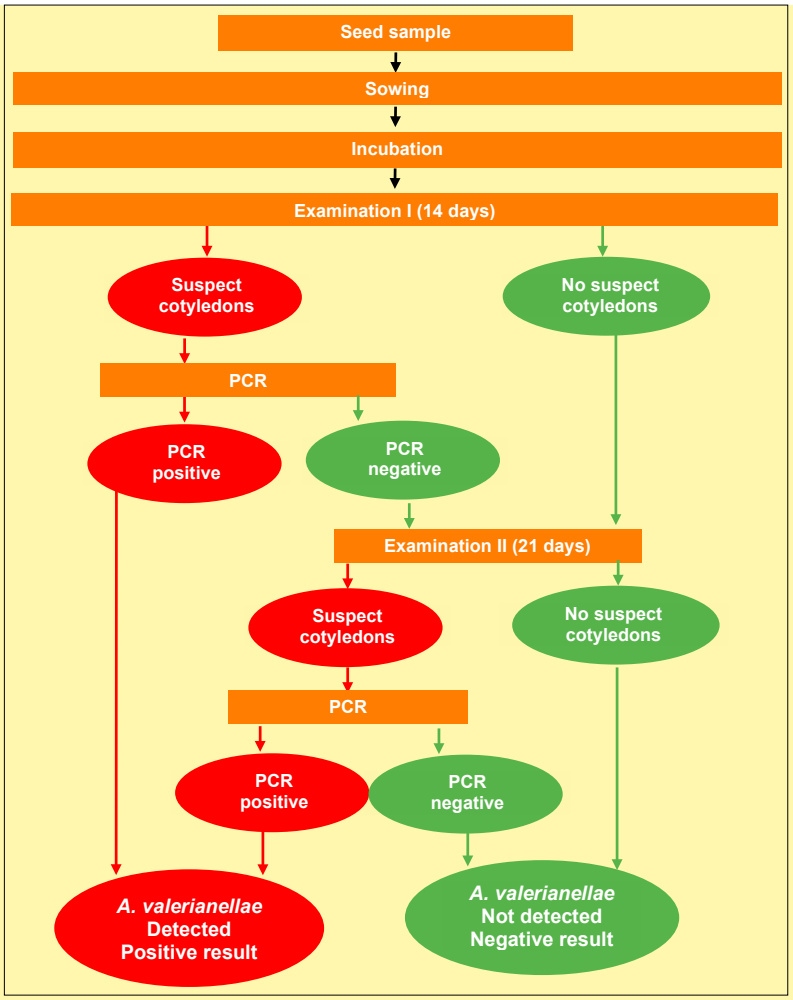
Sample preparation

This can be done in advance of the assay.

It is vital to exclude any possibility of cross-contamination between seed samples. It is therefore essential to disinfect all surfaces, containers, hands, etc. both before and after handling each sample. This can be achieved by swabbing/spraying equipment and gloved hands with 70 % ethanol.

1. Count the number of seeds in a known weight. Estimate the thousand-seed weight (TSW) as:
   TSW = (weight of seeds / number of seeds) × 1000
2. Based on the estimated TSW, weigh out subsamples of the required size into new, clean polyethylene bags or containers.

Follow the same procedure to weigh out the positive and negative control subsamples from the known positive and negative seed samples.



Methods

Critical control points are indicated by CCP.

1. Grow-out in a sweatbox

1.1 Take one sweatbox for each seed subsample and for the positive and negative control samples.

1.2 Disinfect the sweatbox with 1 % chlorine or equivalent disinfectant and rinse well.

1.3 Add 1 l of distilled/deionised water in each sweatbox. In case of high (suspected) saprophytic load in the subsamples, it is recommended to dissolve Thiram fungicide (80 % WP (bis(dimethylthiocarbamoyl) disulphide, $C_6H_{12}N_2S_4$) at a ratio of 4 g Thiram per kg of seeds (CCP) into the 1 l distilled/deionised water, to suppress fungal saprophytes that can hamper the test. However, in cases with low saprophytic load, Thiram treatment is not a necessity.

1.4 Add 2 l of clean and fresh vermiculite or potting soil to the solution in each sweatbox by spreading it evenly over the whole surface.

1.5 Spread the seeds of each subsample and of the positive and negative control samples evenly over the substrate of the corresponding sweatbox. When potting soil is used, cover the seeds with a thin layer of vermiculite. An extra layer of vermiculite under the seeds to help prevent saprophytic development in the sweatbox is optional.

1.6 Close the sweatboxes with the lid and incubate at 20 ±2 °C with 12 h light/dark or at 25 ±2 °C with 12 h light alternating with 18 ±2 °C with 12 h dark (CCP).

1.7 On the 14th day after sowing, inspect cotyledons for typical *Acidovorax valerianellae* symptoms and foci development by comparison with the positive and negative control cotyledons (CCP).

1.8 Typical *Acidovorax valerianellae* symptoms on the cotyledons are the small, circular, black water-soaked spots that turn black on cotyledons (Figs. 1–3). Cotyledons may rot due to *Acidovorax valerianellae* infection. One focus can be one single seedling with symptoms, or consist out of several seedlings together. A focus has a round shape showing the origin of contamination from one single seed. Foci become confluent over time (CCP).

1.9 If cotyledons with typical and/or doubtful symptoms appear, count the number of foci in each sweatbox

(optional) and confirm symptoms in the PCR test (see section 2).

1.10 If positive results are given in the PCR test, end the incubation and record the PCR result as the final result for the seed subsamples.

1.11 If negative results are given in the PCR test or no cotyledons with typical symptoms appear, prolong the incubation of all sweatboxes until the 21st day after sowing.

1.12 On the 21st day after sowing, inspect cotyledons for typical symptoms and foci development by comparison with the positive and negative control cotyledons (CCP).

1.13 If cotyledons with typical and/or doubtful symptoms appear, count the number of foci in each sweatbox (optional) and confirm symptoms in the PCR test. The final result of the seed subsamples will be given by the PCR test result (negative or positive confirmation).

1.14 If no cotyledons with symptoms have appeared on the 21st day after sowing, the final result of the seed subsamples will be given by the grow-out test result (negative), as no PCR test confirmation is needed.

1.15 If more than 20 % of the corn salad seedling population is damped off due to fungal infection and **NO** *Acidovorax valerianellae* symptoms can be observed on the cotyledons, this must be noted and indicated on the test result (CCP). In this case it is recommended to repeat the test with sufficient seeds to compensate for seedling loss with the option of a Thiram-treated substrate.

2. Collection of symptomatic cotyledons for PCR test confirmation

2.1 Collect 1–8 cotyledons with typical *Acidovorax valerianellae* symptoms from each sweatbox from different foci. Pool cotyledons in a suitable container (e.g. extraction bags from Universal, Art. No. 430100, BioReba, Switzerland). In case of doubtful symptoms, more than 8 cotyledons can be collected per sweatbox.

2.2 Collect an equal number of symptomatic cotyledons from the positive control sample and place them in separate containers.

2.3 Repeat the collection for the negative control sample and place the collected symptomless cotyledons in separate containers.

2.4 Add 3 ml of 0.85 % sterile saline in each container and grind the cotyledons with a grinder.

2.5 Transfer 1 ml of each cotyledon extract in separate Eppendorf tubes.

2.6 Transfer 1 ml of 0.85 % sterile saline in a separate Eppendorf tube to use as process control.

3. Polymerase chain reaction (PCR)(CCP)

3.1 Centrifuge the extracts including the process control for 5 min at 6000–10 000 *g*. Discard the supernatant and re-suspend the pellet with 200 µl of sterile water. Incubate for 5 min at 100 °C, place on ice for 5 min and make a 10-fold dilution with sterile water in a separate tube. Extracts can be stored at −20 °C until identification.

3.2 Use the following *Acidovorax valerianellae* specific primers (J. Heldens, Enza Zaden BV, 2004, pers. comm.) that will give a product of 346 bp:
ZTO57-F: GAT CGT GGG GGA TAA CGG A
ZTO58-R: GTC ATG AGC TCT CTT TAT TAG AAA CAG

3.3 Prepare the reaction mixture. Carry out PCR reactions in 0.2 ml thin walled PCR tubes in a final volume of 25 µl (24 µl reaction mixture + 1 µl bacterial suspension). Include a PCR positive control (e.g. DNA of *Acidovorax valerianellae* known strain) and a PCR negative control (sterile water).

3.4 PCR profile: An initial 2 min incubation at 94 °C followed by 35 cycles of 30 s at 94 °C, 1 min at 63 °C and 1 min at 72 °C. A final 5 min incubation at 72 °C and infinity at 4 °C.

3.5 Fractionate 10 µl of the PCR products, the negative process control and sterile water (negative PCR control) by gel electrophoresis in a 1.5 % agarose gel in 1× Tris acetate EDTA (TAE buffer) (CCP). Include a 100 bp ladder. Stain with ethidium bromide in a bath and rinse in water.

3.6 Analyse the amplification products for an *Acidovorax valerianellae*-specific product of 346 bp (Fig. 4). When no bands of 346 bp are visible in the test samples, the presence of *Acidovorax valerianellae* cannot be confirmed and the test result is regarded as negative, provided that the positive controls show the specific band of 346 bp.

General methods

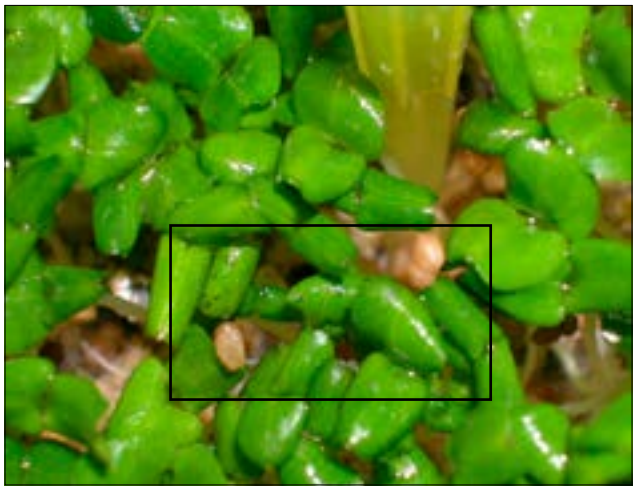
Reporting results: The result of a seed health test should indicate the scientific name of the pathogen detected and the test method used. When reported on an ISTA Certificate, results are entered under ‘Other Determinations’.

The report must indicate the number of seeds tested.

In the case of a negative result (pathogen not detected in any subsample), the results must be reported as ‘not detected’.

In the case of a positive result, the report must indicate the number of positive subsamples out of the total number tested.





Figures 1, 2. Symptoms of *Acidovorax valerianellae* on corn salad cotyledons.

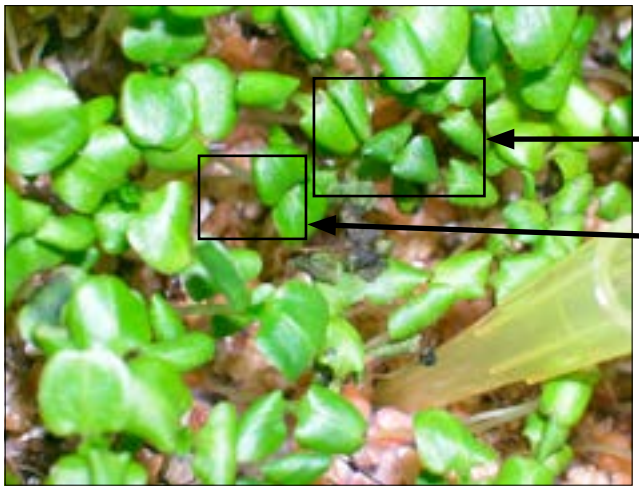


Figure 3. Symptoms of *Acidovorax valerianellae* (A) and fungal development on corn salad cotyledons (B).

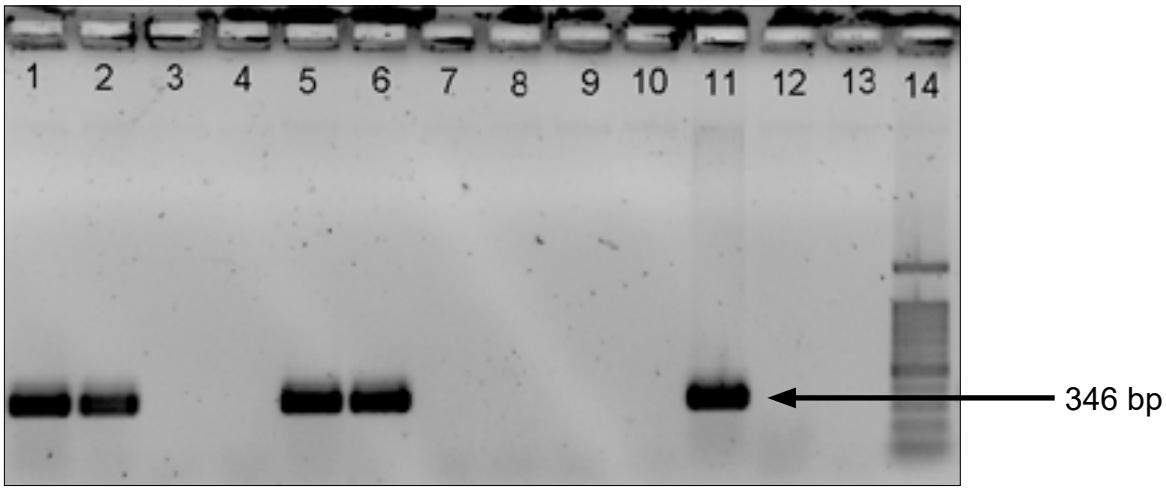


Figure 4. Agarose gel showing *Acidovorax valerianellae* amplification products. 1, 2: pure extract and 1/10 dilution for a positive subsample. 3, 4: pure extract and 1/10 dilution for a negative subsample. 5, 6: pure extract and 1/10 dilution for the positive control sample. 7, 8: pure extract and 1/10 dilution for the negative control sample. 9, 10: pure extract and 1/10 dilution for the process control. 11: PCR positive control (DNA of *Acidovorax valerianellae* strain). 12, 13: PCR negative control (sterile water); no reaction. 14: 100 bp ladder.

Quality assurance

A record should be kept of the date and results of pipette calibration checks.

It is essential that operators have received appropriate training and use automatic pipettes correctly.

Critical control points (CCP)

- Thiram should not be used at concentrations >4 g per kg of seeds (G. Hiddink, pers. comm.) as it will reduce the symptom expression of *Acidovorax valerianellae* on corn salad seedlings (Step 1.3).
- A high relative humidity (>90 %) should be maintained in the sweatboxes throughout the incubation process to enable development of *Acidovorax valerianellae* symptoms (Step 1.5).
- The light duration can be adapted to light quality of the growth chamber throughout the incubation process, provided that the test is validated by comparison with the cotyledons of the positive control seed sample (Step 1.6).
- The cotyledons of the positive control seed sample should give typical symptoms and the cotyledons of the negative control sample should give no symptoms in the grow-out test (Steps 1.6 and 1.11).
- The grow-out test should be carried out by trained operators, as recognition of symptoms requires experience (Steps 1.7 and 1.11).
- Damping-off on corn salad cotyledons caused by fungi can mask the *Acidovorax valerianellae* symptoms. If more than 20 % of the corn salad seedling population is damped-off due to fungal infection and no *Acidovorax valerianellae* symptoms can be observed on the cotyledons, the test result must be considered invalid. If, however, *Acidovorax valerianellae* symptoms are observed on the cotyledons and confirmed in the PCR, the test result is valid and the seed subsample is infected. (Steps 1.13 and 1.15).
- Positive PCR control (DNA of a known *Acidovorax valerianellae* strain or reference material) should be included in every PCR test (Step 3.3).
- The preparation of PCR mixture (Step 3.3), the amplification PCR programme (Step 3.4) and the preparation of agarose gel for electrophoresis (Step 3.5) should be adapted to available material and equipment of individual laboratories testing for *Acidovorax valerianellae* under the condition that results will be validated by PCR controls.
- The positive control seed sample should give a positive result in the PCR test and the negative control seed sample should give a negative result in the PCR test (Step 3.6).
- The positive PCR control (DNA of *Acidovorax valerianellae* known strain or reference material) should give the expected amplification product in the PCR test (Step 3.6).
- The process control and the negative PCR control (sterile water) should give a negative result in the PCR test (Step 3.6).

Media and solutions

Sterile saline

Sodium chloride (NaCl): 8.5 g
Distilled/deionised water: 1000 ml

Preparation

1. Weigh out all ingredients into a suitable container.
2. Add 1000 ml of distilled/deionised water.
3. Dissolve and dispense into final containers.
4. Autoclave at 121 °C and 15 psi for 15 min.

Storage

Provided containers are tightly closed, may be stored for several months before use.

Example of reaction mixture preparation for PCR

Compound	Final concentration	Volume in 25 µl (µl)
Sterile Milli Q		19.8
*Taq Buffer 10×	1×	2.5
dNTP (20 mM total, 5 mM each)	0.1 mM each	0.5
ZTO57-F (20 pmol/µl)	0.4 pmol/µl	0.5
ZTO58-R (20 pmol/µl)	0.4 pmol/µl	0.5
Taq polymerase (5U/µl)	0.05 U/µl	0.2
DNA		1.0

* 10× concentrated PCR reaction buffer (with Mg+), containing 10 mM Tris HCl, 15 mM $MgCl_2$ and 500 mM KCl

Example for visualisation of PCR products

Tris acetate EDTA (TAE) buffer 1×

Compound	Amount/l
Tris acetate EDTA (TAE 50×)	20 ml
Distilled/deionised water	1000 ml

1.5 % agarose gel for electrophoresis

Compound	1 agarose gel (25 × 15 cm)	Amount/l
Tris acetate EDTA (TAE 1×)	300 ml	1000 ml
Agarose	4.5 g	15.0 g

Preparation

1. Make sure that the gel tray is clean and dry before use. Use the gel caster. Place the gel comb(s) in position in the gel tray.
2. Weigh the desired amount of agarose and place in an Erlenmeyer flask with a measured amount of electrophoresis buffer, e.g. for a 300 ml gel add 4.5 g of agarose and 300 ml of 1× TAE buffer to a 500 ml flask. The larger flask ensures the agarose will not boil over.
3. Dissolve the agarose in a microwave oven. All the grains of agarose should be dissolved and the solution clear.
4. Allow the medium to cool down to approx. 60 °C.
5. After the gel is completely set carefully remove the gel comb(s).
6. Remove the gels and place them in the electrophoresis unit.
7. The same electrophoresis buffer used in the gel must also be used for the running buffer.

Note: The amount of 1.5 % agarose gel for electrophoresis to be prepared depends on the available electrophoresis apparatus of a laboratory.

References

ESIS: European Chemical Substances Information System (http://ecb.jrc.ec.europa.eu/esis/).

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