# Development of a MicH-specific immunoassay for MIC detection and diagnosis

Sven Lahme and Jorge Mantilla Aguas

ExxonMobil Technology and Engineering Company, Spring, Texas, USA

**Abstract**

Microbially-influenced corrosion (MIC) is a costly problem across several industries. The steadily rising use of advanced molecular biological methods to investigate MIC allowed ever deeper insights in the underlying microbial community structure and function. However, currently available technologies do not allow accurate detection and diagnosis of MIC in the field. Our team has recently developed a qPCR assay for a first MIC biomarker (*micH*) specific to corrosive methanogenic archaea. The *micH* gene encodes the large subunit of a special [NiFe] hydrogenase involved in MIC. Here we describe the development of recombinant antibody that enable the specific and sensitive detection of the MicH protein in western blot immunoassays. Using a recombinant MicH protein we determined the lower limit of detection per assay to be around ~0.3 fg MicH (~$3 \cdot 10^3$ protein molecules). The novel immunoassay was able to detect a strong signal for the MicH protein in a *micH* positive cultures of *Methanobacterium* sp. strain IM1 that was cultivated on iron granules, but the signal was 500 times lower in a *micH* negative *Methanococcus maripaludis* S2 culture. To further evaluate the ability to differentiate corrosive from non-corrosive microbial communities, we tested 10 oil field enrichment cultures that showed a wide range of corrosion rates (0.02 – 0.48 mm/yr). We detected the MicH protein in planktonic ($2.2 \cdot 10^6$ – $9.3 \cdot 10^7$ MicH/mL) and carbon steel biofilm samples ($1.7 \cdot 10^7$ – $6.4 \cdot 10^8$ MicH/cm$^2$) from corrosive methanogenic enrichments (0.17 – 0.48 mm/yr) but did not detect MicH in any of the non-corrosive tests (<0.08 mm/yr) despite methanogenic activity in some. The results indicate that corrosion was likely caused by methanogenic archaea expressing a corrosive [NiFe] hydrogenase and that the newly developed MicH immunoassay can be used to detect and monitor their activity. Here we showed that an antibody can be applied to detect a

MIC biomarker on the protein level and differentiate between corrosive and non-corrosive methanogenic communities. The development of a specific and sensitive immunoassays to detect MIC biomarkers added another technology to the toolbox of corrosion scientists to improve monitoring and detection of corrosive microorganisms and paves the way for development of rapid in-field testing.

## Introduction

Microbially influenced corrosion (MIC) negatively affects steel infrastructures, causing significant costs across multiple industries ever year. Localized corrosion rates due to the activity of certain microorganisms have been reported in some instances to range several millimeters per year (mm/yr) (Enning et al. 2012, Suarez et al. 2019, Lahme et al. 2021).

In anaerobic systems, such as pipelines used for the production and transportation of mixtures of oil, water and gas, MIC is often linked to the presences and activity of sulfate-reducing bacteria (SRB) and methanogenic archaea (Enning and Garrelfs 2014, Okoro et al. 2014, Mand et al. 2016, Lahme et al. 2021). Recent research identified specific gene islands in genomes of corrosive methanogenic archaea that are absent in non-corrosive close relative strains. The gene islands encode among other genes a unique and phylogenetically highly conserved bidirectional [NiFe] hydrogenase (termed 'MIC hydrogenase'), a Tat system for protein translocation as well as enzymes potentially involved in protein glycosylation (Tsurumaru et al 2018, Lahme et al. 2021, Holten et al. 2021, Kawaichi et al. 2024). The 'MIC hydrogenase' was only detected in corrosive pure cultures and corrosive oil field enrichment cultures but not the ones showing low corrosion and therefore explains the corrosive character of some methanogenic archaea.

We recently proposed to use the large subunit of this 'MIC hydrogenase' as biomarker (termed *micH*) to detect and monitor MIC in the field (Lahme et al. 2021). Utilizing specific qPCR assays, we observed good correlation with MIC in both laboratory tests and field applications (Lahme et al. 2021, Lahme et al. 2022). Here, we discuss the development of a MicH-specific recombinant antibody to detect and monitor the MicH protein in laboratory tests aiming to study corrosive methanogenic archaea. In addition, the antibody could

be used to screen field samples for methanogenic archaea actively expressing the 'MIC hydrogenase'. In addition, protein detection may reduce potential false positive that could be associated with qPCR-based detection. A recombinant antibody able to detect MIC protein biomarker would also allow the development of a field-friendly rapid test assays (e.g. lateral flow tests) to support and accelerate MIC monitoring and detection in the field.

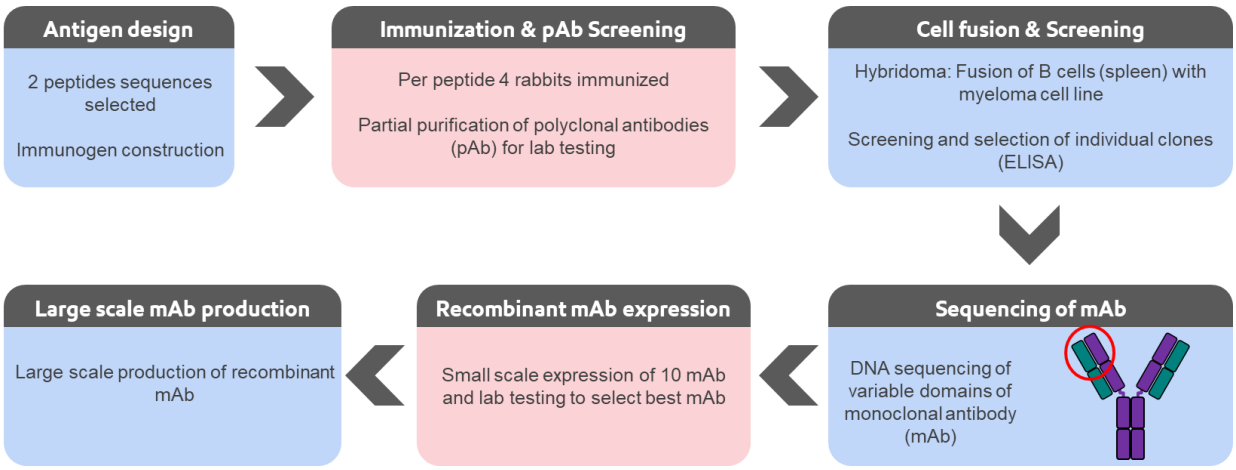



*Figure 1 Workflow of recombinant antibody development.*

## Materials and Methods

*Growth and corrosion bottle tests*

Pure cultures and enrichments used in this study were routinely grown in the presence of carbon steel granules in anaerobic synthetic brine that was modelled after an oil field produced water as described previously (Lahme et al. 2021). Corrosion tests were performed in one liter glass bottles that were filled with 600 ml anoxic synthetic brine to which three API 5L X52 steel coupons in a polyether ether ketone coupon holder as described earlier (Lahme et al. 2021). The bottles were sealed with butyl rubber stoppers using sterile Hungate techniques and a $N_2/CO_2$ (79:21) headspace. Test were incubated on a rotary shaker at 32°C for 8 weeks. Planktonic and biofilm samples were collected for protein and DNA analysis using sterile syringes, 0.2 µm filters or sterile swabs and immediately frozen at −80°C until further analysis (see

66 Lahme et al 2021). Weigh loss corrosion analysis of X52 coupons was performed at the end of the test as
67 described earlier (Lahme et al. 2021).

68 *Recombinant antibody development*

69 The recombinant antibody development workflow was conducted by Genscript Biotech (Piscataway, NJ)
70 and briefly outline below. For the initially development of polyclonal antibodies, two peptide sequences
71 were chosen from the protein sequence of the target gene *micH* of the corrosive methanogen *Methanococcus*
72 *maripaludis* strain OS7 (see supplementary material) and conjugated to the metalloprotein keyhole limpet
73 hemocyanin (KLH). Per peptide four rabbits were immunized with three additional consecutive boosters.
74 After four months, blood was harvested from the rabbits and polyclonal antibodies partially purified for
75 evaluation. Tests were performed by indirect enzyme-linked immunosorbent assay (ELISA) using either of
76 the two peptides as target and via Western blotting using protein extracts from *Methanobacterium* sp. strain
77 IM1 as MicH-positive control (Dinh et al. 2004) and *Methanococcus maripaludis* strain S2 was used as
78 MicH-negative control (Tsurumaru et al 2018). Animals with best performing polyclonal antibodies were
79 selected for cell fusion of spleen B cells with myeloma cell line. Screening and selection of clones were
80 performed via immunoassays as described above. The best performing monoclonal antibody 'A11-1' was
81 chosen for DNA sequencing (see supplementary material) and recombinant antibody production.

82 *Protein extraction and detection via Western blot*

83 Proteins were extracted from filters or swabs by placing them in sterile 2 ml screw cup microcentrifuge
84 tubes. 900 µl lysis buffer (10 mM HEPES, 1% Triton X-100, 5 mM EDTA, 100 mM DTT and 100mM
85 $Na_2CO_3$, 1% SDS, 12% sucrose) were added to the filters or swabs and placed in a heating block (105°C)
86 for exactly 2 min. The tubes were removed and cooled to room temperature. The tubes were then vigorously
87 vortexed and subsequently centrifuged for 5 min at 21,100 g. 200 µl of the supernatant was further
88 processed to remove detergents using Pierce™ HiPPR Detergent Removal Spin Column Kit (Thermo Fisher
89 Scientific, Waltham, MA) according to manufacturer's protocol. The detergent-free protein samples were
90 further processed using Pierce™ Protein Concentrators with a 10 kDa molecular weight cut-off polyether

91 sulfone membrane (Thermo Fisher Scientific, Waltham, MA). Protein samples were concentrated to an
92 approximate volume of 25 µl and either directly used for Wester blotting or stored at −80°C.

93 Western blotting was performed on a Protein Simple JESS Western blot platform (Protein Simple, San Jose,
94 CA) using the 12-230 kDa Separation Module and the Jess Anti-Rabbit Detection Module. Protein analysis
95 was performed according to the manufacturer protocol using a 1:20 antibody dilution (approx. 25 µg/ml)
96 of A11-1. Protein Simple's Total Protein module was used for normalization. As positive control and for
97 quantification purposes a recombinant MicH protein was purchased from Genscript Biotech (Piscataway,
98 NJ) developed following a large-scale *E. coli* expression protocol and His-tag purification.

99 *DNA extraction and qPCR analysis*

100 For DNA extraction cells were collected via filtration and filters transferred to 5 ml cryo vials containing 1
101 ml DNA/RNA shield (Zymo Research, Irvine, CA) as preservative. Filters were stored at −80°C until
102 further analysis. DNA extraction and *micH* qPCR analysis were performed by Microbial Insights Inc.
103 (Knoxville, TN) as described previously (Lahme et al. 2021).

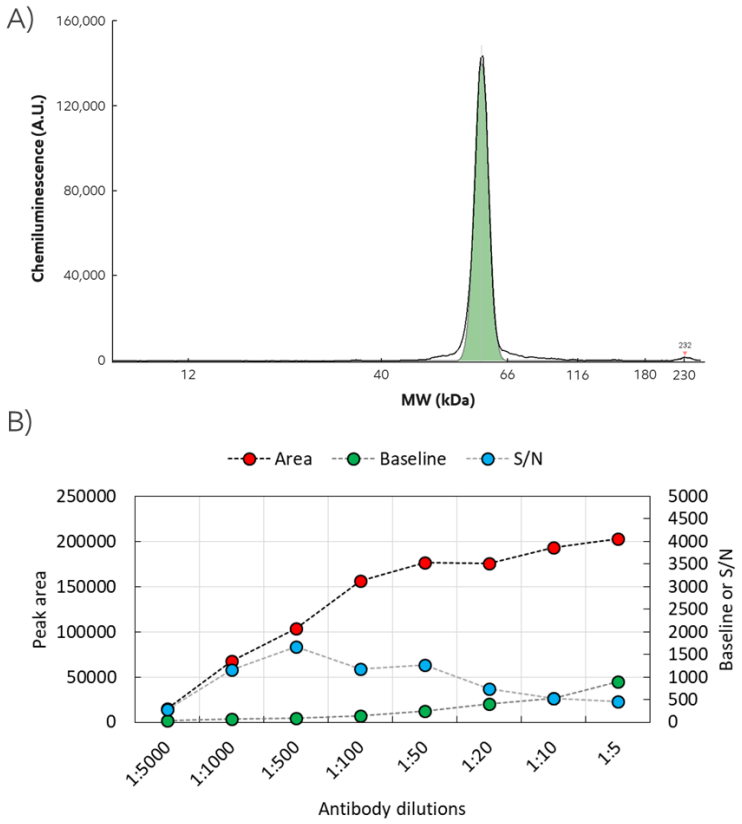



*Figure 2 Performance of recombinant antibody Anti-MicH using a recombinant MicH protein as target. A) Chemiluminescence measured as arbitrary units (A.U.) showing signal at the expected molecular weight of $MicH_{rec}$. Test was performed with a 1:20 dilution of the antibody. B) Results of tests performed with varying dilutions of Anti-MicH and 1 ng/ml $MicH_{rec}$.*

*Gas chromatography*

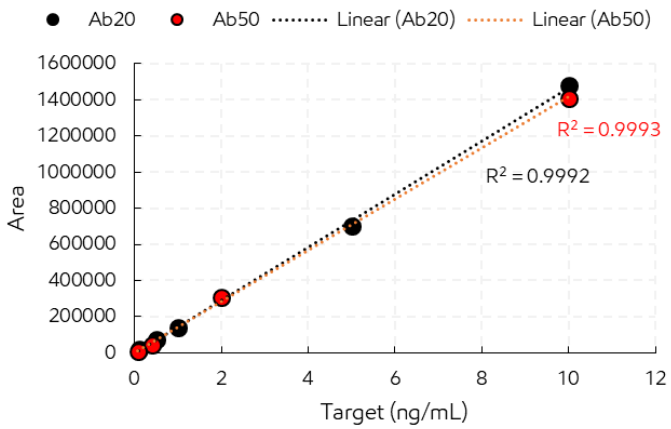
Gas chromatographic analysis of methane was performed on an Agilent 490 Micro gas chromatograph using a thermal conductivity detector. Separation was achieved on a heated Agilent PoraPlot U column (50°C, 22.1 lb/in2, and 10 m) in argon gas.

## Results and Discussion

*Recombinant Anti-MicH antibody showed excellent affinity for recombinant MicH protein.*

In order to optimize the signal to noise ratio (S/N) we performed first a dilution series with the Anti-MicH antibody and a fixed concentration of 1 ng/ml of $MicH_{rec}$. For $MicH_{rec}$ a signal was detected between approximately 57 - 61 kDa molecular weight that is in good agreement with the expected molecular weight

117 of the MicH protein containing the His-tag used for purification (54.5 kDa; Fig. 2A). The shift in molecular
118 weight may have been the result of residual detergent in the protein extracts that is known to interfere with
119 Western Blot analysis. Test performed with different antibody dilutions of Anti-MicH showed best results
120 for S/N ratio and low baseline with dilutions 1:50, 1:20 and 1:10 (Fig. 2B). For subsequent tests an antibody
121 dilution of 1:20 was chosen to minimize potential background noise while achieving an overall strong
122 signal.

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124 *Figure 3 Western Blots performed with dilution series of recombinant MicH protein and Anti-MicH antibody dilutions of 1:20*
125 *and 1:50 showed excellent linear response.*

126 In addition, a calibration curve of recombinant MicH protein showed excellent linear response with
127 concentrations between 10 ng/ml and 20 pg/ml (Fig. 3 and Fig. S2). A clearly detectable signal with
128 acceptable S/N ration (>30) was still detected at 20 pg/ml $MicH_{rec}$ therefore the Western blot assay is able
129 to detect as little as 0.3 fg of recombinant MicH that correspond to (~$3 \cdot 10^3$ protein molecules).

130 *MicH-specific immunoassay showed excellent selectivity for corrosive methanogenic cultures*

131 Unlike qPCR assays, immunoassays are less selective and may show potential background signal due to
132 cross-reactivity with non-target proteins or peptides in absence or low concentration of their specific target.
133 To test for assay specificity and the ability to distinguish between corrosive and non-corrosive methanogens

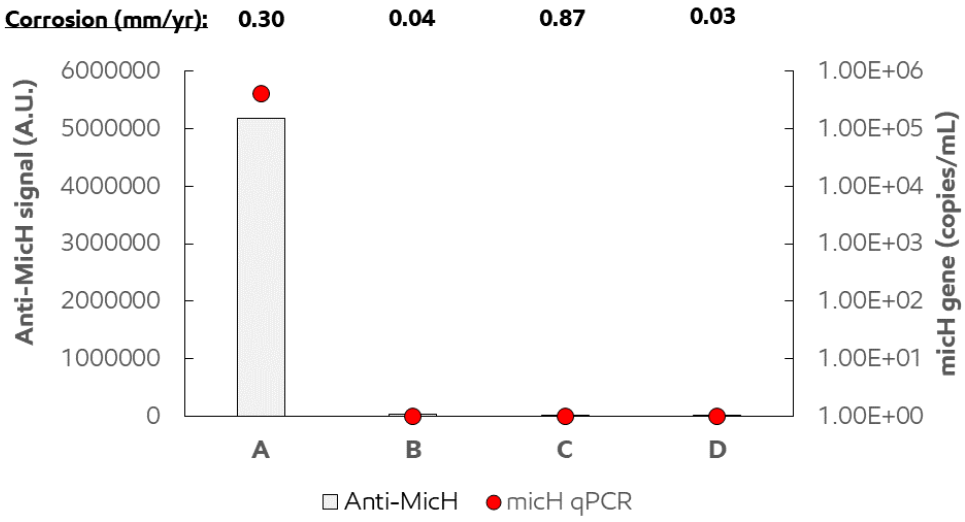
134 and non-methanogenic cultures, we performed Western blot analysis using the MicH-specific immunoassay
135 on protein extracts from non-corrosive methanogenic and various sulfate-reducing cultures (Fig. 4).

136 Protein extract of *Methanobacterium* sp. strain IM1 obtained from cultures that inflicted significant
137 corrosion of 0.3 mm/yr showed a strong signal in the Western blot (Fig. 4). Presence of the *micH* gene in
138 strain IM1 was further confirmed by qPCR confirming presence and expression of the MIC hydrogenase
139 in strain IM1. In contrast, the MicH-specific assay showed more than 500x lower signals for protein extracts
140 from *Methanococcus maripaludis* strain S2, know to lack the 'MIC hydrogenase' and the *micH* gene
141 (Tsurumaru et al. 2018). Similar low signals were observed in protein extracts from corrosive and non-
142 corrosive sulfate-reducing cultures. DNA analysis via qPCR confirmed absence of the *micH* gene in those
143 cultures (Fig. 4).

144 Next, we wanted to better understand the performance of the MicH-specific immunoassay and selectivity
145 of the Anti-MicH antibody in more complex context of oil field produced water enrichment cultures.
146 Therefore, protein extract from ten field enrichment cultures that showed varying degree of MIC activity
147 (Fig. 5) were compared in terms of MIC corrosion and expression of the MicH protein. Five of the ten
148 cultures were classified as "MIC(+)" showing averaged corrosion rates ranging from 0.48 to 0.17 mm/yr,
149 which according to the Association for Materials Protection and Performance (AMPP) is considered high
150 to severe (AMPP-SP0775-2013). The other five cultures were classified as MIC(-) showing rates between
151 0.02 to 0.08 mm/yr, considered as low to moderate corrosion according to AMPP. Significant quantities of
152 the MicH protein were detected in planktonic ($2.2 \cdot 10^6$ – $9.3 \cdot 10^7$ MicH/mL) and carbon steel biofilm
153 samples ($1.7 \cdot 10^7$ – $6.4 \cdot 10^8$ MicH/cm$^2$) from MIC positive tests, whereas the MicH protein was not detected
154 in the MIC(-) enrichments.

155 The results with pure and complex field cultures suggest that the Anti-MicH antibody is highly selective
156 for the MicH protein found in certain corrosive methanogenic archaea and showed little to no cross-
157 reactivity with proteins from other corrosive and non-corrosive microorganisms, including enrichment
158 cultures showing methanogenic activity (Fig. 5). In addition, the assay confirmed that corrosive

159 methanogenic cultures indeed expressed the MIC hydrogenase and thereby accelerating iron oxidation of
160 carbon steel as previously shown in pure cultures and genetically modified *Methanococcus maripaludis*
161 strains (Tsurumaru et al. 2018, Holten et al. 2021).

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163 *Figure 4 Analysis of protein extracts with MicH-specific Western blot immunoassay and DNA extracts with micH qPCR assay. A)*
164 *Methanobacterium sp. strain IM1, B) Methanococcus maripaludis strain S2, C) Desulfovibrio ferrophilus IS5, C) Desulfovibrio*
165 *alaskensis G20.*

166 **Conclusion**

167 Monitoring of MIC in the field is challenging and requires obtaining multiple lines of evidence to accurately
168 diagnose MIC (Knisz et al. 2023). Here we presented the development of a recombinant antibody that
169 showed strong affinity for the large subunit of a specific [NiFe] hydrogenase ('MIC hydrogenase') found
170 exclusively in corrosive methanogenic archaea (Tsurumaru et al 2018, Lahme et al. 2021). The antibody
171 was able to detect the MicH protein in a corrosive methanogenic pure culture of strain IM1 as well as five
172 methanogenic field enrichment cultures, while showing nearly no cross-reactivity with non-corrosive
173 methanogenic or sulfate-reducing cultures. In addition, the detection of the MicH protein not only in
174 biofilms but also planktonic cells suggest that the assay may be applicable to both MIC monitoring in
175 biofilms as well as water samples. The latter is often readily available in the field, while it is often

challenging to obtain relevant biofilm samples in the field to monitor pipeline corrosion. The MicH-specific immunoassay and the development of a recombinant Anti-MicH antibody provides field practitioners with additional ways to monitor corrosive microorganism in the field and may be applicable beyond oil and gas operations.

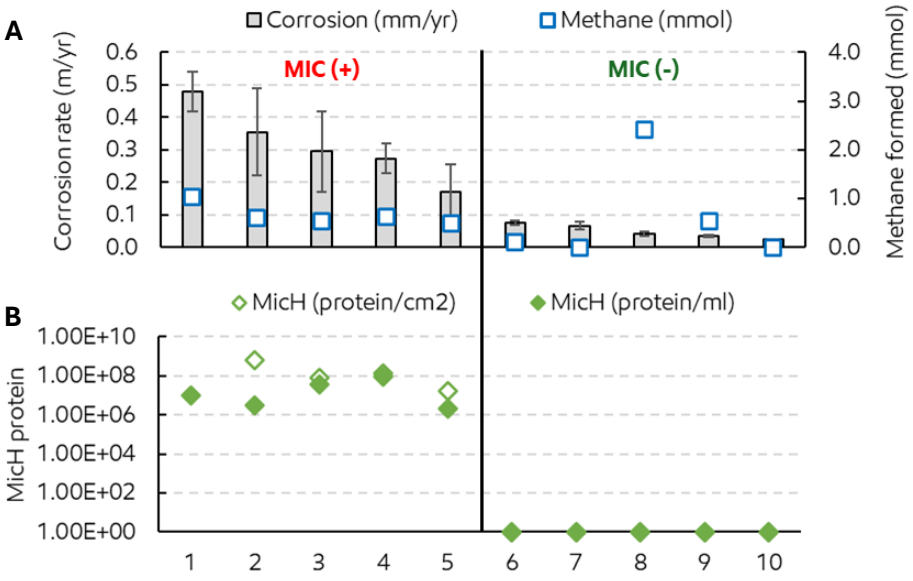


*Figure 5 A) Weight loss corrosion rates and methane formation in oil field produced water enrichment cultures classified as corrosive ['MIC(+)'] and non-corrosive ['MIC(-)']. B) MicH protein numbers detected in corrosion coupon biofilms (open diamonds) and planktonic cells (closed diamonds).*

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