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An Electrochemiluminescence-Based Assay for MeCP2 Protein Variants

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Vienna, 20.01.2020

Dear Dr. Xiaoyan Cao,
please find enclosed a revised version of the manuscript now entitled:

"Employing an electrochemiluminescence based assay for MeCP2 protein variants."

In this manuscript we present the development and validation of an electrochemiluminescence-based immunoassay (ECLIA) to quantify both endogenous as well as recombinant MeCP2 protein levels in mice brain tissues, as well as in cell culture. This method is a very useful tool in developing novel strategies for the treatment of Rett Syndrome, a neurodevelopmental disease, brought about by mutations in the coding region of the MeCP2 protein.
We have addressed the reviewers' comments to the best of our ability and incorporated the appropriate modifications into our manuscript. We have also obtained an explicit permission from Meso Scale Discovery to include the adapted figure 1 in our paper. The corresponding email has been attached.

We would very much appreciate your consideration for a publication in JoVE.

With kind regards,
Hannes Steinkellner

TITLE:

An Electrochemiluminescence-Based Assay for MeCP2 Protein Variants

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KEYWORDS:

ECLIA, Sulfo-TAG, MeCP2, TAT fusion protein, TAT-MeCP2, Rett syndrome, protein replacement therapy

SUMMARY:

The electrochemiluminescence immunoassay (ECLIA) is a novel approach for quantitative detection of endogenous and exogenously applied MeCP2 protein variants, which produces highly quantitative, accurate and reproducible measurements with low intra- and inter-assay error over a wide working range. Here, the protocol for the MeCP2-ECLIA in a 96-well format is described.

ABSTRACT:

The ECLIA is a versatile method which is able to quantify endogenous and recombinant protein amounts in a 96-well format. To demonstrate ECLIA efficiency, this assay was used to analyze intrinsic levels of MeCP2 in mouse brain tissue and the uptake of TAT-MeCP2 in human dermal fibroblasts. The MeCP2-ECLIA produces highly accurate and reproducible measurements with low intra- and inter-assay error. In summary, we developed a quantitative method for the

evaluation of MeCP2 protein variants that can be utilized in high-throughput screens.

INTRODUCTION:

The electrochemiluminescence immunoassay (ECLIA) is based on a process that utilizes labels designed to emit luminescence when electrochemically stimulated. It is a broadly applicable technique for the quantitative detection of biological analytes in basic industry and academic research, food industry as well as in clinical diagnostics[1]. Commonly, a disposable 96-well plate with carbon ink electrodes is used. These electrodes act as a solid-phase carrier for the immunoassay. A secondary antibody is conjugated to an electrochemiluminescent label and when electricity is applied to the system, light emission of the chemical label is triggered. An ultra-low noise charge-coupled device (CCD) records the light intensity which is directly proportional to the antigen bound to the capture antibody resulting in the quantification of the targeted analyte of the sample[2]. Compared to the enzyme-linked immunosorbent assay (ELISA), ECLIA is considered to be advantageous as it offers higher sensitivity and reproducibility as well as better automation and consistency[3].

Here we analyzed the methyl-CpG binding protein 2 (MeCP2) levels in samples of human and murine origin as well as different variants of the recombinant protein using the newly developed ECLIA system. MecP2 is an X-linked nucleic acid-binding protein known to interact with methylated DNA sequences. This protein has been implicated in the regulation of gene expression[4,5]. Loss-of-function mutations in the gene which encodes this protein are the main culprits causing Rett syndrome (RTT), a severe neurodevelopmental disorder[6]. Another MeCP2-related disorder, *MECP2* duplication syndrome, also leads to neurological symptoms that can overlap with those of RTT[7]. Notably, females are mostly affected by RTT while males are mostly afflicted by *MECP2* duplication syndrome[6,7].

These disorders are associated with insufficient or excess MeCP2 levels respectively in the central nervous system (CNS). Hence, treatment options for RTT that involve increasing MeCP2 levels in the CNS would need to avoid the detrimental effects associated with excess of MeCP2[7]. Due to this fact, a highly sensitive and accurate quantification of MeCP2 protein levels, as provided by the ECLIA system, is crucial for the advancement of RTT as well as *MECP2* duplication syndrome research. The precise measurements of endogenous and exogenous MeCP2 levels from human cell lines and mouse tissue samples as well as a recombinant protein consisting of human MeCP2 isoform B (also known as isoform e1), and a minimal N-terminal HIV-TAT transduction domain (TAT-MeCP2) that has the potential to cross the blood-brain-barrier[8,9] are presented in this work.

PROTOCOL:

Approval for skin biopsy procurement for research purposes was obtained from the Human Research Ethics Committee of the Children's Hospital at Westmead, Australia. Consent for animal experiments was obtained from the Austrian Federal Ministry of Science, Research and Economy, which were performed in accordance with local animal welfare regulations (GZ: 66.009/0218-II/3b/2015).

NOTE: The principle of the ECLIA system is depicted in **Figure 1**.

1. Antibody selection

1.1. Evaluate the signal-to-noise ratio for a range of various MeCP2 antibodies and identify the best signal strength and highest specificity within the combination of a primary mouse monoclonal MeCP2 antibody (clone Mec-168) and rabbit polyclonal MeCP2 detection antibody (custom made). For the secondary antibody, use a system-specific antibody (**Table of Materials**), which was also experimentally verified.

NOTE: **Table 1** gives an overview of all verified antibodies during MeCP2-ECLIA development and their tested dilution ranges.

[Place **Table 1** here]

1.2. Alternatively, use each pair of MeCP2-antibodies that works well with conventional ELISA.

2. Treatment of HDFs with TAT-MeCP2 fusion protein

NOTE: TAT-MeCP2 was recombinantly expressed in *Escherichia coli* and purified using standard chromatographic techniques as previously described[8] and stored at -80 °C.

2.1. Plate 1 x 10^6 human dermal fibroblast (HDF) cells in 100 mm dishes and grow the cells overnight at 37 °C with 5% CO_2 until they reach about 90% confluency.

2.2. Take out one vial of TAT-MeCP2 fusion protein. Thaw on ice and mix gently.

2.3. Dilute TAT-MeCP2 to a final concentration of 500 nM in 50 mL of HDF culture media.

2.4. Remove the media from 100 mm dishes and incubate the cells with 500 nM TAT-MeCP2 at 37 °C for various incubation periods up to 24 h.

2.5. Remove the TAT-MeCP2 solution from the cells. Wash the cells 2x with pre-warmed Dulbecco's phosphate-buffered saline (DPBS).

2.6. Treat the cells with 2 mL of 0.05% trypsin-EDTA solution for 5 min at 37 °C[10].

2.7. Add 6 mL of HDF culture media to inactivate the trypsin and collect the cells in 15 mL tubes.

2.8. Pellet the cells by centrifugation at 500 x *g* for 5 min at room temperature.

2.9. Remove the supernatant and wash the cells 2x with ice-cold DPBS. Store the pellet on ice

and proceed with sample preparation.

3. Sample preparation

3.1. HDF lysates

3.1.1. Determine the highest protein levels of MeCP2 using the REAP method for subcellular fractionation into the cytoplasmic and nuclear subcellular compartments according to Suzuki et al.[11]. Limit fractionation to no more than six samples per experiment.

3.2. Mouse brain lysates

3.2.1. Prepare 100 mL of each hypotonic lysis reagent and extraction buffer.

3.2.1.1. For the hypotonic lysis reagent, mix well 10 mM HEPES, 1.5 mM magnesium chloride and 10 mM potassium chloride.

3.2.1.2. For the extraction buffer, mix well 20 mM HEPES, 1.5 mM magnesium chloride, 0.42 M sodium chloride, 0.2 mM EDTA, and 25% (v/v) glycerol.

3.2.1.3. Adjust the pH of both buffers to 7.9.

3.2.1.4. Add 1 mM dithiothreitol (DTT) and 1x protease inhibitor cocktail to both buffers freshly. Chill on ice before use.

3.2.2. Suspend 100 mg of total mouse brain (strain: C57BL/6J, wildtype male and female and B6.129P2(C)-Mecp2$^{tm1.1Bird}$/J, hemizygous male and heterozygous female; age: 4–8 weeks old) in 1 mL of ice-cold hypotonic lysis reagent.

3.2.3. Homogenize the brain with a pre-chilled Dounce all-glass tissue homogenizer by 15 strokes of homogenizer A (loose, for large clearance) and B (tight, for small clearance), respectively.

3.2.4. Transfer the homogenized cells to a clean tube and centrifuge at 10,000 x *g* for 20 min at 4 °C. Discard the supernatant (or keep it as cytoplasmatic fraction for further use).

3.2.5. Resuspend the pellet in 140 µL of extraction buffer per 100 mg of starting material. Place the tube in a pre-cooled thermoblock and mix gently for 30 min at 4 °C.

3.2.6. Centrifuge the tube at 16,000 g for 10 min at 4 °C. Transfer the supernatant (i.e., the nuclear fraction) to a new pre-chilled tube and store at -80 °C until use.

NOTE. The protein concentration of lysates derived from mouse brain and HDFs can be assessed by the BCA protein assay.

4. MeCP2-ECLIA protocol

4.1. Preparation of washing solution, blocking buffer and assay diluent solution (day 1)

4.1.1. Add 500 µL of Tween 20 to 1 L of PBS to prepare a 0.05% Tween 20 in PBS solution, mix vigorously and label as “washing solution”.

NOTE: Ensure that only freshly prepared washing buffer is used.

4.1.2. Prepare a blocking solution of 3% blocker A (**Table of Materials**) in phosphate-buffered saline (PBS). Mix by gentle stirring, filter sterilize and keep them in the fridge until use for a maximum of two weeks.

4.1.3. Add 5 mL of blocking solution to 10 mL of PBS to prepare assay diluent solution (1% blocker A in PBS).

4.2. Coating of high bind plates

4.2.1. Take out a 96-well multi-array single spot high bind plate.

NOTE: High bind plates have a greater binding capacity and therefore a larger dynamic range than standard plates with hydrophobic surfaces.

4.2.2. Thaw the monoclonal mouse anti-MeCP2 antibody on ice and mix 0.67 µL of the antibody with 4 mL of PBS (1:6,000 antibody dilution in PBS). Vortex the antibody solution to mix well and label the tube as “coating solution”.

4.2.3. Carefully dispense 25 µL of coating solution in the bottom corner of each well using a multichannel pipettor; this is called the solution coating method. Tap the 96-well plate gently on each side to ensure that the coating solution covers the bottom of each well.

4.2.4. Seal the plate with an adhesive foil and incubate the plate in the fridge at 4 °C overnight (12−16 h).

4.3. Blocking (day 2)

4.3.1. Take out the plate from the fridge and remove the foil.

4.3.2. Remove the antibody coating solution by flicking it into the waste basket and tap the plate on a paper towel to remove all the coating solution from the wells.

4.3.3. Add 125 µL of blocking solution per well. Seal the plate again and place it on an orbital microplate shaker.

4.3.4. Incubate the plate for 90 min at room temperature with constant shaking at 800 rpm.

4.4. Preparations of standards and samples

4.4.1. During the incubation time, prepare the MeCP2 and/or TAT-MeCP2 protein standards and various samples.

NOTE: Lysis buffer used for standard dilution must be the same as that used in the analyzed samples.

4.4.2. Take out one vial of MePC2 and/or TAT-MeCP2 protein stock solution (250 µg/mL), mouse brain lysates and HDF lysates from -80 °C. Thaw them on ice.

4.4.3. Dilute the standard stock solution (MeCP2 and/or TAT-MeCP2) in clean tubes according to **Table 2**.

[Place **Table 2** here].

4.4.4. Dilute the samples in lysis buffer as follows: 1−20 µg of mouse brain lysate per 25 µL of lysis buffer, and 0.25−1 µg of HDF lysate per 25 µL of lysis buffer. Prepare enough volume of each sample to carry out analysis in triplicate.

4.5. Adding the samples and standard solutions

4.5.1. Remove the blocking solution by flicking it into the waste basket and tap the plate on a paper towel to remove all the blocking solution from the wells.

4.5.2. Wash the plate 3x with 150 µL of washing solution by adding the washing solution and immediately removing it.

4.5.3. Add 25 µL of standards and samples directly to the center of the working electrode surface. Pipetting in other areas may breach the dielectric.

4.5.4. Seal the plate and incubate the plate for 4 h at room temperature with constant shaking at 800 rpm.

4.6. Unlabeled detection antibody

4.6.1. Thaw the polyclonal rabbit anti-MeCP2 antibody on ice. Dilute the antibody 1:6,000 in assay diluent solution.

4.6.2. Remove the standards and samples by flicking it into the waste basket and tap the plate on a paper towel.

4.6.3. Wash the plate 3x with 150 µL of washing solution by adding the washing solution and immediately removing it.

4.6.4. Add 25 µL of unlabeled detection antibody to each well with the multichannel pipettor. Seal the plate and incubate it for 1 h with constant shaking at 800 rpm at room temperature.

4.7. Specific conjugated antibody

4.7.1. Take out the specific secondary antibody (**Table of Materials**) from the fridge and place it on ice. Dilute the antibody 1:666.67 in assay diluent solution and mix gently.

4.7.2. Remove the free unlabeled secondary antibody by flicking it into the waste basket and tap the plate on a paper towel.

4.7.3. Wash the plate 3x with 150 µL of washing solution by adding the washing solution and immediately removing it.

4.7.4. Add 25 µL of specific conjugated antibody (**Table of Materials**) to each well with the multichannel pipettor. Seal the plate and incubate for 1 h with constant shaking at 800 rpm at room temperature.

4.8. Reading the plate

4.8.1. Remove the free conjugated antibody (**Table of Materials**) by flicking it into the waste basket and tap the plate on a paper towel.

4.8.2. Wash the plate 3x with 150 µL of washing solution.

4.8.3. Add 150 µL of 1x Tris-based read buffer T (**Table of Materials**) with surfactant containing tripropylamine as a co-reactant for light generation to the plate. Avoid any air bubbles by using reverse pipetting techniques.

4.8.4. Place the plate on the microplate detection platform (**Table of Materials**) and start the measurement immediately. Use the settings for 96-well plate acquisition.

4.8.5. Capture the electrochemiluminescence signals by a built-in CCD camera in an electrochemiluminescence detection system (**Table of Materials**) and record the signal counts, which correspond to relative light units (RLU) and are directly proportional to the intensity of light.

NOTE. Upon electrochemical stimulation, the ruthenium label bound to the carbon electrode emits luminescence light at 620 nm. Analyze data with the instrument-accompanied software (**Table of Materials**).

REPRESENTATIVE RESULTS:

The principle of the ECLIA system is described in **Figure 1**. Standard curves for two MeCP2 variants are shown in **Figure 2**. Accurate quantification was possible over a wide range of concentrations (1−1,800 ng/mL). In **Figure 3**, MeCP2 levels of lysates derived from mouse brain and HDFs were analyzed. MeCP2 expression in brain nuclear lysates from heterozygous, wildtype and knockout mice were compared in **Figure 3A**, while in **Figure 3B** no MeCP2 protein was detected in the MECP2-deficient human fibroblasts (c.806delG) using the ECLIA. The uptake of TAT-MeCP2 by the MECP2-deficient cell line (c.806delG) was also investigated over time (**Figure 4**). Finally, inter- and intra-assay precision was demonstrated as shown in **Table 3**.

FIGURE AND TABLE LEGENDS:

Figure 1: Diagram of MeCP2 electrochemiluminescence assay. This figure was adapted from www.meso-scale.com.

Figure 2: MeCP2 standard curve generated from human MeCP2 in multiple measurements. The lower limit of detection (LLOD), defined as 2.5 standard deviations (SDs) above the blank, is 1.00 ng/mL. Recombinant human MeCP2 (Abnova) could be accurately quantified over a range from 1.00 ng/mL (LLOD) to 1,800 ng/mL (upper limit of detection [ULOD]) with $R^2 = 0.996$. Error bars represent the standard error of n = 3. The figure has been modified from Steinkellner et al.[8].

Figure 3: MeCP2 levels in mouse brain and HDFs. (**A**) MeCP2-protein levels were measured in brain nuclear lysates from heterozygous (grey, HET) and female wild type mice (black, wildtype) (n = 4) and one *Mecp2*-knockout mouse (RTT); (**B**) Cell lysates from MeCP2-deficient fibroblast cell line (c.806delG) derived from a male patient with neonatal encephalopathy as model for RTT syndrome were conducted to assess the MeCP2 protein levels in humans and a healthy control (black). The presented data are mean ± SD of triplicate wells (n = 3). No MeCP2 protein was detected (below detection range) in the mutant cell lines by immunofluorescence or using the ECLIA, further demonstrating that it is a highly sensitive system for further uptake studies with TAT-fusion proteins. This figure has been modified from Steinkellner et al.[8].

Figure 4: Time dependent uptake of TAT-MeCP2. MeCP2 levels of nuclear fractions in c.806delG HDFs were treated with 500 nM recombinant TAT-MeCP2 fusion protein. Analysis was performed with the MeCP2-ECLIA. At stipulated time points, the cells were washed with DPBS and incubated with 0.05% trypsin-EDTA for 5 min to eliminate extracellular-bound TAT-MeCP2. Trypsinization was stopped by adding media with serum. The cell suspension was centrifuged at 500 x *g* for 5 min. After washing the cell pellet 2x with ice-cold DPBS, the sample was prepared for extraction of nuclear fraction as described in the protocol section. This figure has been modified from Steinkellner et al.[8].

Table 1: List of antibodies and used working dilutions.

Table 2: Standard series from 0 to 1,800 ng/mL.

Table 3: Determination of inter-assay precision on three consecutive days of HDF and wildtype mouse brain lysates. Per well 1−10 µg protein of cell lysate was applied. [a]SD, standard deviation; [b]SEM, standard error mean; [c]CV, coefficient of variation. This table has been modified from Steinkellner et al.[8].

DISCUSSION:

To measure endogenous MeCP2, recombinant MeCP2 and TAT-MeCP2 levels, a 96-well plate ECLIA was developed. It has been shown that loss of MeCP2 protein function leads to RTT syndrome[6], for which treatment is currently limited to symptom management and physical therapy. One promising treatment avenue is the so-called protein replacement therapy, where MeCP2 levels can be titrated up to their needed concentration[12-15]. The potential of TAT-fusion proteins to cross the blood-brain barrier has proven to be successful over the last two decades[12-15]. As such, this method for MeCP2 delivery may be useful in context of protein replacement therapy administration. In order to assess the potential of TAT-fusion proteins and other treatments to restore MeCP2 protein levels, developing an efficient and affordable assay that can quantify them is of utmost importance. The assay described in this work, the ECLIA, is able to determine levels of MeCP2 accurately as well as consistently, with favorable intra- and inter-assay values (**Table 3**).

For the following MeCP2-ECLIA protocol, mouse brain lysates and HDFs were employed as the cells of interest. However, this protocol may be used with all other cell types of at least human and murine origin. Moreover, HDFs were treated with TAT-MePC2 fusion protein to show the capability of this assay to measure various MeCP2 protein variants. During sample preparation, avoiding or minimizing reducing agents in the lysis buffer such as DTT or β-mercaptoethanol, is crucial to preserving the efficiency of the technique. Additional critical steps in this method involve plate coating with a mouse anti-MeCP2 antibody, plate blocking, and sample addition, followed by an 4 h incubation with a rabbit anti-MeCP2 antibody. Subsequent incubation with a specific secondary antibody (**Table of Materials**) and addition of reagents necessary for the luminescence reaction to take place, comprise the final important steps of this procedure.

In order to optimize ECLIA performance, the following steps can be undertaken. The maximum output for the signal for this assay should not exceed one million counts. In order to prevent the fluid from spreading beyond the electrode, a technique called spot coating can be used to introduce the coating solution into the well. This technique requires high precision pipetting or the use of a pipette robot. In addition, testing various antibody concentrations could be useful to both increase assay specificity and reduce background signal. To address the latter, various blockers (such as MSD, Blocker D-M) can also be used to a final concentration of 0.1%. In order to optimize the signal quality, testing various incubation times (shaking at or above 300 rpm) is recommended. Finally, to increase recovery and dilution linearity in specific media such as serum, plasma, urine or cerebrospinal fluid, several diluents can be tested.

Semi-quantitative western blot and commercially available MeCP2-ELISA are normally used to

study MeCP2 protein levels. The working principle behind the ELISA is similar to that of our ECLIA with the notable difference being the detection mode. Compared to these methods, the MeCP2-ECLIA is faster and more convenient. The ECLIA was used to assay for wildtype, heterozygous and Mecp2-knockout mouse brain samples (**Figure 3A**), with findings compared to MeCP2 amounts from the same samples obtained by western blotting (data not shown). A marked difference was observed in MeCP2 levels of female wildtype and heterozygous mouse brain samples measured by the ECLIA which was not detected as statistically significant by the western blot. This higher ECLIA assay accuracy can be important in the search for novel compounds that can elevate MeCP2 protein levels.

In addition, the MeCP2-ECLIA is less costly than its MeCP2-ELISA counterpart and due to its high dynamic range from 1 ng/mL to 1,800 ng/mL (R^2 = 0.996), can be used with samples containing low MeCP2 amounts. When compared to all the commercially available ELISA kits, the ECLIA greatly outperforms them. The ECLIA possesses a more favorable dynamic range from 1−1,800 ng/mL compared to its ELISA counterparts, which were found to be 0.312−20 ng/mL (mouse, Cloud-Clone Corp.) and 0.156−10 ng/mL (human, Cloud-Clone Corp.). Due to the absence of an explicit definition of a lower limit of detection, its direct comparison between those two assays is not possible for the purposes of this work.

In summary, it has been shown that the MeCP2-ECLIA can accurately determine MeCP2 amounts in vivo and in vitro. While replenishing MeCP2 protein levels in the neurons of RTT-affected patients is indeed a promising treatment avenue, presence of excessive MeCP2 may also result in severe neurological symptoms, associated with MECP2 duplication syndrome[16-18]. As such, this method can be of integral importance in optimizing the amount of exogenously introduced MeCP2 during protein replacement therapy.

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DISCLOSURES:
The authors have nothing to disclose.

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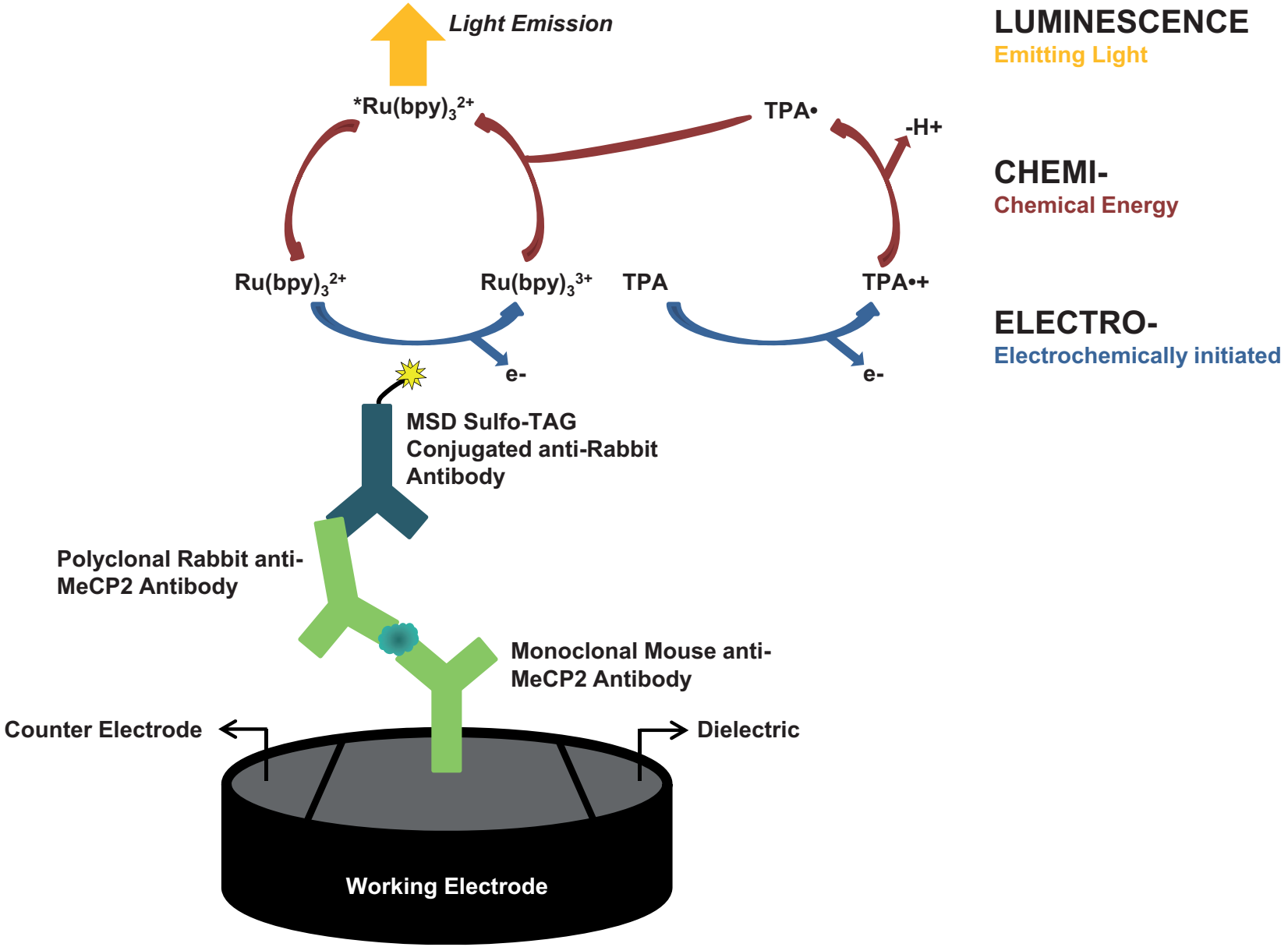

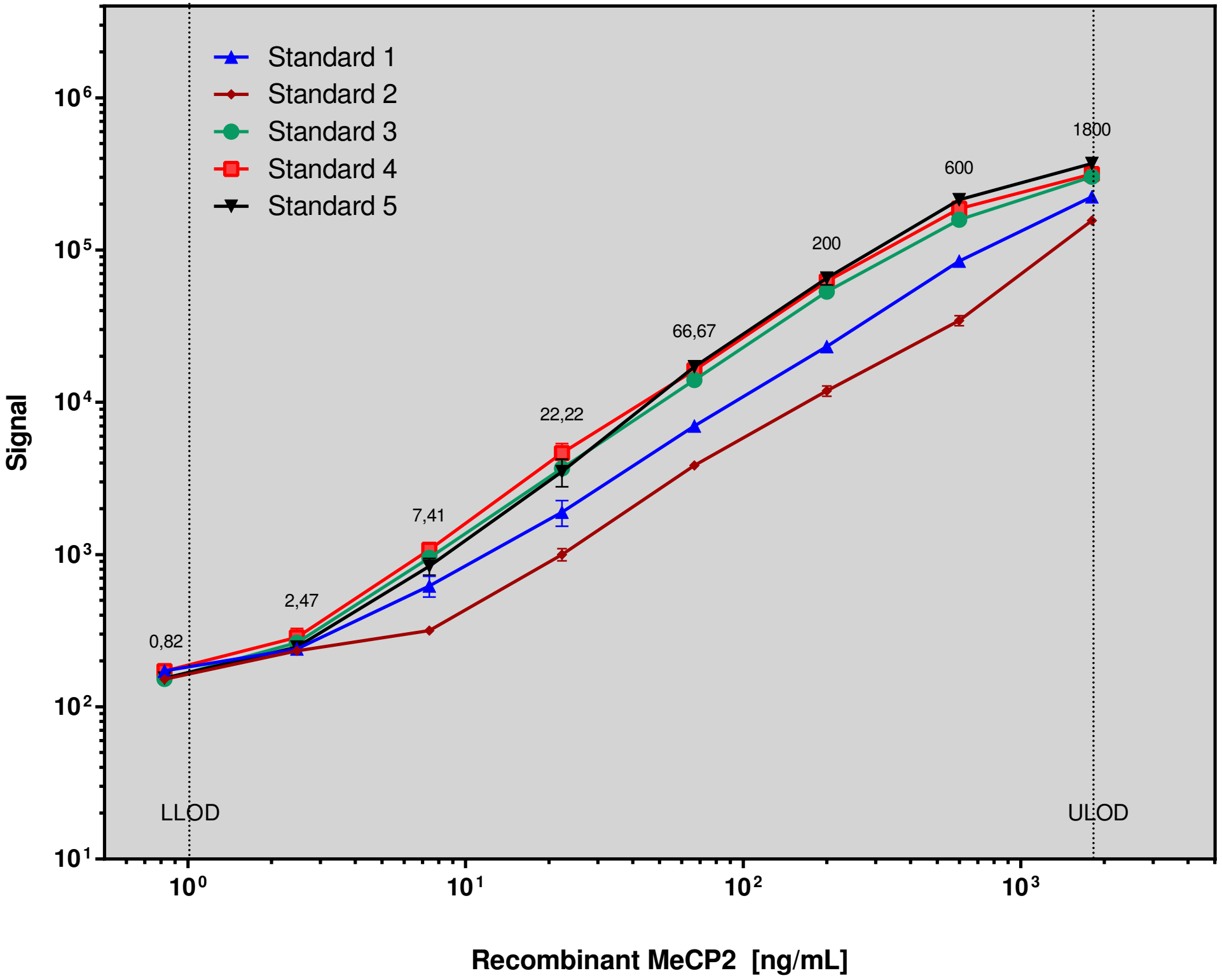

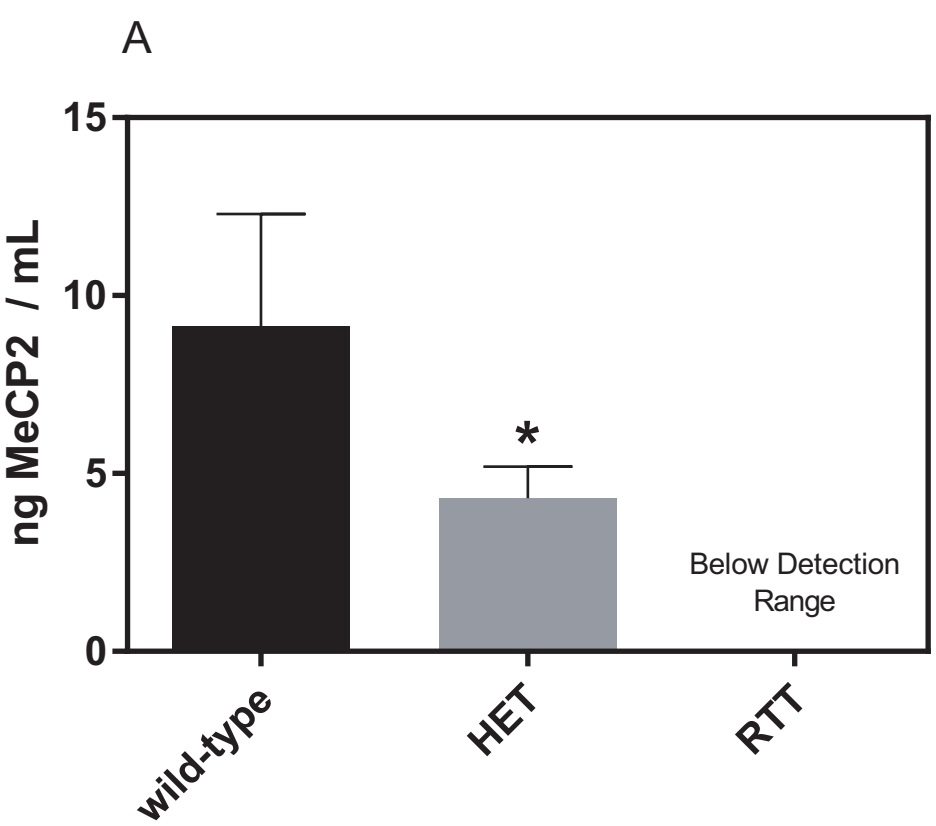

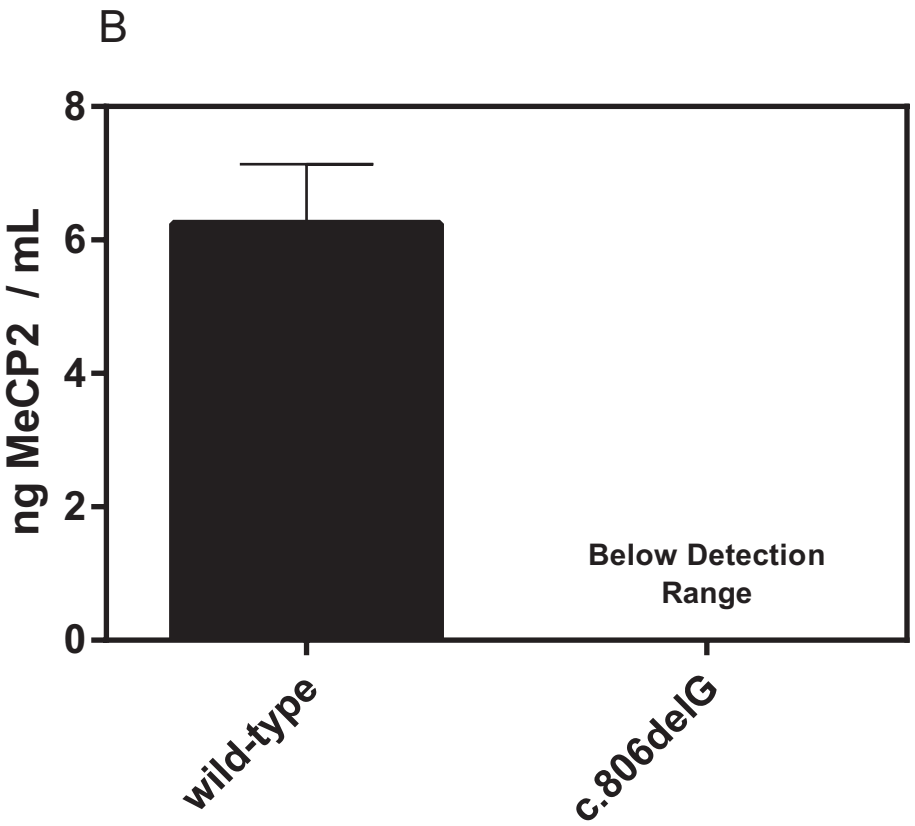

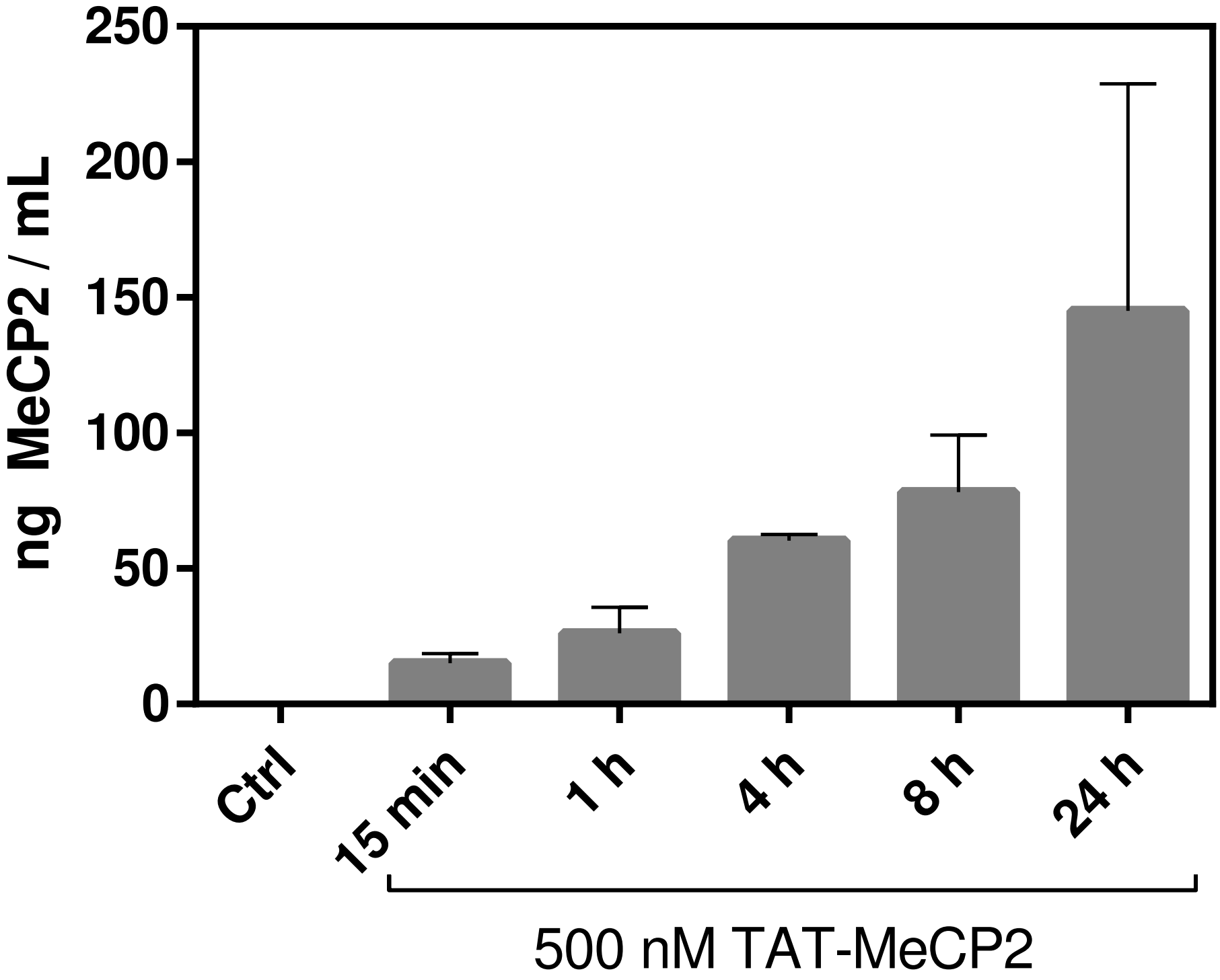

Function	Name	Clone	Dilution
Primary	Mouse, anti-MeCP2	Mec-168	1:500−1:10,000
Primary	Mouse, anti-MeCP2	4B6	1:250−1:4,000
Primary	Mouse, anti-MeCP2	Men-8	1:500−1:4,000
Primary	Mouse, anti-MeCP2	1B11	1:500−1:4,000
Primary	Rabbit, anti-MeCP2	D4F3	1:500−1:4,000
Secondary	Rabbit, anti-MeCP2	polyclonal	1:2,000−1:20,000
Secondary	Rabbit, anti-MeCP2	polyclonal	1:2,000−1:20,000
Detection	SULFO-TAG labeled anti-rabbit	polyclonal	1:500−1:1,000

Standard	Concentration	Dilution
Standard 1	1,800 ng/mL	1.08 µL Standard stock solution + 148.92 µL Lysis buffer
Standard 2	600 ng/mL	50 µL Standard 1 + 100 µL Lysis buffer
Standard 3	200 ng/mL	50 µL Standard 2 + 100 µL Lysis buffer
Standard 4	66.67 ng/mL	50 µL Standard 3 + 100 µL Lysis buffer
Standard 5	22.22 ng/mL	50 µL Standard 4 + 100 µL Lysis buffer
Standard 6	7.41 ng/mL	50 µL Standard 5 + 100 µL Lysis buffer
Standard 7	2.47 ng/mL	50 µL Standard 6 + 100 µL Lysis buffer
Standard 8	0.82 ng/mL	50 µL Standard 7 + 100 µL Lysis buffer
Standard 9	0.27 ng/mL	50 µL Standard 8 + 100 µL Lysis buffer
Standard 10	0 ng/mL	150 µL Lysis buffer

Table3

		INTRA ASSAY							INTER ASSAY			
		ng MeCP2 per mL protein							ng MeCP2 per mL protein			
		Well 1	**Well 2**	**Well 3**	**Mean**	**SD[a]**	**SEM[b]**	**%CV[c]**	**Mean**	**SD[a]**	**SEM[b]**	**%CV[c]**
	Human fibroblasts	6.42	6.30	6.45	6.39	0.08	0.05	1.24	6.61	0.64	0.37	9.71
	Mouse brain lysate	9.92	10.16	10.36	10.14	0.22	0.13	2.17	11.22	0.94	0.54	8.33
	[a]Standard Deviation, [b]Standard Error Mean, [c]Coefficient of Variation											

Name of Material/ Equipment	Company
1,4-Dithiothreitol (DTT)	Sigma-Aldrich
Bio-Rad Protein Assay Dye Reagent Concentrate	Bio-Rad Laboratories Inc.
Detection AB SULFO-TAG labeled anti-rabbit	Meso Scale Diagnostics
Discovery workbench 4.0	Meso Scale Discovery
DMEM (1X)	gibco by Life Technologies
Dulbecco's PBS (sterile)	Sigma-Aldrich
EDTA	Sigma-Aldrich
Fetal Bovine Serum	Sigma-Aldrich
Glycerol	Sigma-Aldrich
Gold Read Buffer T (1x) with surfactant	Meso Scale Diagnostics
HEPES	Sigma-Aldrich
KIMBLE Dounce tissue grinder set	Sigma-Aldrich
Laboratory Shaker, rocking motion (low speed)	GFL
Magnesium chloride hexahydrate (MgCl*6H20)	Sigma-Aldrich
MeCP2 (Human) Recombinant Protein (P01)	Abnova Corporation
Microseal B seal	Bio-Rad Laboratories Inc.
Monoclonal Anti-MeCP2, produced in mouse, clone Mec-168, purified immunoglobulin	Sigma-Aldrich
MSD Blocker A	Meso Scale Diagnostics
MSD SECTOR Imager 2400	Meso Scale Diagnostics
Multi-Array 96-well Plate	Meso Scale Diagnostics
Penicillin-Streptomycin	gibco by Life Technologies
Polyclonal Anti-MeCP2, produced in rabbit	Eurogentec S.A.
Potassium chloride (KCl)	Merck KGaA
Primary AB Mouse, anti-MeCP2 (1B11)	Sigma-Aldrich
Primary AB Mouse, anti-MeCP2 (4B6)	Sigma-Aldrich
Primary AB Mouse, anti-MeCP2 (Mec-168)	Sigma-Aldrich
Primary AB Mouse, anti-MeCP2 (Men-8)	Sigma-Aldrich
Primary AB Rabbit, anti-MeCP2 (D4F3)	Cell Signaling Technology
Protease Inhibitor Cocktail (100X)	Sigma-Aldrich
Secondary AB, Rabbit, anti-MeCP2	Eurogentec S.A.
Secondary AB, Rabbit, anti-MeCP2	Merck
Sodium chloride (NaCl)	Sigma-Aldrich

SULFO-TAG Labeled Anti-Rabbit Antibody (goat)	Meso Scale Diagnostics
TAT-MeCP2 fusion protein	in-house production
Trypsin EDTA 0.25% (1X)	gibco by Life Technologies
Tween 20	Sigma-Aldrich

Catalog Number	Comments/Description
D9779	Hypotonic lysis reagent, Extraction Buffer
500-0006	Sample preperation
R32AB-1	Antibody
	Software
41966-029	Sample preperation
D8537-500ML	Sample preperation, Washing solution, Coating solution
EDS	Extraction Buffer
F9665	Sample preperation
G2025	Extraction Buffer
R92TG	MeCP2 ECLIA protocol
H3375	Hypotonic lysis reagent, Extraction Buffer
D8938	Sample preperation
3014	MeCP2 ECLIA protocol
M2670	Hypotonic lysis reagent, Extraction Buffer
H00004204-P01	Cell treatment
MSB1001	for plate sealing
M6818-100UL; RRID:AB_262075	Antibody, Coating solution
R93BA-4	Blocker
I30AA-0	MeCP2 ECLIA protocol
L15XB-3/L11BX-3	MeCP2 ECLIA protocol
15140122	Sample preperation
custom-designed	Antibody
1049361000	Hypotonic lysis reagent
SAB1404063; RRID:AB_10737296	Antibody
WH0004204M1; RRID:AB_1842411	Antibody
M6818; RRID:AB_262075	Antibody
M7443; RRID:AB_477235	Antibody
3456S; RRID:AB_2143849	Antibody
8340	Hypotonic lysis reagent, Extraction Buffer
custom	Antibody
07-013	Antibody
S3014	Extraction Buffer

W0015528S	Antibody
	Cell treatment
25200-056	Cell treatment
P9416	Washing solution

0.25% Trypsin EDTA (1X)
1,4-Dithiothreitol (DTT)
Bio-Rad Protein Assay Dye Reagent Concentrate
DMEM (1X)
Dulbecco's PBS (sterile)
EDTA
Fetal Bovine Serum (1)
Glycerol
HEPES
Laboratory Shaker, rocking motion (low speed)
Magnesium chloride hexahydrate ($MgCl*6H_20$)
MeCP2 (Human) Recombinant Protein (P01)
Monoclonal Anti-MeCP2, produced in mouse, clone Mec-168, purified immunoglobulin
MSD Blocker A
MSD SECTOR® Imager 2400
Multi-Array® 96-well Plate
Penicillin-Streptomycin
Polyclonal Anti-MeCP2, produced in rabbit
Potassium chloride (KCl)
Protease Inhibitor Cocktail (100X)
Read Buffer T (4x) with surfactant
Sodium chloride (NaCl)
SULFO-TAG™ Labeled Anti-Rabbit Antibody (goat)
Tween® 20

gibco by L	25200-056	
Sigma-Ald	D9779	
Bio-Rad La	500-0006	
gibco® by	41966-029	
Sigma-Ald	D8537-500ML	
Sigma-Ald	EDS	
Sigma-Ald	F9665	
Sigma-Ald	G2025	
Sigma-Ald	H3375	
GFL		3014
Sigma-Ald	M2670	
Abnova Cc	H00004204-P01	
Sigma-Ald	M6818-100UL	
Meso Scal	R93BA-4	
Meso Scal	I30AA-0	
Meso Scal	L15XB-3/L11BX-3	
gibco® by		15140122
Eurogente	custom-designed	
Merck KGa		1049361000
Sigma-Ald		8340
Meso Scal	R92TC-3	
Sigma-Ald	S3014	
Meso Scal	W0015528S	
Sigma-Ald	P9416	

MS NO: JoVE61054

MS TITLE: "Development and validation of an electrochemiluminescence based assay for MeCP2 protein variants"

Detailed response to the editor and reviewer's comments

We thank all reviewers for carefully reading our manuscript as well as for their helpful comments. We have revised the manuscript according to their suggestions and highlighted the changes in green:

Answers to editorial comments:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.

   Response: We thank the editor for this suggestion. The manuscript has been proofread to avoid any spelling errors.

2. Please revise lines 36-40, 63-64, 310-312, 328-332, 335-338, 339-345, 346-350 to avoid textual overlap with previously published work.

   Response: The appropriate portions of the text have been revised to avoid overlap with previous work.

   Ms page 2, line 41-46, ms page 2, line 65-68, ms page 8, line 323-325, ms pages 8, line 326-331, ms pages 8 and 9, line 347-355, ms page 9, line 357-364, ms page 9, line 365-370.

3. Keywords: Please provide at least 6 keywords or phrases.

   Response: Two more keywords / phrases have been added to the manuscript.

   Ms page 1, lines 29-30

4. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials. You may use the generic term followed by "(Table of Materials)" to draw the readers' attention to specific commercial names. Examples of commercial sounding language in your manuscript are: Sulfo-TAG, Sector Imager, Meso Scale Discovery, etc.

   Response: We thank the editor for drawing our attention to this issue. This problem was corrected. Commercial terms have been removed and generic names with reference to Table of Materials are now used.

Ms page 3, line 96
Ms page 6, lines 232, 239, 243, 249 and 253
Ms page 8, line 334

5. Please revise the Protocol text to avoid the use of personal pronouns (e.g., I, you, your, we, our) or colloquial phrases.

   Response: The protocol was revised not to include personal pronouns or colloquial phrases.

   Ms page 3, line 96

6. Please revise the Protocol to contain only action items that direct the reader to do something (e.g., "Do this," "Ensure that," etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as "could be," "should be," and "would be" throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a "NOTE." Please include all safety procedures and use of hoods, etc. However, notes should be used sparingly and actions should be described in the imperative tense wherever possible. Please move the discussion about the protocol to the Discussion.

   Response: We thank the editor for drawing our attention to this issue. The issues were corrected accordingly. All actions have been changed to the imperative tense and phrase such as "could be", "should be" and "would be" are not used. Any text that could not be written in the imperative tense has been added as "NOTE."

7. Protocol: Please revise it to be a numbered list following the JoVE Instructions for Authors: step 1 followed by 1.1, followed by 1.1.1, etc. Each step should include 1−2 actions and contain 2−3 sentences. Use subheadings and substeps for clarity if there are discrete stages in the protocol. Please refrain from using bullets, dashes, or indentations.

   Response: The numbering of the protocol steps was changed following the JoVE Instructions for authors.

   Ms pages 3-6

8. Please add more details to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol. Please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. See examples below.

   Response: More details have been added to the protocol steps so that viewers can easily reproduce it.

9. Line 107: Please specify the growth conditions.

   Response: The growth conditions are now specified in the manuscript.

   Ms page 3, line 108

10. Line 110: What is the dilution factor used here?

Response: The dilution factor was indicated in the manuscript.

Ms page 3, line 110

11. Line 112: Please specify the incubation conditions (temperature & time) and the concentrations of TAT-MePC2 used.

    Response: The incubation conditions and the concentration of TAT-MeCP2 were specified in the manuscript.

    Ms page 3, line 113

12. Line 115: At what temperature?

    Response: The temperature was indicated in the manuscript.

    Ms page 3, line 116

13. Line 127: Please list an approximate volume of solutions to prepare.

    Response: The approximate volume was listed in the manuscript.

    Ms page 4, line 132

14. Line 136: Please specify the source of the mouse brain (age, gender and strain of mouse).

    Response: The source of the mouse brain was specified in the manuscript.

    Ms page 4, line 142

15. Line 139: Please provide the composition of homogenizer A and B. If they are purchased, include product information in the Table of Materials.

    Response: The composition of homogenizer A and B was provided in the manuscript.

    Ms page 4, lines 146-148

16. Please combine some of the shorter Protocol steps so that individual steps contain 2-3 actions and maximum of 4 sentences per step.

    Response: Some shorter protocol steps were combined throughout the manuscript.

17. Figure 4: Please include a space between numbers and their corresponding units (15 min, 1 h, 4 h, 8 h, 24 h, 500 nM).

Response: We thank the reviewer for drawing our attention to this issue. Figure 4 was adapted accordingly.

18. Table 1: Please delete the "Brand" column which contains commercial information. All commercial products should be sufficiently referenced in the Table of Materials.

    Response: The "Brand" column in table 1 has been removed. The antibodies have been added to the Table of Materials.

19. Table 2 and Table 3: Please include a space between numbers and their corresponding units (1800 ng/mL, 50 µL, etc.). Please abbreviate liters to L (mL, µL) to avoid confusion.

    Response: Spaces between numbers and their corresponding units were introduced in table 2 and table 3. The appropriate abbreviations were also included.

20. Table of Materials: Please ensure that it has information on all relevant supplies, reagents, equipment and software used, especially those mentioned in the Protocol. Please remove any ™/®/© symbols and sort the materials alphabetically by material name.

    Response: The Table of Materials has been revised to meet the described parameters.

21. References: Please do not abbreviate journal titles; use full journal name.

    Response: The references were revised and now all include the full journal name.

    Ms page 9-10

22. Please obtain explicit copyright permission to reuse Figure 1.

    Response: The Company has informed us that no explicit copyright permission is required for this adapted figure.

<u>Answers to the comments of Reviewer 1:</u>

Major concerns:

1. "The present Protocol is not intended to describe the development nor the validation of the technique to quantify MECP2 protein. This was done in the original paper by Steinkellner, H. et al., 2019. Therefore, the title should more precisely reflect the content of the manuscript. For example: electrochemiluminescence based assay for MeCP2 protein variants.

Response: We are grateful for this insightful suggestion and have changed the title of the manuscript to: "Employing an electrochemiluminescence based assay for MeCP2 protein variants"
Ms page 1, line 2

2. Still in the title, the present Protocol, using conventional anti-MeCP2 antibodies will detect both endogenous MeCP2 and recombinant MeCP2 proteins added to the cells. It is not clear to me how the specific detection of MeCP2 variants is made. The title and introduction need to reflect this point.

   Response: The MeCP2-ECLIA cannot distinguish between endogenous and exogenously applied MeCP2. For the detection of exogenously applied MeCP2 fusion protein, we are using MeCP2 knock out cell lines or a MeCP2 knock out mouse model.

3. This reviewer understands the importance of determining correct MECP2 protein levels, especially in patients undergoing MECP2 protein replacement therapy. However, contrary to what is mentioned in line 68 in the introduction, the vast majority of patients of RTT have SNP mutations that, although affecting protein function, do not alter protein levels in general. Antibodies aimed at detecting the therapeutic recombinant proteins should them be used, what is not the object of detection in this Protocol. Authors should discuss this point in the intro and discussion sections. In the same note, the authors should also discuss how scalable the technique is for expanding into different protein targets. For example, have the group tried to implement the quantification technique for other protein targets? What is the success rate?

   Response: We thank the reviewer for this insight. We acknowledge the fact that point mutations in the *MECP2* gene lead to the translation of misfolded and/or dysfunctional MeCP2 and we address this issue in the introduction section. However, there are no antibodies which are known to detect dysfunctional MeCP2 variants that lead to RETT syndrome onset, such experiment would not be possible at the time.

4. Another critical aspect relevant for the applicability of the technique, the way it is currently presented, is how the authors envision using it in a treatment setup. Precisely, how MeCP2 protein would be measured upon protein replacement therapy using the described technique since the collection of brain samples for protein lysates pre and post-treatment is not an option? Please expand the discussion to include this point.

   Response: We thank the reviewer for this comment. While measuring MeCP2 levels during actual protein replacement therapy in humans poses a challenge which has to be addressed in future work, it is beyond the scope of this manuscript.

Minor concerns:

5. The table 1 described on page 8 "Determination of inter-assay precision" probably refers to table 3 in the manuscript, please clarify.

   Response: We thank the reviewer for raising this issue and have clarified it.

Ms page 8, line 306

6. How can lysate preparation methods affect the performance of the technique? Please discuss?

   Response: Avoiding and/or minimizing reducing agents (DTT, ß-mercaptoethanol) in the lysis buffer is critical in order to maintain technique performance. We discussed this in the manuscript.

   Ms page8, line 329-331

7. The commercial antibodies and recombinant protein items in the table of materials should ideally come with RRID #

   Response: We are grateful for this suggestion, and RRID numbers were added in the List of Materials where applicable and available.

8. Section 2 - points e and g should specify the amount or range of recombinant protein used, or refer to a figure. Still in point g, should specify the incubation time.

   Response: Many thanks for this suggestion. It was incorporated into the manuscript.

   Ms page 3, lines 110-113

9. Section 3. Sample preparation -
   a. i. which subcellular fractionation was actually used?
   b. iii. Which glass tissue homogenizer was used? Presumably a Dounce, but which configuration: loose or tight? Was glass homogenizer pre-chilled?

   Response: We thank the reviewer for these queries. They are now addressed in the manuscript.

   Ms page 4, lines 146-148

10. Section 4 - MeCP2-ECLIA protocol
    a. i. Please spell out the desired final Tween-20 final concentration.

    b. ii. Which volume of monoclonal mouse anti-MeCP2 antibody diluted in PBS is desired?

    b vi. Which adhesive foil is used? Not described in the list of material table.

    e. iii. How does one not "breach the dielectric" while pipetting the samples? Be more descriptive here, please.

    h. iv. Machine and software setup should be mentioned.

    Response: Thanks for pointing this out. We have incorporated all above mentioned points in the manuscript. Additionally, the used adhesive foil was added to the list of materials.

Ms page 4, line 164

Ms page 5, lines 177-178

Ms page 6, line 218

Ms page 6, lines 249-257

11. Line 306. The abbreviation BBB is not defined in the text. The term blood-brain-barrier is used in the introduction, but the abbreviation is not presented. Since it is used only twice in the whole manuscript, the authors should avoid abbreviation to keep simple for the general readership.

    Response: We thank the reviewer for drawing our attention to this issue. It was adapted accordingly. The abbreviation BBB has been changed to the term blood-brain barrier.

12. The paragraph on line 318, the authors mean "to expand to other protein targets and optimize performance…"

    Response: The sentence the reviewer refers to serves as an opening part of a paragraph outlining crucial steps in the ECLIA protocol.

    Ms page 8, line 337

Answers to the comments of Reviewer 2:

Major concerns:

1. Manuscript Summary:
   Overall, the article gives a bench-level protocol for quantifying MeCP2 protein levels in cell and tissue lysates. They discuss how this method is more accurate and high-throughput than other quantification methods currently used in the field. While the focus is on the ECLIA technique, some more background on protein replacement therapy and its advantages over other strategies (e.g. gene replacement therapy) to cure Rett syndrome may be of interest to the reader. It would also be helpful to remind the reader how ELISA works, to better discuss the differences between the two methods.

   Response: We thank the reviewer for this comment. A brief description on the principle behind ELISA has been added to the discussion section.

   Ms pages 8-9, lines 348-349

2. *Are the title and abstract appropriate for this methods article?
   Yes, the except lines 40-41: "To demonstrate its broad applicability, this assay was used to analyze mouse brain tissue and human dermal fibroblast to study the uptake of TAT-MeCP2". Brain tissue was not treated with TAT-MeCP2 in this study, as this sentence suggests.

   Response: We acknowledge this inconsistency and modified this part of the abstract accordingly.

Ms page 2, lines 41-44

3. *Are there any other potential applications for the method/protocol the authors could discuss?
This method could be used to analyse MeCP2 protein levels in different cell fractions, e.g. chromatin-bound vs nuclear.

Response: As MeCP2 is a transcription factor and is largely localized to the nucleus, evaluating its levels there is integral to its functionality and activity.

4. *Are all the materials and equipment needed listed in the table? (Please note that any basic lab materials or equipment do not need to be listed, e.g. pipettes.)
Yes, could the last 2 tables which both list all the reagents be combined?
The authors list several antibodies that they have shown work in this assay, but not which pairs work together. This information would help others who want to use this assay with other antibodies than their recommended pair.

Response: The two tables list antibodies and reagents and contain different contents. The antibody pair described here are optimal for this assay to analyse MeCP2 and TAT-MeCP2, respectively. Other antibodies display significantly reduced efficiency in this assay. However, all antibodies mentioned in this manuscript have been added to the list of materials.

5. *Do you think the steps listed in the procedure would lead to the described outcome?
Yes, I just have a couple of queries.
Do the authors have a reference for trypsin treatment removing membrane-bound TAT-MeCP2? I am also concerned that the extraction method described would not get all the MeCP2 off the DNA: the extraction buffer contains 420 mM NaCl, but Goffin et al., 2012 (and others) show that more MeCP2 is extracted at 700 mM than 350 mM NaCl. An alternative to high salt is sonication/degradation of the DNA (e.g. with benzonase).
How does the machine read the plate - i.e. what is the delay between the activation of the Sulfo-tag and the light recording? Does this vary across the plate (does the stability of the light emission matter)?

Response: We thank the reviewer for this insight! We included the corresponding reference in the text. We acknowledge that increasing sodium chloride concentration in the lysis buffer may lead to increased MeCP2 recovery. For future versions of the protocol sonication will be used to address this issue.

Ms page 3, line 117

6. *Are the steps listed in the procedure clearly explained?
Yes. An earlier reference to figure 1 would help the reader follow the description of ECLIA.

Response: We acknowledge the reviewer's remark regarding the importance of referencing Figure 1 earlier and added the appropriate reference.

Ms page 3, line 85

7. *Are any important steps missing from the procedure? Are all the critical steps highlighted?
There are two instances where the steps are not given and a previous paper is cited. While this is acceptable in a lot of journals, it is troublesome for the reader and stops this protocol being 'ready to go'.
One reagents table has 4x Read buffer and one has 1x Read buffer, which is confusing.
Are the authors sharing their plasmids to make TAT-MeCP2 with the community, e.g. via Addgene?

   Response: We use 1x Read buffer both in the text and in the tables. The plasmids and their sequences are available upon request.

8. *Are appropriate controls suggested?
Yes.

9. *Is there any additional information that would be useful to include?
While the ECLIA can be performed in a 96-well format, how high-throughput can you make the cell fractionation step? What is the maximum number of samples that can be handled?
Can lysates be frozen (at -80C) before the ECLIA analysis?

   Response: We recommend limiting cell fractionation to six samples per experiment. Lysates can be flash frozen and stored at -80°C prior to analysis on a later date. We included this information in the manuscript.

   Ms page 4, line 129

10. *Are the anticipated results reasonable, and if so, are they useful to readers?
Yes, as long as membrane-bound MeCP2 is removed in the trypsin step.

    Response: We acknowledge that removing membrane bound MeCP2 is of importance as described by Koutsokeras A. et al. and included the appropriate reference in the text.

    Ms page 3, line 117

11. *Are any important references missing and are the included references useful?
The readers should reference Van Esch et al. 2005/2012 when introducing MECP2 duplication syndrome (line 66).
None of the references in line 305 (references 11-14) are about MeCP2, add a RTT reference here.

    Response: We appreciate the reviewer's remark and the references were adapted to more accurately reflect the discussed topics.

    Ms page 2, line 67

Minor concerns:

12. Line 67: not explained why RTT affects females and MECP2 duplication syndrome affect males. Mention MECP2 is X-linked.

    Response: We thank the reviewer for this remark. The X-linkage of *MECP2* was added to facilitate better understating of the text.

    Ms page 2, line 65

13. Line 68: 'as well as' is incorrect - reads as if both diseases are associated with both insufficiency AND excess MeCP2.

    Response: The wording was corrected.

    Ms page 2, line 72

14. Line 75: isoform B is also known as e1.

    Response: Both names are now mentioned in the text.

    Ms page 2, line 79

15. Line 94: detection not secondary antibody.

    Response: We thank the reviewer for this remark and made the appropriate correction in the text.

    Ms page 3, line 95

16. Line 100: Replace "However" with "Alternatively"?

    Response: “However” was replaced with “Alternatively”.

    Ms page 3, line 101

17. Line 104: coli has a lower case c.

    Response: This has been corrected.

    Ms page 3, line 105

18. Line 106: this step is written in the passive voice but all subsequent steps are written as instructions.

Response: The sentence has been changed to "Place 1x10^6 HDF cells in 100 mm dishes.".

Ms page 3, line 107

19. Line 112: 'various concentrations' - range? Only 500 nM shown. This should be in step e.

    Response: We made the appropriate correction in the text.

    Ms page 3, line 112

20. Line 112 and 192: "MePC2" typo.

    Response: This typo has been corrected.

    Ms page 3, line 113

21. Line 118: "with 500 g" should be "at 500 g".

    Response: The word has been changed to "at 500 g".

    Ms page 3, line 120

22. Line 136: Dissolve is not the right word.

    Response: The word has been changed to "Suspend"

    Ms page 4, line 142

23. Line 139: A and B = loose and tight?

    Response: We thank the reviewer for this comment and specified A and B in the manuscript.

    Ms page 4, line 146-148

24. Line 171: which is the 'solution' being referred to?

    Response: The solution in question refers to the antibody solution in 4.2.2. The text was adapted to state this more clearly.

    Ms page 5, line 178

25. Line 207,217,228,236: how long are the washing steps? Or incubation not required?

Response: No incubation is required. The information was added to the protocol.

Ms page 5, line 216; page 6, line 227, 238, 250

26. Line 218: detection not secondary antibody.

    Response: Detection and secondary antibody are two separate antibodies.

27. Line 251: I don't understand where 1.002 and 1800 came from. These numbers are different from those on the graph. Why 1.002 not 1?

    Response: We thank the reviewer for pointing out this inconsistency. Figure 2 and the corresponding legend have been modified to address this issue.

28. Line 257, 293: Table 3?

    Response: The text was changed to reference the right table.

    Ms page 7, lines 270; page 8, line 306

29. Line 304: delete 'the' before 'so-called'

    Response: The "the" before "so-called" was removed.

    Ms page 8, line 317

30. Line 315: It is crucial that the coating and detection antibodies are different species. Monoclonal vs polyclonal doesn't matter.

    Response: The species of the antibodies were added.

    Ms page 8, lines 332-333

31. Line 319: What does one million counts mean? What is the equivalent ng/ml?

    Response: The term "Counts" is used in this technique for "relative light units" (=RLU) and is directly proportional to the intensity of light. However, neither one of these terms are directly related to any concentration. We added the appropriate statement in the manuscript.

    Ms page 6, lines 254-255

32. Line 330: not "similar findings" if the western blots couldn't differentiate between WT and Het. You should be able to detect a 2-fold change by western - could the westerns have been overload?

    Response: Many thanks for this comment. We made the appropriate corrections in the manuscript.

    Ms page 9, line 352-353

33. Line 345: Other assays (ELISA and western) also enable processing of both mouse and human samples. I therefore do not understand this point.

    Response: Many thanks for this comment. We removed this sentence from the manuscript.

34. Figure 1: SUFLO should be Sulfo.

    Response: "SUFLO" was changed to "Sulfo" in Figure 1.

35. Figure 4: add spaces between numbers and units. Delete spaces before % signs in method. Nomenclature: human gene = MECP2, mouse gene = Mecp2, protein = MeCP2. This includes MECP2 duplication syndrome.

    Response: Spaces between numbers and units were added and the nomenclature of MeCP2 verified.

In summary we attempted to fulfil all requests from the reviewers and hope that the manuscript is acceptable in its revised form.

Betreff: Fwd: Request for MSD image in article
Von: "Duerr, Mark" <MDuerr@meso-scale.com>
Datum: 08/01/2020, 18:11
An: Hannes Steinkellner <hannes.steinkellner@meduniwien.ac.at>

Hi Hannes,

Weiter unten findest Du die Antwort unseres Legal Departments zu Deinem Bild. Ich hoffe, das Statement reicht aus.

Lg
Mark

Dr. Mark Dürr

Regional Manager, Field Applications – Central Europe

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Anfang der weitergeleiteten Nachricht:

Von: "Duncan, Nicholas" <nduncan@meso-scale.com>
Datum: 27. November 2019 um 14:09:11 GMT-5
An: "Duerr, Mark" <MDuerr@meso-scale.com>
Kopie: "Beterams, Gertrud" <GBeterams@meso-scale.com>
Betreff: RE: Request for MSD image in article

Hi Mark,

Received confirmation if the customer is utilizing the attached hand drawn graphic an agreement is not required. Please let me know if the use of our image will be required for initiating the agreement request and we will need the final draft of the paper for review.

Thank you,

Nick

From: Duerr, Mark <MDuerr@meso-scale.com>
Sent: Monday, November 25, 2019 5:11 AM
To: Duncan, Nicholas <nduncan@meso-scale.com>
Cc: Beterams, Gertrud <GBeterams@meso-scale.com>
Subject: RE: Request for MSD image in article

Hi Nick,

Please find attached the final version of the self-drawn graphic from Hannes Steinkellner.
Please let us know, if he can use the graphic.

Thanks
Mark

From: Duerr, Mark
Sent: Thursday, November 21, 2019 17:44
To: Duncan, Nicholas <nduncan@meso-scale.com<mailto:nduncan@meso-scale.com>>
Subject: RE: Request for MSD image in article

Hi Nick,

1. The specific corporate name (entity) that the license will be issued?

Medical University of Vienna

2. The complete address?
Währingerstrasse 10, Inst of Medical Genetics
1090 Vienna

AT

3. Title of the article: Development and Validation of an Electrochemiluminescence Based Assay for MeCP2 Protein Variants (original publication attached)

4. Expected due date?

Monday, Nov 25th (next Monday)

5. Confirm the paper provide is in its final form for review for executing the agreement?

Final version is not yet done.

My customer would like to know, if he could use his own drawing instead of using our picture, and if this would also need approval. I attached his drawing to this email. Please let me know, if that can be used (maybe without official approval process).

Thanks
Mark

Office Location

Mailing Address
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