LABORATORY PROTOCOL

PCR for plasmid-mediated colistin resistance genes, *mcr-6, mcr-7, mcr-8, mcr-9,* and variants (multiplex)

(protocol optimized at National Food Institute, Denmark)

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Version 1

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PROTOCOL

PCR protocol for the *mcr-6, mcr-7, mcr-8, mcr-9* genes and variants (multiplex)

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Materials

Equipment

- PCR thermocycler
- Pipettes for 1 µL to 1000 µL
- Electrophoresis unit
- Microwave or autoclave
- Eppendorf centrifuge
- Photo camera
- UV-transilluminator
- Water bath 50°C

Materials

- Molecular marker (e.g. GeneRuler^{TM}100 bp Plus DNA ladder–100- 3000 bp)
- Ethidium bromide solution (1%), or preferable dyes
- Staining bath
- Electrophoresis buffer TAE or TBE (see composition of media and reagents)
- Eppendorf tubes
- Tips (filter) for pipettes 1 µL to 1000 µL
- Agarose
- Primers stock solutions (10 µM)
- Dream Taq Green PCR Master Mix (2×) (Thermo Fisher)
- DNA template (boiling lysates)
- TE buffer (Tris:EDTA 10:1)
- TrisHCl buffer
- Crushed ice
- Mineral oil (if necessary)

Safety

Carry out all procedures in accordance with the local codes of safe practice.

For staining the DNA, ethidium bromide can be used. This dye is carcinogenic. Therefore, gloves and proper clothes should be worn (Appendix 1: Laboratory Safety). Alternatively, dyes which are less toxic, or the E-Gel^{TM} Power Snap Electrophoresis System (Invitrogen) can be used. (https://www.thermofisher.com/dk/en/home/life-science/dna-rna-purification-analysis/nucleic-acid-gel-electrophoresis/dna-stains). Visualization of the stained DNA is done by use of UV transilluminator. UV light is harmful for skin and eyes. Therefore, proper protection (facemask, glasses) should be worn (Appendix 1: Laboratory Safety).

PCR detection of *mcr-6, mcr-7, mcr-8* and *mcr-9* variants (multiplex)

Previously, a multiplex PCR protocol for detection of *mcr-1, mcr-2, mcr-3, mcr-4 and mcr-5* colistin resistance genes was developed by Rebelo *et al*, 2018. In 2018, a novel *mcr* homologue encoding the phosphoethanolamine-lipid A transferase, *mcr-6* was found in a *Moraxella* strain and the sequence uploaded to the NCBI nucleotide archive (Genbank acc. no. NG_055781.1). In addition, the *mcr-7* (Yang *et al,* 2018) and *mcr-8* (Wang *et al*, 2018) variants have been reported in *Klebsiella pneumonia* and both genes encode a phosphoethanolamine-lipid A transferase, conferring phenotypic resistance to colistin. In 2019, *mcr-9* was discovered in a *Salmonella Typhimurium* strain which notably was found to be phenotypically sensitive when tested against colistin (Carroll *et al*, 2019). In contrast, when *mcr*-9 was cloned into the colistin-susceptible *E. coli* host under an isopropyl-*β*-D-thiogalactopyranoside-induced promoter, the expression of *mcr-9* was found to mediate resistance to colistin at concentrations of; 1, 2, and 2.5 mg/L colistin (Carroll *et al*, 2019).
This protocol is an update of the previous multiplex PCR protocol developed at DTU Food, for detection of the *mcr-1* to *mcr-5* homologues (Rebelo *et al*, 2018), and this new version of the protocol aims to detect all *mcr-6, mcr-7, mcr-8* and *mcr-9* homologues and was originally developed by Borowiak *et al*. 2020.

Control strains

E. coli carrying Top10F' pCR2.1 -*mcr-6* obtained from Borowiak *et al,* 2020
E. coli carrying Top10F' pCR2.1 -*mcr-7* obtained from Borowiak *et al,* 2020
E. coli carrying Top10F' pCR2.1 -*mcr-8* obtained from Borowiak *et al,* 2020
S. Agona carrying *-mcr-9.1* (27683) obtained from Central Veterinary Laboratory, Algete Madrid

DNA extraction

The template DNA used consisted of boiling lysates prepared from the strains. A brief description: a loopful of culture was suspended in 100 µL of sterile TE buffer, boiled 10 min at 100ºC, centrifuged 5 min at 6000 G. For use as template in the PCR, the DNA supernatant was further diluted at 1:10 in TrisHCl buffer.

Primers

Target gene	Primer sequences (5'-3')	Product length (bp)	Reference
mcr-6	*mcr6*_fw: AGCTATGTCAATCCCGTGAT *mcr6*_rev: ATTGGCTAGGTTGTCAATC	252	Borowiak *et al.* 2020
mcr-7	*mcr7*_fw: GCCCTTCTTTTCGTTGTT *mcr7*_rev: GGTTGGTCTCTTTCTCGT	551	Borowiak *et al.* 2020
mcr-8	*mcr8*_fw: TCAACAATTCTACAAAGCGTG *mcr8*_rev: AATGCTGCGCGAATGAAG	856	Borowiak *et al.* 2020
mcr-9	*mcr9*_fw: TTCCCTTTGTTCTGGTTG *mcr9*_rev: GCAGGTAATAAGTCGGTC	1011	CVL, Algete Madrid

Reaction mix

Prepare the following mix in a microcentrifuge tube (for a 25µL reaction; see the example of PCR set up). Additionally, prepare a blank reaction without template DNA as negative control. This version of the PCR protocol is optimized for the Dream Taq Green PCR Master Mix (2×) (https://tools.thermofisher.com/content/sfs/manuals/MAN0012704_DreamTaq_Green_PCR_MasterMix_K1081_UG.pdf) which can be replaced by another polymerase, although the protocol might need some optimization to adjust for the particular conditions at your laboratory in which we can give you some assistance.

Dream Taq PCR Master Mix*	12.5 µL
Primer Mix**	4 µL
DNA template	2 µL
Water up to	25 µL

*This PCR at the EURL-AR laboratory is optimized for the Dream Taq Green PCR Master Mix (Thermo Fisher) which already contains $MgCl_2$.

**The primer mix contains 0.5 µL of each primer stock solution (10 µM)

Conditions for the PCR

95ºC 3 min + 30× (95ºC 30 sec + 55ºC 30 sec + 72ºC 60 sec) + 72ºC 10min.

References

- Rebelo AR, Bortolaia V, Kjeldgaard JS, Pedersen SK, Leekitcharoenphon P, et al. Multiplex PCRfor detection of plasmid-mediated colistin resistance determinants, *mcr-1, mcr-2, mcr-3, mcr-4* and *mcr-5* for surveillance purposes. Euro Surveill. 2018; 23(6).
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- Carroll LM, Gaballa A, Guldimann C, Sullivan G, Henderson LO, et al. 2019. Identification of novel mobilized colistin resistance gene *mcr-9* in a multidrug-resistant, colistin-susceptible *Salmonella enterica* serotype Typhimurium isolate. mBio 10:e00853-19.
- Yang Y-Q, Li Y-X, Lei C-W, Zhang A-Y, Wang H-N. Novel plasmid-mediated colistin resistance gene *mcr-7.1* in *Klebsiella pneumonia*. J Antimicrob Chemother. 2018 (73).
- Wang X, Wang Y, Zhou Y, Li J, Yin W, et al. Emergence of a novel mobile colistin resistance gene, *mcr-8*, in NDM-producing *Klebsiella pneumonia*. Emerg. microbes & infect 2018 (7) 122, 7:1.

Detailed Procedure	Theory / comments
Preparation of the samples 1. Transfer 100 µL of TE buffer to a 1.5 mL Eppendorf tube. Using a disposable inoculation loop (white; 1µL), pick a loop full of bacteria from a plate and transfer to the Eppendorf tube. 2. Boil the suspension (or heat at 95°C) for 5-10 minutes. 3. Centrifuge at 6000 G for 5 min. 4. Dilute the supernatant lysed DNA 10- fold in TrisHCl	Make a homogeneous cell suspension in an Eppendorf tube, using a loop or cotton swab. Shake or vortex suspension just before use. Only little cell mass is needed and if too many bacteria are used, it might cause inhibition of the PCR. TE is used because it contains EDTA that binds divalent ions needed by enzymes that would be able to degrade DNA. Since EDTA can inhibit the PCR reaction, dilute in TrisHCl. Boiling breaks down the bacterial cell wall and allows release of DNA. Make a ventilation hole in the lid of the eppendorf tube using a needle, alternatively the eppendorf tubes can be capped with a lidlock.
Preparation of the mix 1. Check the number of samples and calculate the amount of PCR master mix needed. 2. Prepare the PCR master mix (you maydo it in a tray of crushed ice as mentioned in Appendix 2). 3. Aliquot the PCR master mix to the required number of PCR tubes (23 µL per tube). 4. Depending on the PCR machine that is used, one drop of mineral oil could be added.	Always prepare mix for at least 1-2 additional samples (n mix = n samples + 1 or 2). Use crushed ice if the PCR master mix is prepared at temperatures above 25°C as this may affect the result. If the PCR machine has no heat in the lid use oil as a lid to avoid the mixture to vaporise and condensate in the lid of the tubes.

Running the PCR 1. Add 2 µL of sample to the sample tube and close the lid. 2. Add 2 µL of water to the negative control tube and close the lid 3. Finish the procedure by adding the positive control DNA and close the lid. 4. Place the tubes into the PCR thermocycler. 5. Program the PCR thermocycler (or select the requested program) as mentioned in appendix 2 (Example of PCR set-up). 6. Run the program.	Always end the set up with the positive control DNA to avoid contaminations of the test tubes, causing false positive results. If oil is used, make sure DNA is dispensed below the oil phase. For PCR a positive control and a negative control (sterile water) should be taken along.
Preparation of the agarose gel **Note**: Other solutions can be used (e.g. SYBR Safe staining, E-gel^{TM}. See p. 3) 1. Assemble the gel tray and make a proper set-up. 2. Prepare a 1.5% agarose solution in TBE buffer 1× by boiling the solution a few minutes until completely dissolved by the use of a water bath or microwave oven. 3. Cool the agarose to 40-50°C in a water bath and pour the agarose into the gel tray. 4. Let the gel solidify for 15-30 minutes. 5. Prepare a staining bath containing a final concentration of 5 µg/mL ethidiumbromide.	Depending of electrophoresis system use TBE or TAE buffer. Wear suitable protection for working with ethidium bromide (gloves, clothes). Appendix 1: laboratory safety.

Assembling the results	
1. Put the gel into the electrophoresis unit and if necessary refill with buffer.	TBE contains boric acid. See Appendix 1 for safety sheet.
2. Load 8 µL of each PCR sample into the wells of the gel. Finish off by loading at least one molecular marker.	The Dream Taq Green Master Mix contains two tracking dyes and a density reagent that allows the direct loading of PCR product. It is important to use a proper marker in order to notice whether the PCR product has the right size.
3. Replace the lid of the unit and run the gel by starting the electrophoresis process.	Electrophoresis can be done at different voltages/amperages. Normally, 130V seems to be fine. Running time depends on several parameters like buffer composition, resistance, current.
4. After a complete run of 30-45 minutes, remove the lid of the unit and place the gel in a staining-bath for about 30 minutes. Rinse shortly in water before visualizing the gel / bands.	Be extremely careful and wear the correct protective gloves when dealing with ethidium bromide. Read safety precautions in Appendix 1
5. Place the gel / tray on top of the UV-transilluminator.	UV light is harmful for skin and eyes. Wear proper protection (facemask). See Appendix 1.
6. Visualize the results by switching on the UV-lamp. 7. Look for the presence of specific bands (Appendix 2: Example of PCR set up).	The whole PCR process is very sensitive towards contaminations that can affect the result as false positive results. It is therefore recommended to perform the different steps, if possible, in different rooms e.g.: Room 1: Preparing the PCR master mix into tubes. Room 2: Adding the samples to the tubes and running the samples in the PCR thermocycler. Room 3: Running the electrophoresis and visualising the DNA.

Composition and preparation of media and reagents

Reagents can be made as described below and/or are commercially available from companies like Invitrogen Life Technologies and Roche Applied Science.

TAE (Tris-Acetate EDTA) buffer

Working solution

- 0.04 M Tris Acetate
- 0.001 M EDTA

Concentrated stock solution (50×) – per liter:

- Tris base 242 g
- Glacial acetic acid 57.1 mL
- 0.5 M EDTA (pH 8.0) 100 mL

TBE (Tris-Borate EDTA) buffer

Working solution

- 0.089 M Tris borate
- 0.089 M boric-acid
- 0.002 M EDTA

Concentrated stock solution (5×) – per liter:

- Tris base 54 g
- Boric acid 27.5 g
- 0.5 M EDTA (pH 8.0) 20 mL

Tris-EDTA buffer (TE 10:1) 1 L (pH 8)

- 1 M Tris-HCl (pH 8) 10 mL
- 0.5 M EDTA (pH 8.0) 2 mL
- Water 988 mL

Tris-HCl buffer 1 L (pH 8)

1 M Tris-HCl (pH 8) 10 mL

Water 990 mL

Ethidium bromide (10mg/mL)

- Add 1 g of ethidium bromide to 100 mL of H_2O. Stir on a magnetic stirrer for several hours to ensure that the dye has dissolved. Wrap the container in aluminum foil or transfer to a dark bottle and store at 4°C.
- Ethidium bromide can be bought ready-made as a 10 mg/mL solution

APPENDIX 1 (Laboratory Safety)

Safe Work Procedure **ETHIDIUM BROMIDE**

Use

Ethidium bromide is added to electrophoresis gels for visualization of nucleic acids

Hazards

Class 6 – Toxic. Potent mutagen

Risk control measures

Only use ethidium bromide (EtBr) after receiving safety training (laboratory induction / authorization). Wear safety glasses when using ethidium bromide. Avoid skin contact; ethidium bromide may be absorbed through the skin. Wear latex gloves, laboratory coat. Always dispose of gloves after use. Do not touch equipment, door handles, phone, keyboard, etc.

Weighing solid - Powder may cause irritation when inhaled - wear dust mask and use in ventilated area. Use designated micropipette, only, when dispensing the liquid.

Engineering/Ventilation controls

Ensure access to a safety shower and eye wash in areas where ethidium bromide is used. Preferably weigh the solid in a fume hood.

Storage requirements

Store in a cool, dry place away from strong oxidizing agents. Keep containers tightly closed. Use with adequate ventilation.

First aid/Spill control procedures

Wash off immediately with copious amounts of cold water (at least 10 minutes). Ethidium bromide is absorbed through the skin so follow the cold water washing with a thorough washing with warm water and soap. Contaminated clothing should be removed as soon as possible and thoroughly washed.

In case of contact with eyes, immediately flush eyes with copious amounts of water for at least 15 minutes (eye wash).

Seek medical attention.

If the spill is on equipment, use ultraviolet light (wear appropriate eye protection) to locate spill, then use the decontamination procedure outlined below.

Wear protective clothing.

Small spill: If in solution, absorb freestanding liquid using vermiculite or Polyzorb from Spill Kit. Use ultraviolet light to locate spill. Follow instructions on Spill Kit.

Large Spill: Notify others in the area of spill. Evacuate area. Barricade area with tape (in Spill Kits) to prevent entry until arrival of response personnel. Provide assistance and information to spill clean up crew.

Waste

Store waste; liquid: In proper waste container

Store waste; Solid: In proper waste container

Ethidium bromide liquid disposal

1. Add 10g activated charcoal per 2.5L waste

2. Leave for 1 hour, with occasional shaking

3. Filter contents through Whatman Number 1 filter paper.

4. Filtrate may be disposed of down the sink.

5. Charcoal & paper is treated as solid hazardous waste and disposed in the EtBr Solid Waste Bucket.

If Using 'Green Bag' (Bio-101 Cat. No. 2350-200):

1 For 10mg Ethidium Bromide (max) add 1 'Green Bag' to the waste bottle with a magnetic flea.

2. Place waste bottle onto a magnetic stirrer and mix the solution for 24hours.

3. Dispose of the 'Green Bag' in the dry Ethidium Bromide waste. The remaining solution may be disposed of in the sink.

Staining gels

During electrophoresis, add EtBr after boiling up the agarose - let it cool down before adding EtBr

Afterwards, soak gel in a well-marked plastic container - put name and date on container as it is possible to re-use the staining solution.

Safe Work Procedure **<u>ULTRA VIOLET SOURCE</u>**

Ultraviolet light

Ultraviolet radiation is the portion of the electromagnetic spectrum that falls in the region of 100 to 400nm. This spectrum has been divided into three regions:

A: 400nm to 315nm known as Near-UV or UV-A
B: 315nm to 280nm known as Mid-UV or UV-B
C: 280nm to 100nm known as Far-UV or UV-C

Hazards

Two categories of hazard are involved in the use of high intensity UV lamps: those inherent in the radiation itself and those associated with operation of the lamps. All radiation of wavelength shorter than 250 nm should be considered dangerous.

- Damage to eyes and skin caused by exposure to UV radiation. Repeated overexposure of skin to UV has been linked with premature aging, wrinkles and most seriously, skin cancer. Eye damage can result in corneal scarring and cataract formation.
- Burns caused by contact with a hot UV lamp.
- Fire ignited by hot UV lamp.
- Interaction of other nearby chemicals with UV radiation.

Damage caused to apparatus placed close to UV lamp

Risks

Damage to vision is likely following exposure to high intensity UV radiation.

Who is likely to be injured?

The user or anyone exposed to the UV light as a result of faulty procedure. Injuries may be slight to severe.

Control measures operating precautions

Lab-coats, gloves and safety glasses or other appropriate eye/skin protection such as UV protective glasses or a UV protective face shield must be worn.

Reactions using UV lamps: external irradiation sources

- These operations must never be attempted by an untrained person.
- These operations must never be attempted by a single person.
- These operations must never be attempted out of normal working hours.
- Use of UV lamps must be carried out in a fume hood with boarded up windows.
- As far as possible, the UV source should be contained in a closed radiation box.
- The fume hood sash must remain closed while the UV lamp is switched on.
- The fume hood may contain only the UV lamp and associated apparatus and chemicals. No other chemicals are to be stored in the fume hood and no other reactions are to be performed in the fume hood.
- Reaction vessels containing flammable solvents must be at least 20 cm away from the lamp to avoid excessive heating.
- Flammable equipment (*e.g.* rubber/plastic tubing) must be positioned at least 10 cm away from the lamp.
- After the UV lamp is switched off, unless the reaction mixture requires immediate attention, the fume hood sash should remain closed for 30 minutes to allow the UV lamp to cool.

Reactions using UV lamps: low/medium pressure Hg lamps in an immersion well

- These operations must never be attempted by an untrained person.
- These operations must never be attempted by a single person.
- Low/ Medium pressure lamps are to be used ONLY in approved, water-cooled immersion well apparatus.
- The UV lamp power supplies must incorporate an electrical cutout that activates in the event of disruption to cooling water.
- The UV lamp must not be switched on until:
    - The glassware is shrouded in Al foil
    - The immersion well set-up is shielded by the appropriate metal case
    - The boarded up fume-hood doors are closed

- The UV lamp must NEVER be switched on/connected outside of the shrouded immersion well apparatus.

Training

For the use of high intensity UV sources, new users must be trained by another member of the laboratory who, in the opinion of the member of staff in charge of the laboratory, is sufficiently competent to give instruction on the correct procedure. Newly trained users should be overseen for some time by a competent person.

Emergency Procedures

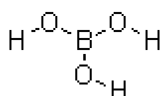
UV exposure: Act according to local procedures and provide first aid to the injured. If necessary prepare a report for working accidents.

Burns: Act according to local procedures and provide first aid to the injured. If necessary prepare a report for working accidents.

Hazard sheet BORIC ACID

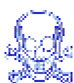
Synonyms: Boric Acid, MB Grade (1.12015); Boron; Boric acid high purity; Boric acid whitextl

Molecular Formula: $\mathbf{H_3BO_3}$
Formula Weight: 61.83
Registry number: 10043-35-3



Registry number: 10043-35-3
Density: 1.43
Melting point: 169 °C

Hazard Symbol

 Toxic

Risk Description
R60 May impair fertility.

Safety Description
S45 In case of accident of if you feel unwell, seek medical advice immediately (show the label where possible).
S53 Avoid exposure - obtain special instructions before use.

IR

Analysis	Result
Miscellaneous	545 (29.51); 641 (30.64); 813 (26.79); 1193 (21.05); 1456 (17.55); 3215 (15.75)

APPENDIX 2 (Example of PCR set up)

Project number: Date:

Primers Forward:		
	mcr6_252bp_fw	5’-AGCTATGTCAATCCCGTGAT-3’
	mcr7_551bp_fw	5’-GCCCTTCTTTTCGTTGTT-3’
	mcr8_856bp_fw	5’-TCAACAATTCTACAAAGCGTG-3’
	mcr9_1011bp_fw	5’-TTCCCTTTGTTCTGGTTG-3’
Primers Reverse:	mcr6_252bp_rev	5’-ATTGGCTAGGTTGTCAATC-3’
	mcr7_551bp_rev	5’-GGTTGGTCTCTTTCTCGT-3’
	mcr8_856bp_rev	5’-AATGCTGCGCGAATGAAG-3’
	mcr9_1011bp_rev	5’- GCAGGTAATAAGTCGGTC-3’

DNA polymerase: DreamTaq™ Green PCR Master Mix

PCR product lengths: *mcr-6* 252bp; *mcr-7* 551bp; *mcr-8* 856bp; *mcr-9* 1011bp

Remarks: Use 2µl boiling lysate as templatePrimer stock solutions at 10 µM

Positive controls:
E. coli + Top10F' pCR2.1 -mcr-6
E. coli + Top10F' pCR2.1 -mcr-7
E. coli + Top10F' pCR2.1 -mcr-8
S. Agona (27683) carrying *-mcr-9*

Volumes can be adjusted!

Number of samples	1	**10**
PCR H_2O	6,5	**65**
2×Green PCR Master Mix	12,5	**125**
Primer mix (0,5 µL of each)	4	**40**
Total volume	23	**230**

1. 3 min at 95 °C
2. 30 Cycles
   - 30 sec at 95 °C
   - 30 sec at 55 °C
   - 60 Sec at 72 °C
3. 10 min at 72 °C
4. ______ hold at 4 °C

Well	**Sample**
2	O’GeneRuler 100 bp Plus
3	*E. coli* + Top10F' pCR2.1 *-mcr-6.1*
4	*E. coli* + Top10F' pCR2.1 *-mcr-7.1*
5	*E. coli* + Top10F' pCR2.1 *-mcr-8.1*
6	*S. Agona* (27683) carrying *-mcr-9*
7	Mastermix