MCR Procedures

- Reserve the anaerobic tent.

Buffers (and chemicals) for purification:

- **For MCRred1/MCRed2**
  - 2 L KPP buffer (for 2 l: 14.739 g K_2P and 2.014 g KH_2)
  - 1 L 10 mM TrisHCl buffer pH 7.6 + 10 mM CoM
  - saturated $(NH_4)SO_4$ solution + 10 mM CoM
  - **filtrated for FPLC:**
    - 2 L 50 mM TrisHCl (pH 7.6) + 10 mM CoM (buffer A)
    - 1 L 50 mM TrisHCl (pH 7.6) + 10 mM CoM + 2 M NaCl (buffer B)
  - solid $(NH_4)SO_4$

  *HS-CoM is added inside the tent to **all** the buffers.*
    - 10 mM HS-CoM (1.64 g/l)

- **For MCRox1**
  - 2 L KPP buffer (for 2 l: 14.739 g K_2P and 2.014 g KH_2)
  - 1 L 150 mM KPP buffer pH 7.6 (for 1 l: 22.550 g K_2P and 2.794 g KH_2)
  - saturated $(NH_4)SO_4$ solution
  - **filtrated for FPLC:**
    - 2 L 50 mM TrisHCl (pH 7.6) (buffer A)
    - 1 L 50 mM TrisHCl (pH 7.6) + 2 M NaCl (buffer B)
  - solid $(NH_4)SO_4$
  - **Resuspension buffer for MCRox1 cells:**
    - 95 ml 150 mM KPP
    - 5 ml 200 mM Ti(III) citrate stock
    - 17.8 mg CH_3CoM (= 1 mM) or 178 mg (= 10 mM)
    - 2.5 mg methylviologen (0.1 mM)

- **Ti(III) citrate:** ~200 mM
  - Solve 4.4 g Na-citrate·$2H_2O$ in 45 ml water.
  - Add 1.54 g $Ti(III)Cl_3$
  - Add $KHCO_3$ (or $NaHCO_3$). This will cause gas formation. Repeat this step till the gas formation is not that large anymore. The pH should be around 7.0 (**Check this!**).

- **Assay Mix:**
  - 5ml Ti(III) citrate stock
  - 0.5 mM CoB-SS-CoB (1.965 mg)
  - 3 mM cobalamin (20.74 mg)

- **100 mM CH_3-S-CoM solution for assay (35.64 mg/2 ml buffer)**

Molecular weights:

BrCoB	440.84
CH_3-***O***-CoM (Na^+)	162.14
CH_3-***S***-CoM (Na^+)	178.19
HS-CoM ($Na^{+)}$	164.18
KH_2PO_4	136.09
K_2HPO_4	174.18
MOPS	209.27
Na-citrate·$2H_2O$	294.0
NaCl	58.44
$Ti(III)Cl_3$	154.26
Tris	121.14

Growing *M. marburgensis* cells in Culture

- For a 10 liter culture:
    - 90 g KH_2PO_4
    - 27 g NH_4Cl
    - 33 g Na_2CO_3
    - 15 ml trace element solution
    - 0.5 ml 0.2 % resazurine (not more, sticks to MCR!)

  Add the compounds to a small amount of water, ~1 liter, to prevent formation of an insoluble clump.

- For a 2 liter culture use 1/5 of the above amounts.

  - Element solution:
    - 30.0 g nitrilotriacetic acid
    - Add water to a volume of 500 ml
    - Set pH at 6.7 with 10 M NaOH
    - 40 g $MgCl_2$•6 H2O
    - 10 g $FeCl_2$•4 H2O
    - 0.2 g $CoCl_2$•6 H2O
    - 0.2 g $NaMoO_4$•2 H2O
    - 1.2 g $NiCl_2$•6 H2O
    - Add water to a total volume of 1L

Procedure:

- Assemble the glass fermenter including the orange heat blanket. Put the whole thing in the aluminum pot. (This is needed to quickly cool down the culture before harvesting the cells by filling the pot with ice water.) Wrap the white blanket around the exposed part of the fermenter to contain the heat produced by the heating blanket.
- The gas bottles (80% H_2/20% CO_2) are in the cabinet (# 308) in the hallway
- Open the gas bottles
- Open the gas valve on the outside of the hood.
- Turn the valve on the gas regulation panel to start the flow of the H_2/CO_2 gas mixture until the flow meter reads 4 (1200 ml/min), or 2 for a 2 L culture.
- Open the H_2S gas bottle and regulate the flow to one bubble per 10 sec using the bubble counter (flush the system first!).
- Set the stirring motor to 1000 rpm for a 10 L culture or 400 rpm for a 2 L culture.
- Wait 1½ h before inoculation (or until 65 ºC is reached)

- Inoculate with ±200 ml cells of a 10 L cluster or 50 ml cells for a 2 L culture. Use the earlier prepared stock. Cell solution left in the flow-through centrifuge can be used for this, however, not when the cell culture was gassed with H_2 before harvesting.
- Grow cells for ±13 h at 67°C (the OD should be 4 - check the extinction for a 1:10 diluted sample at 568 nm).

Prepare the flow-through centrifuge:

- The rotor is kept at 4ºC.
- The lid and the rotor bottom have to be aligned on the marks on their surface when put together.
- Put the parts together following the scheme on the wall in the fermenter room. **Use a new ball-bearing every time you use the rotor.**
- **Add glycerol (2 x 1ml) to the rotor!**
- Run the centrifuge at 4000 rpm.
- Use the 2 L KPP buffer to make the transfer tubes and the flow-through rotor anaerobe. Pressurize the KPP bottle with N_2 gas. (To completely remove oxygen the rotor can be gassed with N_2 gas before the KPP buffer is run through the rotor.)
- When the rotor is completely filled with liquid, the speed can be increased to 15000 rpm and the cells can be harvested.
- The centrifuge has only three functions that will be used: START, STOP, and LID.

Harvesting the cells:

For MCRred1/red2

- For the MCRred1 and MCRred2 forms, the cell culture is gassed with 100% H_2. After 5 min the MCRred1 form can be detected in cell extracts, after 15 min the MCRred2 form. Gas the cells for 20-25 min (during the purification the MCRred2 form is converted into the MCRred1 form due to loss of coenzyme B).
- Cool the cells down: Connect the fermenter to the small pump which has been placed in an ice bath and fill the bucket around the glass container with ice and water. It takes about 10 min for the cell culture to cool down.
- Pressurize the fermenter with H_2 for the cell transfer. Minimize the flow so that it takes about 40 to 45 min to empty the fermenter. This will give a more solid pellet. (Make sure that the waste water is guided into the sink in the hood since it contains a lot of hydrogen gas).
- After the fermenter has been emptied, bring the rotor into the anaerobic tent and collect the pellet. This should be around 40 to 60 g (wet weight).
- Resuspend the cells in 50 ml 10 mM TrisHCl buffer pH 7.6 + 10 mM CoM.

For MCRox1

- For the MCRox1 form, the cell culture is gassed with 80% N_2/20% CO_2 for 20 min. The gas flow is kept very low. The cells are very sensitive to oxygen. Although the special pure gas is used that should not contain any oxygen there is no need to use a high flow.
- Pressurize the fermenter with 100% N_2 gas from the hood outlet. This does contain low amount of oxygen but since the stirrer is switched off that does not get into the cell suspension.
- Resuspend the cells in 50 ml 150 mM KPP buffer pH 7.6 containing:
    - 10 mM CH_3CoM
    - 10 mM Ti(III) citrate
    - 0.1 mM methylviologen

Purification of MCR

Breaking the cells

- Sonicate the cells in the sonication beaker on ice 3 times 3.5 min (puls 0.5 sec off/0.5 sec on, flat top, 100% power. The tip of the sonicator should be about 1 cm in the solution.
- Centrifuge the cells: Sorvall Ultra Pro 80, ti45 rotor (6x50 ml), 20 min, 35000 rpm, 4°C. Make sure that all the centrifuge tubes have their rubber o-ring. Every tubes should be filled to at least 2/3 of the total volume.

Ammonium sulfate precipitation

- Take the supernatant, measure the volume and add 2.33x this amount of saturated $(NH_4)SO_4$ solution. This will create a 70% saturated solution. The $(NH_4)SO_4$ solution has to be added slowly under stirring of the protein solution on ice.
- Centrifuge the solution: Sorvall Ultra Pro 80, ti45 rotor (6x50 ml), 20 min, 35000 rpm, 4°C.
- Take the supernatant and add slowly solid $(NH_4)SO_4$ (± 25 g) to obtain a 100% saturated solution. (Make sure not to add to much $(NH_4)SO_4$ since that will end up in the pellet and disturb the purification on the Q-Sepharose column.)
- Centrifuge the solution: Sorvall Ultra Pro 80, ti45 rotor (6x50 ml), 20 min, 35000 rpm, 4°C.

For MCRred1/MCRred2:

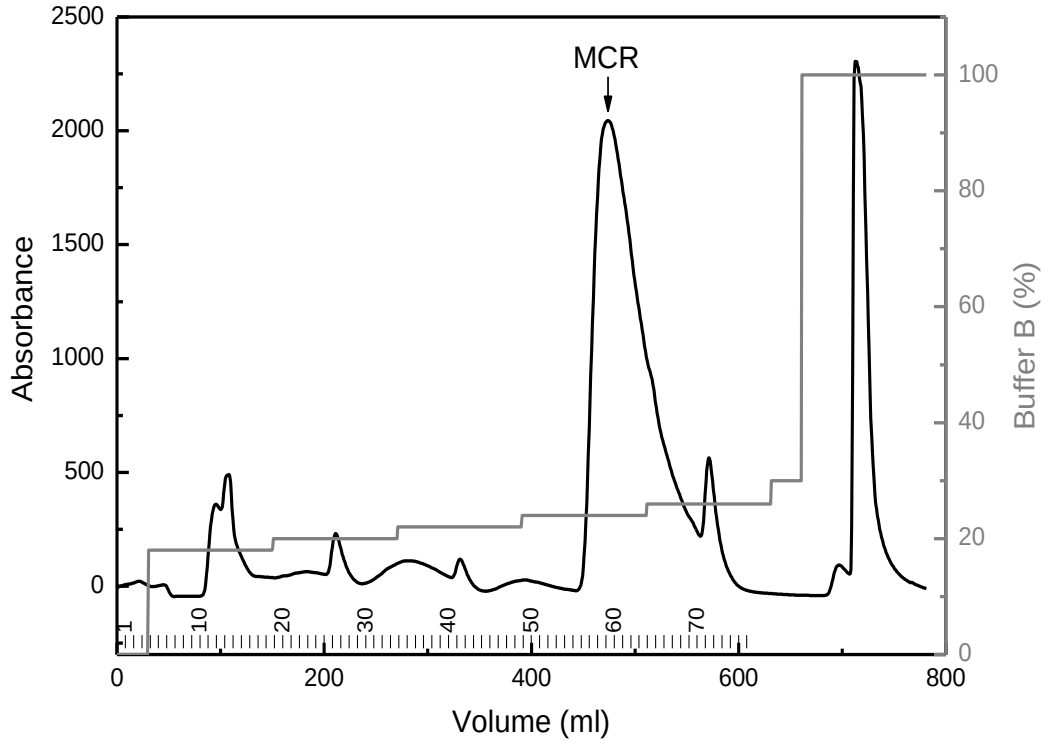
- Resuspend the pellet in 10 mM TrisHCl buffer pH 7.6 + 10 mM ***CoM***.

For MCRox1:

- Resuspend the pellet in 10 mM TrisHCL buffer pH 7.6 + 10 mM ***CH_3CoM***.

Ion-exchange: Q-Sepharose

- Wash the FPLC pumps with Buffer A and Buffer B
- Wash the column (1 column volume buffer A - 1 CV buffer B - 1 CV buffer A)
- Load protein with the P-1 pump
- Clean/replace the waste bottle!
- Run the purification program
  (Program starts with 0% buffer B and increases the concentration to 18, 20, 22, 24, and 26 %, followed with wash steps of 30% and 100% buffer B)
- Collect the tubes that contain MCR and concentrate the protein in the Amicon Concentration Units to about 2-3 ml.
- The protein can be stored in the tent.



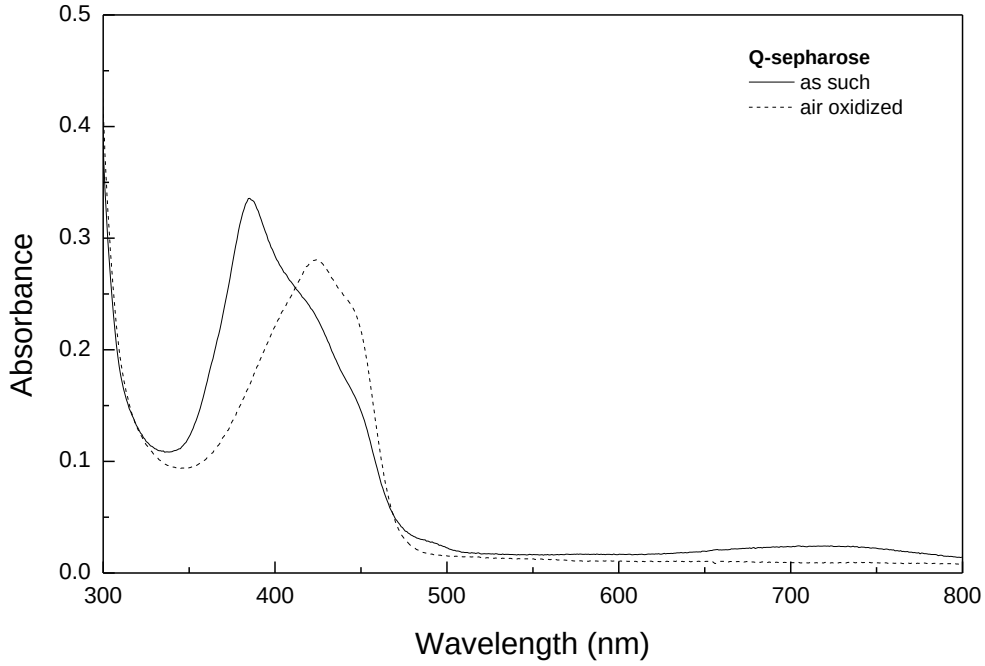
Typical purification profile for the Q-sepharose column. The black line is the absorbance at 280 nm. The gray line represents the % concentration of buffer B used. Note that the % Buffer is what is programmed and is added to the top of the column, while the absorption is measured at the column exit. The 'delay' is about 30 ml. The MCR peak is indicted in the figure.

Purification profile

(35 g of wet cells were used – OD_{568} of 3.0)

	activity in units ($\mu mol \cdot min^{-1}$)	activity (%)	total mg protein	specific activity ($units \cdot mg^{-1}$)
after sonication	6125	100	3750	1.6
cell extract	5460	89	3255	1.7
70% before centrifugation	7990	130	3400	2.1
70% after centrifugation	5550	91	570	9.7
100% before centrifugation	5280	86	594	8.8
100% after centrifugation	3100	51	554	6.0
Q-sepharose	737	12	147	5.0

UV-visible spectrum



EPR spectra