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Leopold-Franzens-Universität Innsbruck
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Dr. Alexander Weiss

To the Editor
Journal of Visualized Experiments

Dear Dr. Werth,
Dear Editor,

March 10th, 2019

Thank you for the opportunity of resubmitting our revised article „*Expression, Purification, Crystallization and Enzyme Assays of Fumarylacetoacetate Hydrolase Domain containing Proteins (FAHD).”* to the *Journal of Visualized Experiments*. As part of the uploaded documents, please find our response to your assessment of the original manuscript JoVE59729.

We would like to thank you and all reviewers for their careful and thorough examination of our article. As requested, we uploaded a revised version of the manuscript, and a version with tracked changes. A point-by-point reply to both your comments and the comments of the original reviewers on manuscript version JoVE59729 is provided.

We kindly ask you to assess if the revised manuscript is now suitable for publication in the *Journal of Visualized Experiments*.

Thank you and with best regards,

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TITLE:

Expression, Purification, Crystallization, and Enzyme Assays of Fumarylacetoacetate Hydrolase Domain-Containing Proteins

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SUMMARY:

Expression and purification of fumarylacetoacetate hydrolase domain-containing proteins is described with examples (expression in *E. coli*, FPLC). Purified proteins are used for crystallization and antibody production and employed for enzyme assays. Selected photometric assays are presented to display the multi-functionality of FAHD1 as oxaloacetate decarboxylase and acylpyruvate hydrolase.

ABSTRACT:

Fumarylacetoacetate hydrolase (FAH) domain-containing proteins (FAHD) are identified members of the FAH superfamily in eukaryotes. Enzymes of this superfamily generally display

multi-functionality, involving mainly hydrolase and decarboxylase mechanisms. This article presents a series of consecutive methods for the expression and purification of FAHD proteins, mainly FAHD protein 1 (FAHD1) orthologues among species (human, mouse, nematodes, plants, etc.). Covered methods are protein expression in *E. coli*, affinity chromatography, ion exchange chromatography, preparative and analytical gel filtration, crystallization, X-ray diffraction, and photometric assays. Concentrated protein of high levels of purity (>98%) may be employed for crystallization or antibody production. Proteins of similar or lower quality may be employed in enzyme assays or used as antigens in detection systems (Western-Blot, ELISA). In the discussion of this work, the identified enzymatic mechanisms of FAHD1 are outlined to describe its hydrolase and decarboxylase bi-functionality in more detail.

INTRODUCTION:

The fumarylacetoacetate hydrolase (FAH)[1,2] superfamily of enzymes describes a group of enzymes that share the highly conserved catalytic FAH domain[3–10]. Despite their common catalytic center, these enzymes are multi-functional, and most are found in prokaryotes, where they are used to break down compounds retrieved from complex carbon sources[3]. Only three members of this family were identified in eukaryotes so far: the name giving FAH[2], as well as FAH domain-containing protein 1 (FAHD1)[11–15] and FAH domain-containing protein 2 (FAHD2). Depletion of FAHD1 has been associated with impaired mitochondrial respiration[13,16] and associated with a reversible type of cellular senescence phenotype[14] that is linked to intermediate potential shortcomings in the electron transport system. Human FAHD1 and its orthologues in model systems (mouse, nematode, cancer cell lines, plants, etc.), as well as selected point mutation variants, have become druggable targets of potential interest. For this research, recombinant protein at high levels of purity, as well as information on catalytic mechanisms guided by crystal structures and selective antibodies are vital.

This manuscript describes methods for FAHD protein expression in *E. coli*, affinity chromatography, ion exchange chromatography, ammonium sulfate precipitation, preparative and analytical gel filtration, crystallization, X-ray diffraction, and photometric assays. The purpose of the methods and protocols described here is to provide guidance for scientists working in diverse fields such as bacteriology, plant biology, as well as animal and human studies, to characterize members of the FAH superfamily, including uncharacterized superfamily members should they become relevant in a particular field. The protocols described here may provide valuable support for projects aiming to characterize other prokaryotic or eukaryotic FAH superfamily members.

The rationale behind the methods described here is the fact that for characterization of poorly described proteins (in particular, metabolic enzymes of unknown physiological relevance), the approach to start with purified recombinant proteins allows the development of invaluable, high-quality research tools such as in vitro active enzyme preparations, high-quality antibodies, and potent and specific pharmacological inhibitors for selected enzymes. The described methods require fast protein liquid chromatography (FPLC) and X-ray crystallography. Alternative methods (e.g., to express protein without chemical induction, or to display protein purification by centrifugation after heat treatment followed by desalting and size exclusion chromatography),

may be found elsewhere[17]. While a broader spectrum of methods is available for the expression and purification of FAH superfamily enzymes[2,7,9,17,18], this work focuses on the expression and purification of FAHD proteins in particular.

In the discussion section of this manuscript, the catalytic mechanisms identified for the FAHD1 protein (hydrolase, decarboxylase)[15] are described in more detail, in order to demonstrate the chemical character of the catalyzed reactions. The data obtained based on previous work[7,15,18] (PDB: 6FOG, PDB:6FOH) imply a third activity of the enzyme as keto-enol isomerase.

PROTOCOL:

1. Expression of FAHD proteins in competent *E. coli*

1.1. Transformation of *E. coli* with vectors for expression of FAHD protein

NOTE: The steps discussed in the following section are summarized in the sketch in **Figure 1A**,**B**. The same protocol applies for any FAHD protein, including point-mutant variants. Such variants may be obtained via site-directed mutagenesis and PCR techniques[19] (such as two-sided SOE PCR[20]) from wild-type cDNA.

[place Figure 1 here]

1.1.1. Obtain competent BL21(DE3) *pLysS E. coli* bacteria and a pET expression vector (see **Table of Materials**). Preferably choose a pET vector that also encodes an *N*-terminal His-tag or related capture tag for convenience to simplify the following purification steps.

1.1.2. Obtain cDNA of the FAHD protein of choice and insert it into the active cloning site of the pET expression vector, in between the T7 promoter and T7 terminator sites, respectively.

1.1.3. After successful plasmid amplification and verification [via sequencing by a commercial supplier (T7 primers may be used with the pET system for convenience: T7 promoter, forward primer: TAATACGACTCACTATAGGG; T7 terminator, reverse primer: GCTAGTTATTGCTCAGCGG)], insert 5–10 ng of plasmid into 100 µL of competent BL21(DE3) *pLysS E. coli* bacteria on ice. Do not aspirate up and down, but slightly tap the tube with in order to mix the content.

1.1.4. Keep the bacteria on ice for 30 min, gently tapping the tube every few min.

1.1.5. Heat a heating device or water bath to 42 °C (exact). Put the tube containing the bacteria into the apparatus and keep them for 90 s (exact). Put them on ice immediately (**Figure 1A**).

1.1.6. After 5–10 min on ice, add 600 µL of NCZYM medium (see **Table of Materials**) and put the tube into a bacteria incubator. Shake the tube at medium speed oriented along the shaking direction at 37 °C for 1 h.

1.1.7. Plate 200 µL of the bacterial culture on a 10 cm LB-agar plate (see **Table of Materials**), containing selection antibiotics of choice [e.g., one specific for the BL21(DE3) *pLysS* resistance (chloramphenicol), and one for the resistance encoded on the *pET* vector (kanamycin or ampicillin, **Figure 1B**)].

1.1.8. Culture the bacteria on the LB-agar plate in a bacterial incubator at 37 °C overnight.

1.2. Expression of FAHD proteins by IPTG induction

NOTE: The first steps discussed in the following section are summarized as a sketch in **Figure 1C**,**D**. The *T7* expression system via combination of the bacterial DE3 cassette and *pET* vector system are summarized in **Figure 2**.

[place Figure 2 here]

1.2.1. After successful colony formation, pick one single colony (without any satellite colonies) and disperse it in 5 mL of NZCYM or LB medium with antibiotics, selected as before (step 1.1.7). Culture in the bacterial incubator at 37 °C overnight (**Figure 1C**).

1.2.2. After successful bacterial growth, amplify the bacteria in 250 mL, 500 mL, or 1 L batches of medium, depending on the demand of protein quantity.

1.2.2.1. Appropriate to the volume, apply antibiotics selected as done in step 1.1.7 and add about 1%–2% of dense bacterial pre-culture (i.e., 2.5–5.0 mL to 250 mL volume of medium, etc.). Take a sample to be used in step 1.2.5 (1 mL or more) and check the optical density (OD) at 600 nm. Culture bacteria in the bacterial incubator at 37 °C for 2–3 h (**Figure 1C**).

1.2.3. After 2–3 h, draw a sample for photometric analysis. If the OD at 600 nm has reached 0.4, apply 200 µM up to 1 mM isopropyl-β-D-thiogalactopyranosid (IPTG, see **Table of Materials**).

NOTE: The actual value is empirical for each FAHD protein or point mutation variant, where 1 mM IPTG is the maximum that should be applied. This induces protein expression (**Figure 1D**, **Figure 2C**).

1.2.4. After 3–5 more hours in the bacterial incubator at 37 °C, protein expression is exhausted.

NOTE: See the discussion section for comments on temperature control. Longer than 5 h of shaking after induction is not recommended. Take a sample for use in step 1.2.5 (1 mL or more) and check the optical density (OD) at 600 nm.

1.2.4.1. Harvest the bacterial pellet *via* centrifugation at 5000 x *g* for 5 min. Discard the supernatant and freeze the pellet at -80 °C for longer storage or -20 °C for brief storage (**Figure 1D**).

1.2.5. Verify induction via the two retrieved photometric samples, that are labelled "-I" (before induction) and "+I" (after induction). After centrifugation and resuspension of the bacterial pellet, analyze the two samples by SDS-PAGE by loading the same amount of total protein.

NOTE: The "+I" sample should display a strong band associated with the molecular weight of the chosen protein, whereas the "-I" sample should not contain this band. A low induction level is a common problem for production of proteins, yet the level of expressed protein is often sufficient for the following steps. A high induction level is an advantage but is not mandatory.

2. Lysis of bacterial pellets and filtration of debris

2.1. Dependent on whether the chosen protein is *His*-tagged or untagged, select Ni-NTA running buffer (*His*-tagged, see **Table of Materials**) or ice-cold HIC running buffer (untagged).

2.2. For each 250 mL of original bacterial suspension, apply 5 mL of the selected buffer to the bacterial pellet (5 mL for 250 mL, 10 mL for 500 mL, etc.). Add 10 µL β-mercaptoethanol (β-ME) per 5 mL of applied buffer. Use a 10 mL Pasteur pipet to mechanically force the pellet into suspension by scratching and pipetting (avoid air bubble formation while pipetting). Eventually transfer all of the suspension into one 50 mL tube.

2.3. Preferably sonicate (6x for 15 s at medium force) the suspension.

2.4. Centrifuge for 30 min at high speed (30,000 x *g*) at 4 °C. Filter the supernatant consecutively with filter units (e.g., 0.45 µm, 0.22 µm) on ice.

NOTE: Depending on the previous centrifugation step, filtration directly through a small filter pore size may be tedious and usually requires pre-filtration through a larger pore size. DNAse may be added for better results.

2.5. Store the sample on ice and proceed immediately with either section 3 or 4, depending on whether the protein is *His*-tagged or untagged.

3. Purification of *His*-tagged FAHD proteins using Ni-NTA affinity chromatography

NOTE: Ni^{2+} ions are bound via nitrilotriacetic acid (NTA) to an agarose resin that is used in affinity chromatography (immobilized metal ion chromatography, IMAC, **Figure 3A**). Poly-histidine amino acid tags bind strongly to this Ni-chelate, and His-tagged proteins can be separated from the majority of remaining proteins. An alternative to the described preparation of Ni-NTA columns is using prepacked Ni-NTA columns and a FPLC system.

[place Figure 3 here]

3.1.1. Proceed from step 2.5 (i.e., the protein is in Ni-NTA running buffer and filtered by 0.22 µm filter units on ice).

3.1.2. Prepare an empty plastic or glass column by washing the empty column and attaching it to a stable retainer. Choose the size of the column depending on the volume of the protein suspension.

3.1.3. For each 10 mL of protein suspension, apply 500 µL of Ni-NTA agarose slurry into the column (shake heavily before usage). Apply the slurry slowly and dropwise onto the bottom filter of the column using a pipette. Let the column settle, which takes a few seconds.

3.1.4. Fill the column completely with Ni-NTA running buffer, ensuring not to disrupt the agarose resin. Let the buffer run through by gravity. The process may be accelerated up by applying thumb pressure onto the liquid (using a lid or glove and thumb pressure), but take care not to distort the agarose resin.

3.1.5. Apply the protein suspension. As before, let the sample run through by gravity. Accelerating this step using thumb pressure is not recommended, as binding of proteins to the column is enhanced if the flow rate is low. Collect the flow-through in a tube (**Table of Materials**).

3.1.6. After the sample has passed through, fill the whole column again with Ni-NTA running buffer. Take care to not disrupt the agarose resin. Let the sample run through by gravity, but in contrast to the previous step, accelerating the process via thumb pressure is recommended, as potential contaminations because of unspecific interactions may be disrupted this way. Collect the washing solution in a tube. Repeat this step.

3.1.7. Place a UV-transparent cuvette below the column and apply 1 mL of Ni-NTA elution buffer. Collect the sample without applying any thumb pressure to the resin.

3.1.8. Check the optical density (OD) of the sample at 280 nm vs. a blank sample (i.e., Ni-NTA elution buffer). Optimally, the sample displays an OD of greater than 2.5. An OD below 0.5 denotes that no significant amount of protein is in the sample.

NOTE: As outlined in the discussion section, salt and imidazole concentrations of the elution buffer may have to be adapted for each FAHD protein individually.

3.1.8. Repeat steps 3.1.7 and 3.1.8 until the OD falls below 0.5. Pool all samples with higher OD in a tube on ice.

3.1.9. Start again with step 3.1.4, using the flow-through from step 3.1.5 as new input for this repetition of step 3.1.5. Repeat this process until the first sample collected in step 3.1.6 displays an OD below 0.5.

NOTE: As outlined in the troubleshooting part of the discussion section, His-tagged proteins may bind insufficiently to the Ni^{2+}-resin. In such cases, repetition of this step or alternative methods (e.g., ion exchange chromatography) are required.

3.1.10. Take samples of all intermediate fractions for SDS-PAGE analysis.

3.1.11. FAHD proteins in Ni-NTA elution buffer will precipitate upon freezing and thawing. Therefore, dialyze the protein against a different buffer (overnight on ice, using 1 µL of DTT per 100 mL of dialysis buffer). Use low-salt buffer based on which type of ion-exchange chromatography should be performed after this step. Use common cellulose tubing with a typical molecular weight cut-off of 14 kDa (**Table of Materials**).

3.1.12. After overnight dialysis, optionally concentrate the protein using ultra-centrifugation filter units. Perform SDS-PAGE analysis (12.5% running gel, 4% stacking gel) to check for potential loss of protein, insufficient elution, and protein purity in general. If all is fine, proceed to section 5.

4. Purification of untagged FAHD proteins via hydrophobic interaction chromatography (HIC)

NOTE: Phenyl-groups on the coating surface of a silica gel in a HIC column for FPLC (**Figure 3B**) enable the separation of proteins according to hydrophobic character. The described steps should be performed with an FPLC system equipped with a 5 mL of HIC-phenyl column. Columns may be washed with 1 M NaOH to be reused for different proteins. However, columns once used for one type of FAHD protein should be reused for only this type of protein.

4.1. Ammonium sulfate (AS) precipitation

4.1.1. Proceed from step 2.5. The protein is in ice-cold HIC running buffer (**Table of Materials**).

4.1.2. Assess the volume of the prepared protein solution precisely to the microliter ($V_{initial}$). Slowly and drop-wise add pre-cooled HIC running buffer AS solution, until a 35 volume-% AS saturation is reached: V_{AS} added = $V_{initial}$ * 0.538. Gently stir the solution for 30 min. Centrifuge for 15 min at high speed (≥30,000 x *g*) at 4 °C.

4.1.3. Filter the supernatant using a 0.22 µm filter unit on ice. Optionally, take a sample for SDS-PAGE analysis: dilute 1:4 and heat immediately at 95 °C for 5 min or else the sample will lump. The sample may be frozen at this point (-20 °C) in order to proceed another day.

4.2. FPLC using a HIC column

4.2.1. Setup the FPLC system and equilibrate a 5mL HIC-phenyl column with 5 column volumes (CV) of 20% EtOH (in H_2O) followed by 5 CV of H_2O.

4.2.2. Mix 260 mL of HIC running buffer (exact) with 140 mL of HIC running buffer AS (exact). This results in a 35 volume-% AS solution. Check the pH (7.0); this is buffer A. Buffer B is 250 mL of running buffer. Add 1 mM DTT to both buffers A and B, then keep them on ice.

4.2.3. Equilibrate the column with 8 mL of buffer A, 8 mL of buffer B, and 8 mL of buffer A in this sequence. Apply the sample prepared in protocol step 4.1. Wash with buffer A, until the baseline optical absorption at 280 nm reaches 1000–500 mAU.

4.2.4. Apply a mixture of buffers A and B, so that the concentration of AS is 33 % (w/v). Wash with 1 CV, resulting in a plateau in the chromatogram. Set up a gradient of buffer B (up to 100% buffer B over time): 1.5 mL of buffer B in 3.8 min (i.e., 5.7% buffer B with 1% B/mL slope). When the UV signal at 280 nm rises, start collecting the fraction and place it on ice immediately.

4.2.5. In the end, wash the column with buffer B. Take samples of all fractions for SDS-PAGE analysis. Freeze all samples using liquid nitrogen, and store them at -80 °C.

4.2.6. Perform SDS-PAGE (and western blot) analysis, to detect the FAHD protein in the collected fractions. Fractions that contain the protein are pooled and applied to further purification, as outlined in the following protocol steps. Wash the column with H_2O and 20% EtOH (in H_2O).

5. Purification of FAHD proteins via ion exchange chromatography

NOTE: Molecules with charged functional groups are bound to a silica particle column for FPLC (**Figure 3C**). This enables the differentiation of proteins according to their ionic character, such as surface charge. The described steps should be performed with an FPLC machine and associated know-how, respectively. The described method is the same for either cationic or anionic exchange chromatography, but the buffers to be used are slightly different.

5.1. Chose the cationic or anionic exchange chromatography system. This choice is empirical and may vary among FAHD proteins. Optimally, both methods can be used consecutively.

5.2. Setup the FPLC system and wash the column with 5 CV of 20% EtOH (in H_2O), followed by 5 CV of H_2O. Equilibrate the column with 1 CV of low-salt buffer, high-salt buffer, and again low-salt buffer in this sequence.

5.3. Apply the sample (dialyzed against the correct low salt buffer from step 3.1.11) onto the column. Collect the flow-through. Wash the column for 1 CV with low salt buffer.

5.4. Setup a gradient elution: 100% high-salt buffer in 30 min at a flow rate of 1 mL/min, or 60 min at a flow rate of 0.5 mL/min. This may be re-selected based on an already known FPLC chromatogram, in order to optimize the purification. Collect all peak fractions.

NOTE: High-salt conditions may vary among FAHD proteins, as outlined in the discussion section.

5.5. After the gradient has finished, run with high-salt buffer until no more peaks are detected over the range of 1 CV (collect the fractions).

5.6. Take samples of all collected fractions and perform SDS-PAGE analysis (12.5% running gel,

4% stacking gel). Freeze the individual samples in liquid nitrogen and store them at -80 °C.

5.7. After SDS-PAGE analysis is complete, pool the samples containing the FAHD protein and discard the others. Optionally, concentrate the protein using ultra-centrifugation filter units.

5.8. Apply 1 mL of 25% SDS in 0.5 M NaOH (or other detergents) to clean the column. Wash the column with H_2O and 20% EtOH (in H_2O).

5.9. Optionally, repeat section 5 with the alternate column (cationic or anionic exchange chromatography). The protein obtained from this method is sufficiently pure to perform basic activity assays or can be used in screening assays for crystallography. For advanced applications, proceed with section 6.

6. Purification of FAHD proteins via size-exclusion chromatography (SEC)

NOTE: Porous particles in a silica gel column for FPLC enable the differentiation of proteins according to molecular size, such as hydrodynamic radius (**Figure 3D**). The described steps are to be performed with an FPLC system, using SEC columns.

6.1. Choose an SEC column, dependent on the molecular weights of contaminations still present, as detected via SDS-PAGE and silver staining. The outlined method is suitable for both columns. Wash the column overnight with 400 mL of H_2O and equilibrate with SEC running buffer. It is recommended to write a program for the FPLC system to automate this step.

6.2. Add 1 mM DTT to 300 mL of SEC running buffer and put it on ice. This is the running buffer. Apply 60 mL of this buffer to the column.

6.3. Centrifuge the protein sample (13,000 x *g* for 10 min) to remove any micro-precipitation. Apply the supernatant to the column. It is generally recommended to filter the supernatant before FPLC.

6.4. Apply the running buffer to the column until all protein is eluted. Collect all peaks in fractions of suitable volume (e.g., 2 mL). Take samples for SDS-PAGE and freeze all fractions using liquid nitrogen. Store the frozen fractions at -80 °C.

6.5. After SDS-PAGE (and western blot) analysis, collect and pool all fractions containing the FAHD protein. Silver staining is recommended to detect minor contaminations that may still be present.

6.6. Use ultra-centrifugation filter units in order to concentrate the protein. Although not mandatory for FAHD proteins, in general a desalting step (e.g., by dialysis) is recommended for enzyme assays and crystallization.

6.7. Repeat steps 6.3–6.6 several times with different flow rates and salt concentrations (empirical) in order to enhance the purity of the FAHD protein. Wash the column overnight with

H_2O and 20% EtOH (in H_2O).

7. Basic FAHD activity assays with substrates oxaloacetate and acetylpyruvate

NOTE: FAHD protein 1 (FAHD1) displays oxaloacetate decarboxylase (ODx) and acylpyruvate hydrolase (ApH) activity. This is outlined in more detail in the discussion section. Because of destabilization by keto-enol tautomerization in aqueous solution (i.e., enolization), oxaloacetate decays by itself over time (auto-decarboxylation) as a function of cofactor concentration and pH. At around a pH of 7 and temperature of 25 °C, this effect is not dramatic, but assays must be blanked to account for both auto-decarboxylation and enzyme concentration. The pipetting scheme is outlined in **Figure 4A**. In general, it is recommended to use well calibrated pipettes for this assay, as it is quite sensitive to minor pipetting errors.

[place Figure 4 here]

7.1. Start up a microplate reader and equilibrate for 30 min at 25 °C. Setup a program for reading 12 wells (as outlined in **Figure 4A**) at 255 nm. It is recommended to use 25 multiple readouts with 5 ms time delay. Setup a cycle to measure 15x every 2 min (30 min total).

7.2. By default, prepare an enzyme assay buffer (see **Table of Materials**) with 1 mM $MgCl_2$ at pH 7.4. Variant FAHD proteins may require different cofactors or pH levels. Mg^{2+} and Mn^{2+} are known cofactors for FAHD1[3,11,12,21].

7.3. Create a 1 µg/µL protein solution, diluting with enzyme assay buffer (**Table of Materials**).

7.4. Set up 1 mL of 20 mM solution of a substrate to be tested (so far identified substrates of FAHD proteins are listed elsewhere[3]) in enzyme assay buffer.

7.5. According to the pipetting scheme displayed in **Figure 4A**, prepare the enzyme blank and sample wells: pipet 80 µL of enzyme assay buffer (**Table of Materials**) into the wells with 5 µL (5 µg) of enzyme solution.

7.6. According to the pipetting scheme displayed in **Figure 4A**, prepare the substrate blank and sample wells: pipet 85 µL of enzyme assay buffer into the wells.

7.7. Right before measuring, apply 5 µL of enzyme assay buffer into the six blank wells. Apply 5 µL of the 20 mM substrate solution to the sample wells. It is recommended to use a multichannel pipette.

7.8. Use a multichannel pipette at 50 µL settings to gently mix all wells. Start with the blanks and proceed with the sample wells. Take care not to create any bubbles. Insert the plate into a microplate reader and measure each well at 255 nm (as outlined in step 7.1).

7.9. Perform the analysis in a spreadsheet. Copy the raw data from the photometer into a spreadsheet, and write all settings (i.e., all documentation) into another sheet. Average the data of the three wells of each of the four preparations. Subtract the blank from the sample. Also compute standard deviations and sum the deviations of blank and sample.

7.10. Plot this data (y: optical density, x: time in min). An exponentially decreasing curve should be displayed. Dependent on the kind of substrate in use, an initial increase within the first 10 min may be observed, after which the signal decreases. This is ascribed to the keto-enol tautomerization of the substrate, as outlined in more detail in the discussion section.

7.11. Divide the optical signal data over time by the maximum value of the plot, in order to scale the data down into the range [0, 1] (an example is provided in **Figure 5A**). Identify the linear range of the curve, starting at the initial decrease, and compute the negative slope (1/min).

7.12. The time course of the decrease in OD is associated to the substrate via its initial concentration: 100 nmol/well * slope. Using the assessed protein concentration c_0, the specific activity is computed: 100 nmol/well * slope * $1/c_0$. Expressing c_0 in µg/well, the specific activity computed this way is expressed using the unit nmol/min/µg, which equals µmol/min/mg.

8. Assessing Michaelis-Menten kinetics of FAHD proteins

NOTE: Assessing Michaelis-Menten kinetics of FAHD proteins is tedious, as the specific protein activity is dependent on both the relative protein-substrate concentration and physical volume in which the reaction is taking place. Steady-state kinetics must be established in order to obtain reliable results. A tested protocol on a 96 well UV transparent plate is outlined in the following steps. Every step needs to be performed with great care, as minor errors usually spoil the experiment. It is recommended to master the assays outlined in section 7 before attempting the more complicated assay described below.

[place Figure 5 here]

8.1. Start a microplate reader and equilibrate for 30 min at 25 °C. Set up a program for reading 72 wells (as outlined in **Figure 4B**) at 255 nm. It is recommended to use 25 multiple readouts with a 5 ms time delay. Set up a cycle to measure 15x each 2 min (30 min total).

8.2. Perform steps 7.2 and 7.3. Then, set up 1 mL of 100 mM substrate solution in enzyme assay buffer.

8.3. Prepare dilutions of the substrate solution in enzyme assay buffer: 40 mM, 20 mM, 10 mM, 6 mM, 4 mM, 2 mM. The assay is performed with pairwise ("adjusted") enzyme/substrate concentrations. For this, prepare the following dilutions of the enzyme solution in enzyme assay buffer: 0.5 µg/µL, 0.4 µg/µL, 2.5 µg/µL, 2 µg/µL, 1.5 µg/µL, 1 µg/µL.

8.4. Into all wells depicted in **Figure 4B** apply 180 µL of enzyme assay buffer. Apply 10 µL of

enzyme assay buffer into all wells for the substrate (blank and sample). Apply 10 µL of the prepared protein dilution series into the wells for the enzyme (blank and sample). Apply 10 µL of enzyme assay buffer into all wells for wells for the substrate blank and the enzyme blank.

8.5. Right before measuring, apply 10 µL of the prepared substrate dilution series into the wells for the substrate sample and the enzyme sample.

8.6. Use a multichannel pipette at 50 µL settings to gently mix all wells, starting with the blanks, proceeding to the sample wells. Take care not to create any bubbles.

8.7. Insert the plate into a microplate reader and measure each well at 255 nm, as outlined in step 8.1. Perform the analysis in a spreadsheet. Copy the raw data from the photometer into a spreadsheet, write all settings (i.e., all documentation) into another sheet.

8.8. Perform individual data analysis per point in the dilution series as outlined in steps 7.11. to 7.14. Eventually, obtain all specific activities and plot against the initial substrate concentration: 2 mM, 1 mM, 0.5 mM, 0.3 mM, 0.2 mM, 0.1 mM.

8.9. Display all data points with individual standard deviations. Computer Michaelis-Menten kinetics *via* non-linear curve fitting, or via Lineweaver-Burk analysis. It may be required to re-measure individual points, and to adapt individual protein-concentration/substrate-concentration pair ratios in steps 8.5 and 8.6. The Michaelis-Menten diagram for human FAHD1 is provided in **Figure 5B**.

9. Crystallization of FAHD proteins

NOTE: Crystallization of FAHD proteins (human FAHD1 described previously[15]) may be achieved by the hanging drop vapor diffusion method in a 24 well format (**Figure 6A**). A step-by-step protocol on crystallization of human FAHD1 using this technique is presented below[15]. A more detailed description is provided in the discussion section.

[place Figure 6 here]

9.1. Ensure that the protein is dialyzed against SEC running buffer. The FAHD1 protein should be available at high concentrations (2–5 mg/mL). At lower concentrations, the protein may not crystallize due to lack of spontaneous nucleation.

9.2 Prepare ≥20 mL of the reservoir solution for crystallization. Make three stock solutions, using distilled or deionized water as a solvent: 1 M Na-HEPES (minimum 25 mL, adjusted to pH 7.5), 50% (w/v) polyethylene glycol 4000 (PEG4k) (minimum 65 mL), and 1 M $MgCl_2$ (10 mL).

9.3. Setup a grid of 4 x 6 (24 total) different 15 mL tubes. Label them according to corresponding positions on the plate (e.g., row (A, B, C, D) vs. column (1–6) like “A1”, “B5”, “D6”, etc.). Pipette 1 mL of 1 M Na-HEPES into each tube.

9.4. Pipette 1 mL of 50% (w/v) PEG4k into row A of the tubes, 2 mL into row B, 3 mL into row C, and 4 mL into row D. Pipette 100 µL of 1 M $MgCl_2$ into column 1 of the tubes, 250 µL into column 2, 500 µL into column 3, 1.0 mL into column 4, 1.5 mL into column 5, and 2.0 mL into column 6.

9.5. Fill all tubes up to a 10 mL volume with distilled or deionized water, where the scale on the tubes is sufficiently accurate.

9.6 Take the human FAHD1 protein sample (~5 mg/mL) from the fridge (or from ice) and spin down at maximum speed with a table top centrifuge at 4 °C for at least 10 min. If co-crystallization with oxalate is desired, add oxalate from a stock solution so that the protein sample contains a final oxalate concentration of 2 mM. Apply 1 mM DTT and store on ice.

9.7. In the meantime, unpack a 24 well crystallization plate, ideally inside a temperature-controlled room at 18 °C. Distribute a thin layer of paraffin oil onto the rim on top of every well of the 24 well plate with the help of a thin glass or plastic rod. Add 800 µL of the prepared crystallization cocktails (A1 to D6) into each corresponding well of the crystallization plate.

9.8. Place fresh 22 mm coverslips onto a clean surface. Avoid contaminating the cover slips with dirt or dust. If necessary, remove any debris from the cover slip using compressed air or a duster spray.

9.9. After centrifugation is complete, avoid shaking the protein sample so that the spun-down aggregates and debris at the bottom of the tube do not float up again. In the following steps, pipette from the protein sample just below the surface of the solution in order to avoid stirring up aggregates and deposits from the bottom.

9.10. For each well (see **Figure 6B**) pipette 1 µL of protein solution onto the center of a cover slip and add 1 µL of the respective reservoir cocktail to the protein droplet, avoiding bubbles. Turn the coverslip upside down and place it onto the top of the well so that the oil seals the well with the coverslip air-tight. Repeat until the 24 well plate is completed.

9.11. Store the plate at 18 °C and observe the drops on a progressive schedule with a proper microscope. Human FAHD1 crystals usually appear overnight (see **Figure 6C**).

REPRESENTATIVE RESULTS:

Starting with a prepared cloning vector and purchased BL21(DE3) *pLysS E. coli*, the plasmid is inserted into the bacteria via heat-shock or any appropriate alternative method (**Figure 1**). After a short period of amplification, the transformed bacteria are plated on LB agar plates, in order to grow overnight. Plates at this point may look different, depending on a variety of potential error sources. Plates may be empty (i.e., no colonies), completely overgrown by bacteria, or something in between, respectively. Two examples of LB agar plates after optimal and non-optimal transformation are depicted in **Figure 7A**. Too many bacterial colonies indicate either that too many bacteria were plated (likely) or that the antibiotics in use may be expired (unlikely). Too

few bacterial colonies may indicate that either not enough plasmid was used for the transformation (use more next time) or that too much antibiotics were used to select the bacteria. In any case, if colonies are present, they should be fine, as using two selective antibiotics implies a rather insignificant chance of untransformed bacteria to grow. No colonies at all, however, indicates that either the bacteria lost their transformation competence (because of wrong storage or storage over longer periods, repetitive freeze and thaw, etc.), the heat-shock was not successful (no plasmid uptake or bacterial death by too much heat), the cloning vector is corrupted, or by mistake a wrong set of selective antibiotics was used (verify the resistance gene on the plasmid vector).

[place Figure 7 here]

Validated colonies are selected and picked. After amplification in nourishing medium, protein expression is triggered by application of the chemical IPTG. The bacterial pellet containing the expressed protein in milligram quantities is harvested, and expression is verified via SDS-PAGE (see for example **Figure 7B**). Some problems may occur during this otherwise simple process. First, some proteins form inclusion bodies, because they apparently somehow interfere with the natural metabolism of the host bacteria. This was observed for some point mutations of human FAHD1 and FAHD2. In such cases, other expression systems like insect cells may be more appropriate and should be considered. After harvesting a pellet from insect cells, for example, purification of the proteins follows the same steps as described in this protocol. Second, the DE3-*pET* system is sometimes found to be "leaky" (i.e., protein is already expressed to some extent before IPTG induction). The potential reason for this is not well-understood, but it may help to express the protein slowly overnight in a cold room incubator. Third, no protein is expressed. This is probably the worst-case scenario, as it likely indicates a corrupted plasmid vector and thus advisable to sequence the plasmid.

If a *His*-tag was used to tag the protein, affinity chromatography with Ni-NTA agarose is an easy and cheap capture method eliminating the majority of contaminations (**Figure 7C**). Similar methods exist for other tag systems (e.g., *STREP-II*). If no tag was used, a combination of ammonium sulfate precipitation and consecutive hydrophobic exchange chromatography may also separate the protein from the majority of other proteins (**Figure 8A**). However, comparing the two methods (**Figure 7C** vs. **Figure 8A**), the superiority of the Ni-NTA methods can be demonstrated by SDS-PAGE analysis. Using *His*-tagged protein is therefore advised.

[place Figure 8 here]

Consecutively, the protein is further separated from leftover contaminations by cation/anion exchange chromatography (for example, see **Figure 8B**), followed by size-exclusion chromatography (for example, see **Figure 8C**). It is advised to set up an initial purification strategy in this order; however, these columns should be used in combination, subsequently and in variation, until the protein is sufficiently pure.

Simple activity assays, in order to test for "yes or no" decisions on active substrates and/or cofactors, may be performed with *His*-tagged proteins after Ni-NTA purification, or untagged proteins after the ionic exchange column. Specific activities and kinetic constants must be determined with protein of highest purity. Crystallization may be attempted with proteins after the ionic exchange column, but the quality of crystals almost always correlates with protein purity. Polyclonal antibodies may be raised against proteins at any stage of the purification protocol; however, here the quality also correlates with the protein purity.

FIGURE AND TABLE LEGENDS:

Figure 1: Amplification of competent *E. coli* and induction of protein expression. (A) Insertion of the *pET* vector into competent BL21(DE3) *pLysS E. coli* bacteria, described in section 1. **(B)** Heat shock protocol and plating of the *pET* transformed *E. coli* bacteria, described in step 1 of the protocol. Transformed bacteria are plated on LB agar plates with antibiotics for selection. **(C)** Amplification of *pET* transformed *E. coli* bacteria, described in section 1. Colonies are picked from an LB agar plate and amplified in nourishing medium (LB or NZCYM) until the bacterial density reached the empirical threshold of 0.4. **(D)** Induction of protein expression via the DE3-IPTG-*pET* system, described in section 1 and sketched in **Figure 2**. Protein production is started by the application of the chemical IPTG. At the end of section 1, the bacterial pellet containing the protein is harvested.

Figure 2: The DE3 cassette/pET vector dual system explained. (A) The sketched genome of *pET* vector transformed BL21(DE3) *pLysS E. coli* bacteria. The native bacterial genome carries a DE3 cassette (see panel B), as well as a lac gene that constantly expresses lac repressor units. The non-native *pET* vector carries the protein gene inserted between a *T7* polymerase promoter and terminator sequence. More details in panel B. **(B)** The DE3 cassette of the native bacterial genome encodes the information for *T7* polymerase in terms of an *E. coli* RNA polymerase operon. This protein, however, is not expressed because the lac repressor unit prevents the RNA polymerase protein from binding. Hence no T7 polymerase is expressed and no exogenous protein is expressed**. (C)** Application of the chemical IPTG (**Table of Materials**) distorts the structure of lac repressor units and prevents them from binding to the DE3 cassette. As a result, RNA polymerase can now bind to the cassette, for which T7 polymerase is expressed, as is exogenous protein eventually.

Figure 3: Sketched illustrations of common types of chromatography. (A) The resin of a Ni-NTA column. NTA holds bivalent nickel ions that are used in terms of immobilized metal ion affinity chromatography (IMAC). Poly-histidine tags bind preferably to this motif and may be eluted by imidazole. **(B)** The typical coating of silica particles in a phenyl-based hydrophobic interaction chromatography (HIC-phenyl). Hydrophobic proteins interact with the coating material and are delayed in their migration while others are not. **(C)** The typical coating of silica particles in ionic interaction chromatography. Polarized and charged proteins interact with the coating material and are delayed in their migration while others are not. **(D)** The resin of a silica gel in size-exclusion chromatography (SEC). Based on defined pores in the silica material, proteins may be separated by their size (in a first approximation corresponding to their molecular mass). Small

proteins permeate the porous column material and are retarded, while large proteins migrate faster around the porous particles.

Figure 4: Sketched pipetting scheme for enzyme assays. (A) A sketched pipetting scheme for basic substrate-based FAHD protein enzyme assays. Substrate blank: -S/-E; substrate sample: +S/-E; enzyme blank: -S/+E; enzyme sample: +S/+E (S: substrate, E: enzyme). See protocol step 7 for more details. **(B)** A sketched pipetting scheme for assessing Michaelis-Menten kinetics of FAHD protein. Substrate blank: -S/-E; substrate sample: +S/-E; enzyme blank: -S/+E; enzyme sample: +S/+E (S: substrate, E: enzyme). See section 8 of the protocol for more details.

Figure 5: Exemplary results of enzyme assays. (A) An exemplary UV absorption curve obtained for basic substrate-based FAHD protein enzyme assays (normalized into the range of 0 to 1) with standard deviation. The optical density (OD) ratio [OD(t)/OD(0)] at any given time t [OD(t)] is normalized to the initial OD [t = 0; OD(0)]. See section 7 of the protocol for more details. **(B)** Exemplary Michaelis-Menten kinetics of the human FAHD1 protein with standard deviation. See section 8 of the protocol for more details.

Figure 6: Crystallization of FAHD proteins. (A) Crystallization plates in standard 24 well or 96 well SBS footprint. See section 9 for more details. **(B)** The basic plate setup process in crystallization of FAHD proteins. This figure is redrawn with permission[23]. See section 9 for more details. **(C)** Human FAHD1 crystals and corresponding diffraction patterns (small inserts). The closest lattice spacing is indicated in the inserts as a measure for diffraction quality of the crystals. Lower numbers indicate higher resolution and thus more informative data. See section 9 of the protocol for more details.

Figure 7: Representative results for bacteria transformation and IMAC. (A) Representative LB agar plates with transformed BL21(DE3) *E. coli*, obtained by following protocol step 1.1. Left: A plate with well distributed colonies (positive example). Right: A plate with only one single colony (negative example). White circles mark good colonies. The red circle marks colonies that are growing too close to each other and should not be picked as long as isolated colonies are available. **(B)** A 12.5% acrylamide SDS-PAGE analysis of a series of induction controls ("-" indicates before IPTG induction; "+" indicates after IPTG induction, before pellet harvest), adjusted to equal amounts of total protein. This is described in step 1.2. **(C)** An exemplary 12.5% acrylamide SDS-PAGE analysis of Ni-NTA purification of *His*-tagged FAHD1 protein. This is described in section 3 of the protocol. The affinity chromatography yields protein of high purity (>70%, black arrow), however, several small contaminations are also observed (red arrows). These contaminations consist of non-FAHD proteins that bind to the column, and from proteins that bind to the FAHD protein.

Figure 8: Representative results for FPLC experiments (HIC, **ion exchange**, **SEC). (A)** A typical chromatogram and 12.5% acrylamide SDS-PAGE analysis of HIC-phenyl chromatography after ammonium sulfate (AS) precipitation of untagged FAHD1 protein, as described in section 4 of the protocol. The green line reflects the gradient of buffer B that does not contain AS. During the process AS is gradually washed out from the system. Comparing this panel to **Figure 7C** displays

the power of Ni-NTA affinity chromatography compared to the HIC-phenyl method, and the advantage of using a *His*-tag system for protein purification. **(B)** An exemplary chromatogram and 12.5% acrylamide SDS-PAGE analysis of cationic exchange chromatography of *His*-tagged FAHD following Ni-NTA purification. Using a salt gradient, the applied sample is separated into individual proteins. **(C)** An exemplary chromatogram and 12.5% acrylamide SDS-PAGE analysis of G75 size exclusion chromatography of *His*-tagged FAHD following cationic exchange chromatography.

Figure 9: Details on the proposed catalytic mechanism of human FAHD1. (A) Oxaloacetate exists in crystalline state as well as in neutral solution mainly in the *Z*-enol form[24]. However, under physiological pH-conditions the 2-keto form is the predominant representation[25]. **(B)** Chemical sketch of the hFAHD1 cavity[15] with Mg-bound oxaloacetate (left) and acylpyruvate (right, with R^1 as organic rest; the red arrow denotes a nucleophilic attack of the adjacent stabilized water molecule) (see discussion). **(C)** Comparison of favored conformations for C^3-C^4 cleavage in decarboxylase (b to c) and hydrolase (b' to c) mechanism of FAHD1: both processes result in Mg-complexed pyruvate-enolate (see discussion). Intermediates b and b' are expected to be stabilized by Q109, as sketched in panel B (see discussion).

DISCUSSION:

Critical steps

FAHD proteins are very sensitive to salt concentrations. At low NaCl concentrations, the proteins may precipitate upon thawing, but they can usually be fully reconstituted at higher salt concentrations. That is, if a FAHD protein precipitates for some reason, it may be recovered or refolded with higher salt concentrations (>300 µM). Some more hydrophobic proteins, however, may not be recovered (for example, human FAHD2), but detergents such as CHAPS (maximum 1%) or glycerol (10%) may be used to keep them in stable solution. In any case, shock-freezing using liquid nitrogen and storage at -80 °C is recommended, as it is a gentle and slow process of thawing.

Some unexpected problems may occur during Ni-NTA purification in step 3.1.10. Of note, a higher OD in the second collected sample than in the first sample indicates a too high volume of the agarose resin (take a note and use less resin in the next experiment). Also, the agarose resin itself leads to an OD signal at 280 nm (i.e., disruption of the agarose resin bed will give artificial signals). In case of doubt, it is advised to use other methods like a Bradford or BSA assay to determine protein concentrations.

In enzymatic assays, there are three critical aspects to be considered. First, assessing the protein concentration is critical to obtain the correct specific activities. The level of purity of the protein is influencing the result and needs to be estimated. In case of tagged protein, the mass of the tag-part has to be computed, and the specific activity has to be correspondingly corrected. For simple assays described in section 7 of the protocol, Ni-NTA purity is sufficient to distinguish between active and inactive substrates, cofactors, etc. In the case of more complex Michaelis-Menten kinetics, all reactant and substrate concentrations must be correctly determined.

Especially when using oxaloacetate (which auto-decarboxylates over time) the enzymatic part of the reaction must be corrected for auto-decarboxylation (under the assumption that both reactions occur simultaneously). Initial changes in the optical density signal addressed to keto-enol tautomerization of the substrate must be considered. Third, concentrations and volumes must be adjusted. A reaction with defined concentrations of enzyme and substrate may give different results dependent on the assay volume. If there is too much enzyme per well, adhesion of the liquid may in fact bias the result.

For assessing Michaelis-Menten kinetics it is recommended to perform initial experiments in 100 µL, 200 µL, and 300 µL batches in order to find the optimal combination. Similar aspects apply to the ratio of enzyme-substrate concentrations for kinetic assays. Too much enzyme per substrate or too much substrate per enzyme put the system outside the linear steady-state Michaelis range. Initial experiments are required to optimize these conditions. Exemplary adjustment for human FAHD1 (wild-type) protein are provided in section 8, resulting in kinetic diagrams (as presented in **Figure 5B**, for example).

For crystallization a droplet of protein solution is pipetted in the center of a coverslip and mixed with a droplet of crystallization cocktail, which is usually composed of a buffer (e.g., Tris-HCl, HEPES) and a precipitant (e.g., polyethylene glycol, ammonium sulfate). A droplet of inhibitor solution for co-crystallization (such as oxalate in this protocol) may optionally be applied. The coverslip is then placed upside down above a well of reservoir containing crystallization cocktail, sealing the well air tight with the help of sealant oil (**Figure 6B**). Ideally, no precipitation occurs within the drop at the beginning of the experiment meaning the protein remains in solution. Since precipitant concentration in the reservoir is higher than in the drop, the drop starts to lose water by evaporation into the atmosphere of the well until equilibrium with the reservoir is reached. The diffusion of water into the reservoir causes a slow volume decrease of the drop which in turn causes an increase of both, protein and precipitant concentration in the drop. If the protein solution reaches the required state of super-saturation and thus meta-stability, spontaneous nucleation followed by crystal growth can occur. Reaching the supersaturated state is a necessary but not sufficient condition for crystallization. Crystallization of proteins needs both, favorable thermodynamic and kinetic conditions, and heavily depends on the unpredictable properties of the protein to be crystallized[22].

Modifications and troubleshooting

Expression of protein in *E. coli* may be inefficient. Varying IPTG concentrations, expression temperature, and amplification time, such as room temperature for several hours or in cold room overnight, may need to be tested for each new protein to find optimal conditions. Precipitation of protein in inclusion bodies is sometimes observed for more hydrophobic FAHD proteins. In such cases, protein expression in other model systems such as insect cells is recommended, as inclusion bodies are less likely to form[26].

As FAHD proteins are sensitive to salt and cofactor concentrations, as well as pH, purification strategies for different homologues, orthologues, and point mutation variants may differ in individual settings. The purification methods described are developed for the wild-type human

and mouse FAHD1 protein. Concentrations of chemicals, such as NaCl and imidazole, as well as pH, may have to be adapted for individual proteins with a different isoelectric point (pI). Also of note, not every *His*-tagged protein may bind well to a Ni-NTA resin. If protein binding to the Ni-NTA column is inefficient, adapted concentrations of NaCl and imidazole, as well as varying pH conditions in the Ni-NTA running buffer may help to improve the quality of the outcome. If not, skipping the Ni-NTA step and proceeding to the step of ionic exchange chromatography may also lead to a successful purification strategy. If a protein binds to the Ni-NTA column but cannot be eluted from the column, addition of some mM EDTA may help disrupt the Ni^{2+} complex.

Concerning the process of crystallization, it needs to be understood that self-organization of large and complex protein molecules into a regular periodic lattice is an inherently unlikely process that depends heavily on difficult to control kinetic parameters. Even small changes in the set-up used for crystallization can dramatically alter the result and no crystals will form. Protein purity is generally of paramount importance. As a rule of thumb, a heavily overloaded SDS-PAGE gel should not show other bands. Also, the sequence in which steps are performed may affect the outcome. As an example, to ensure reproducibility, it is often necessary to keep the pipetting sequence the same, then first add the protein, and finally add precipitant to the crystallization droplet (or vice versa). Whichever method used, it should be kept the same when trying to reproduce or scale-up experiments. If no crystals are observed following this protocol, the chemical precipitant composition, pH, drop size, and protein-to-precipitate ratio can be varied in small increments. Patience and consistent observations of the drops are of virtue.

Remarks to catalytic mechanisms of FAHD1

The presented methods have been developed specifically to obtain FAHD1 proteins of high-quality. This enabled growth of FAHD1 crystals as well as engineering of crystals containing FAHD1 complexed to an inhibitor (oxalate, PDB:6FOG). The X-ray structures provide a 3D architecture of the enzyme's catalytic cavity. These results establish a comprehensive description of residues potentially important for the catalytic mechanisms of this intriguing enzyme. FAHD1 was first described to be able to cleave acylpyruvates (acetylpyruvate, fumarylpyruvate)[11]. Later on, it was found that FAHD1 operates also as a decarboxylase of oxaloacetate[12]. Although the substrates acylpyruvate and oxaloacetate are different chemical moieties, the chemical transformations share mechanistically the strategic cleavage of a common single C^3-C^4 bond, energetically facilitated if the C^3-C^4 bond orbitals stay orthogonal to the π-orbitals of the C^2-carbonyl[15]. Such a conformation allows resonance stabilization of the C^3-carbanion transiently formed during the cleavage process. The FAHD1 substrates (oxaloacetate and acylpyruvates) are flexible molecules and may exist in tautomeric (keto-enol) as well as C^2-hydrated forms (**Figure 9A**). The equilibria between the different species are determined mainly by the nature of buffer composition used, pH and presence of metal ions. In the following we discuss hypothetic mechanistic scenarios inspired from analysis of X-ray crystal structures which disclosed the catalytic center of FAHD1.

[place Figure 9 here]

The decarboxylase activity of FAHD1

Oxaloacetate exists in crystalline state as well as in neutral solution mainly in the *Z*-enol form[24]. But it was shown that under physiological pH-conditions (buffer conditions at pH 7.4) the 2-keto form is the predominant representation of oxaloacetate[25] (**Figure 9A**), and that enolization is not a prerequisite for decarboxylation[27]. Of note, Mg^{2+} ions have no influence on the ratio of the oxaloacetate species at a pH of 7.4 or below[28]. Transposition of the oxaloacetate keto form into the catalytic center of FAHD1 (guided by the bound oxalate in the complexed enzyme (PDB: 6FOG[15])) revealed residue Q109 as a conformational regulator of the bound oxaloacetate[15]. As outlined in another article[15], hydrogen bonding to the carbamoyl group of Q109 stabilizes an oxaloacetate-conformation resulting from rotation around the C^2-C^3 bond (**Figure 9B**, left panel). As a consequence of this rotation, the C^3-C^4 bond (to be cleaved) adopts a close to orthogonal disposition relative to the π-orbitals of the C^2-carbonyl (**Figure 9C**). Carbon dioxide can be released. The primary product of this process would be resonance stabilized Mg-enolate of pyruvate. It is known from investigations of oxaloacetate-Mg complexes that the enolate forms the most stable complex[28,29]. Assuming a comparable stability for a Mg-pyruvate enolate-complex the cofactor of FAHD1 could be blocked, but lysine residue K123 can protonate the pyruvate-enolate in an equilibrium to prohibit loss of the cofactor[15].

The given interpretation suggests pyruvate enol as a distinct intermediate in the catalytic ODx function of FAHD1. At this step in the hypothesized model, experimental data does not provide any further indication as to why the closed lid should open to release the product. It may be deduced, however, that the proposed mechanism looks like an enzyme inhibition by the product: The crystal structure reveals a conserved water molecule held in directional orientation towards the FAHD1 catalytic center by residues H30 and E33 presented in a short helix[15], which is induced upon ligand binding and lid closure. If the primary enol would stay in an equilibrium with the enolate, the resonance stabilized enolate could be quenched to pyruvate by the water molecule. The resulting hydroxyl would be capable to displace the pyruvate from the Mg-cofactor upon which the lid would open. Finally, the catalytic center would be restored in the mitochondrial environment. In this hypothetic scenario, the cavity water molecule would operate as an acid, respectively.

Hydrolase activity of FAHD1

Hydrolase activity of an enzyme implicitly requires the intermediate formation of a hydroxyl nucleophile. This mechanism is usually found in combination with acid-base catalytic activity. The transitional state of the reaction has to be prepared via conformational control by critical amino acid side chains in the cavity. In analogy to the discussion of the decarboxylase function, enzyme-bound acylpyruvate in 2-keto form will be put under conformational control by hydrogen-bonding of the 4-carbonyl oxygen to Q109 (**Figure 9B**, right panel). The crystal structure of oxalate-bound FAHD1 (PDB:6FOG) reveals a conserved water molecule held in directional orientation towards the FAHD1 catalytic center by residues H30 and E33 presented in a short helix[15]. The E33-H30 dyad is competent to deprotonate the directional positioned water and the resulting hydroxyl is in ideal disposition to attack the 4-carbonyl of acylpyruvate presented under conformational control by Q109[15].

Of note, a similar mechanism has been proposed for FAH[18]. Attack by the hydroxyl nucleophile is

expected to result in an oxyanion species, that is stabilized upon orbital controlled C^3-C^4 bond cleavage (**Figure 9C**). In this model, the C^3-C^4 bond rotation (**Figure 9C**) happens after the nucleophilic attack by the formed hydroxyl indicated in **Figure 9B** (i.e., it prepares the acylpyruvate for the bond cleavage). The primary products would be acetic acid and Mg-pyruvate enolate. In this hypothetic scenario, the acetic acid could quench the enol to pyruvate and subsequently assist displacement of the product. Above a pH of 7.5 and in the presence of Mg ions, acylpyruvates exist in an equilibrium between keto- and enol-forms, the latter in slight preference[30]. Most probably both forms are capable to bind to the cofactor of FAHD1 under subsequent lid closure. Processing of enolic acylpyruvate substrates by the enzyme is hampered due to the flat structure of the enol-form. The C^3-C^4 cleavage would result in a vinylic carbanion without resonance stabilization.

Therefore, we propose a catalytic ketonization step to prepare for attack of the hydroxyl nucleophile on the acyl carbonyl. This process of ketonization, however, would require control over proton transpositions by FAHD1 residues, which would attribute an inherent isomerase activity to FAHD1. It is reported that the acidity of Mg-bound enol hydrogen reveals a ten-thousand-fold increase compared to the un-complexed form[28]. A deprotonation of the Mg bound enol-form would be feasible by un-protonated K123. Deprotonation of K123 may be assisted by the carboxylate of D102. A hydrogen bond network formed by residues D102-K47-K123 could operate as the necessary proton relay in the catalytic center of FAHD1[15]. A such-formed intermediate enolate could then be quenched by a E33-H30-H_20 triad under ketonization of the substrate[15]. The 2-keto form would come under conformational control of Q109, and the concomitantly formed hydroxyl would attack the acyl carbonyl. The summarized discussion implies a control of FAHD1 about a water molecule for switching between acid and base through interplay of cavity-forming residues.

Future applications or directions of the method

Future applications of the methods described here are numerous. A plethora of prokaryotic members of the FAH superfamily still awaits functional characterization. Even the available information on the catalytic activities of known FAH superfamily members is scarce and, in most cases, based on theoretical assumptions rather than experimental data. Application of the methods described here for prokaryotic FAH superfamily members depends on the specific research interests in bacteriology. On the other hand, the recent demonstration that eukaryotic FAH superfamily members play essential roles in various cellular compartments (e.g., cytosol vs. mitochondria) highlights the need to better characterize these proteins (three of which have been identified so far), in particular because current data suggest that some uncharacterized proteins may carry out different functions in the context of mitochondrial biology, aging research, and cancer research. It is proposed that the full molecular and physiological characterization of these eukaryotic FAH superfamily members may provide important insight into major fields of contemporary research in the biomedical sector. More research on the mechanisms of FAHD1 (and related enzymes) are needed to better understand mechanisms underlying the bi-functionality of FAHD1, which is still not fully clarified. Additional studies with FAHD1 mutants, NMR-investigations, and structural studies on inhibitor complexes may help resolve the true mechanistic scenarios for which FAHD1 seems to be competent. Furthermore, computer-aided

design of enol mimics capable to bind to the Mg-cofactor will eventually lead to potent inhibitors of FAHD1.

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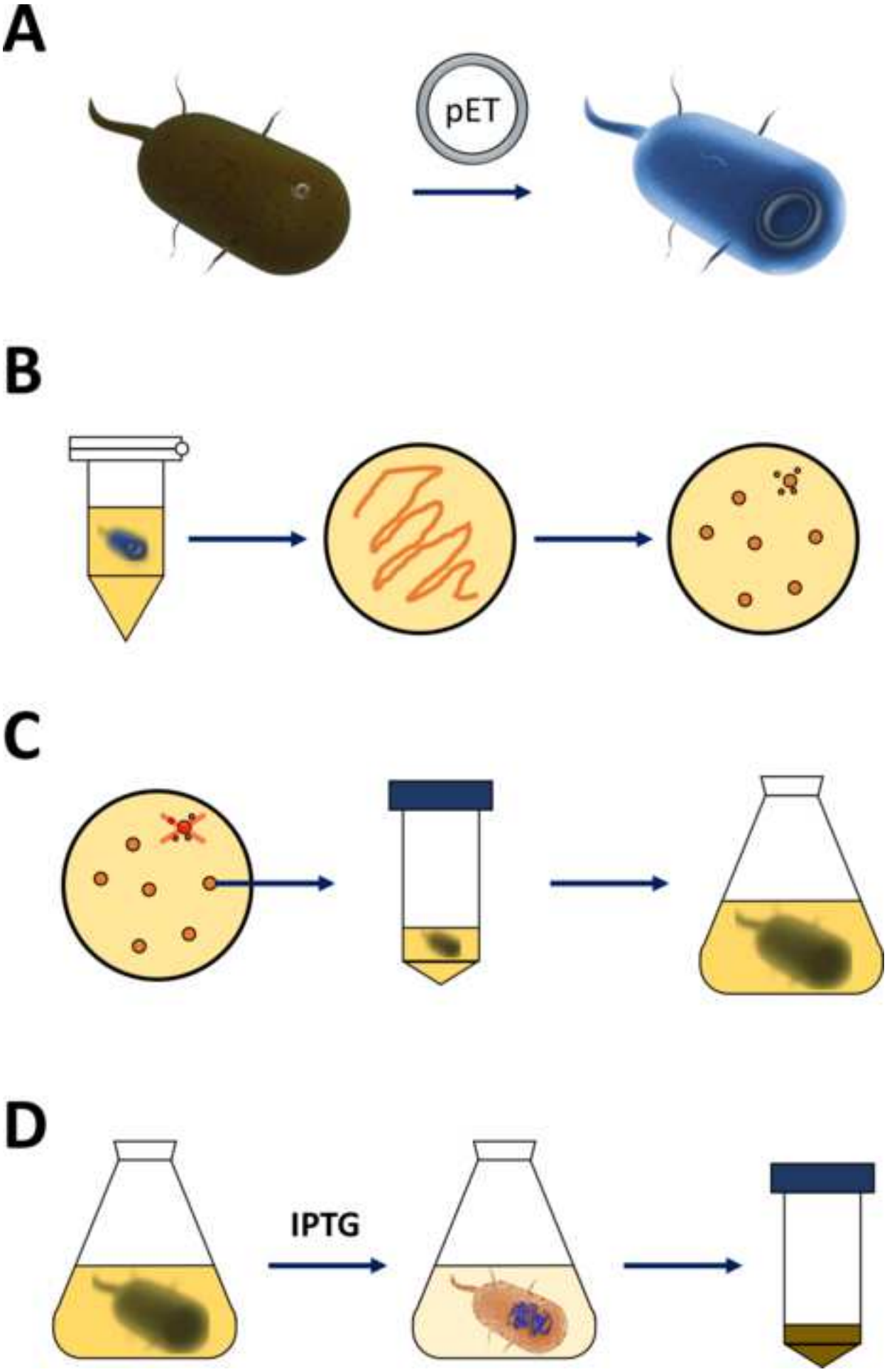

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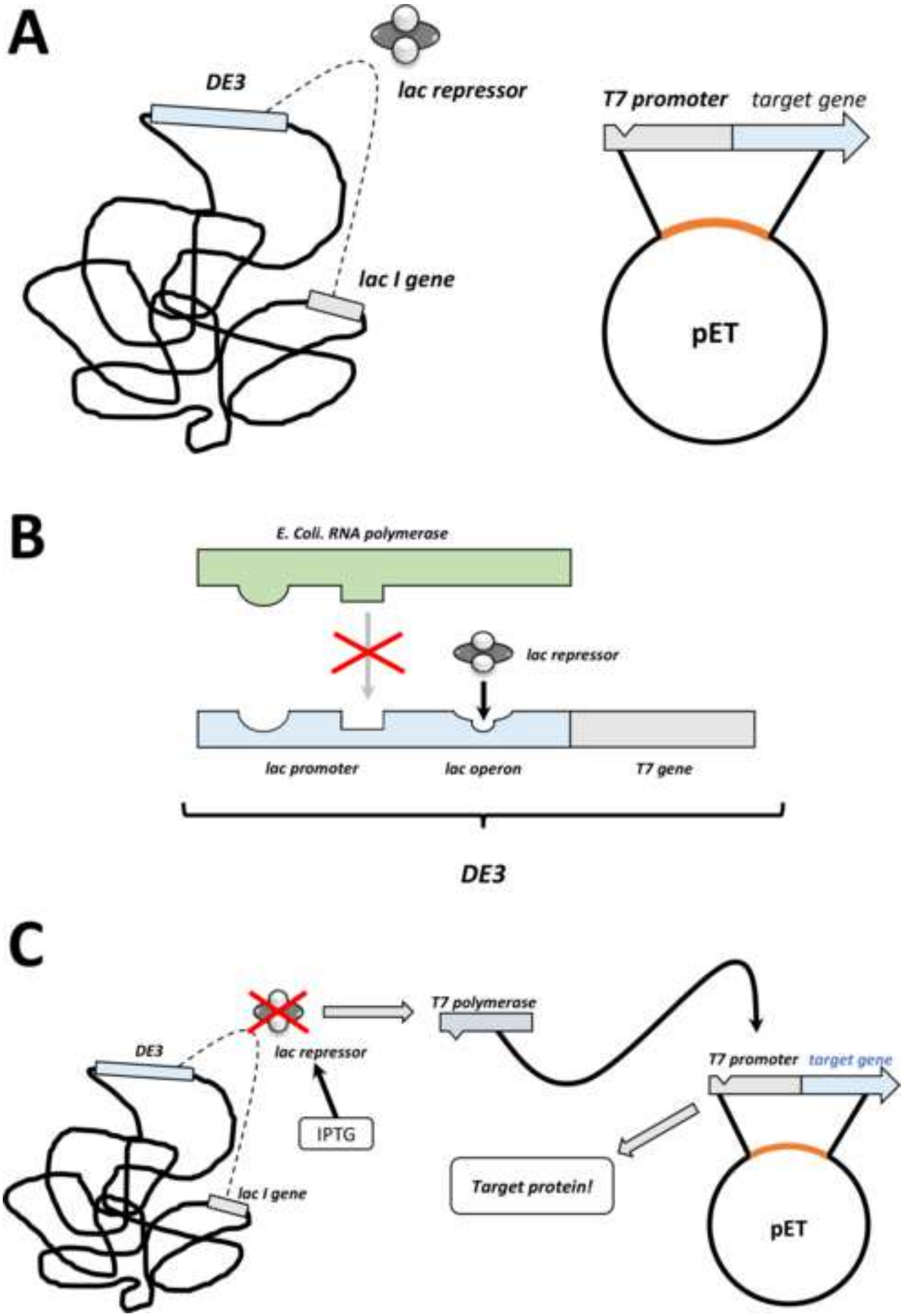

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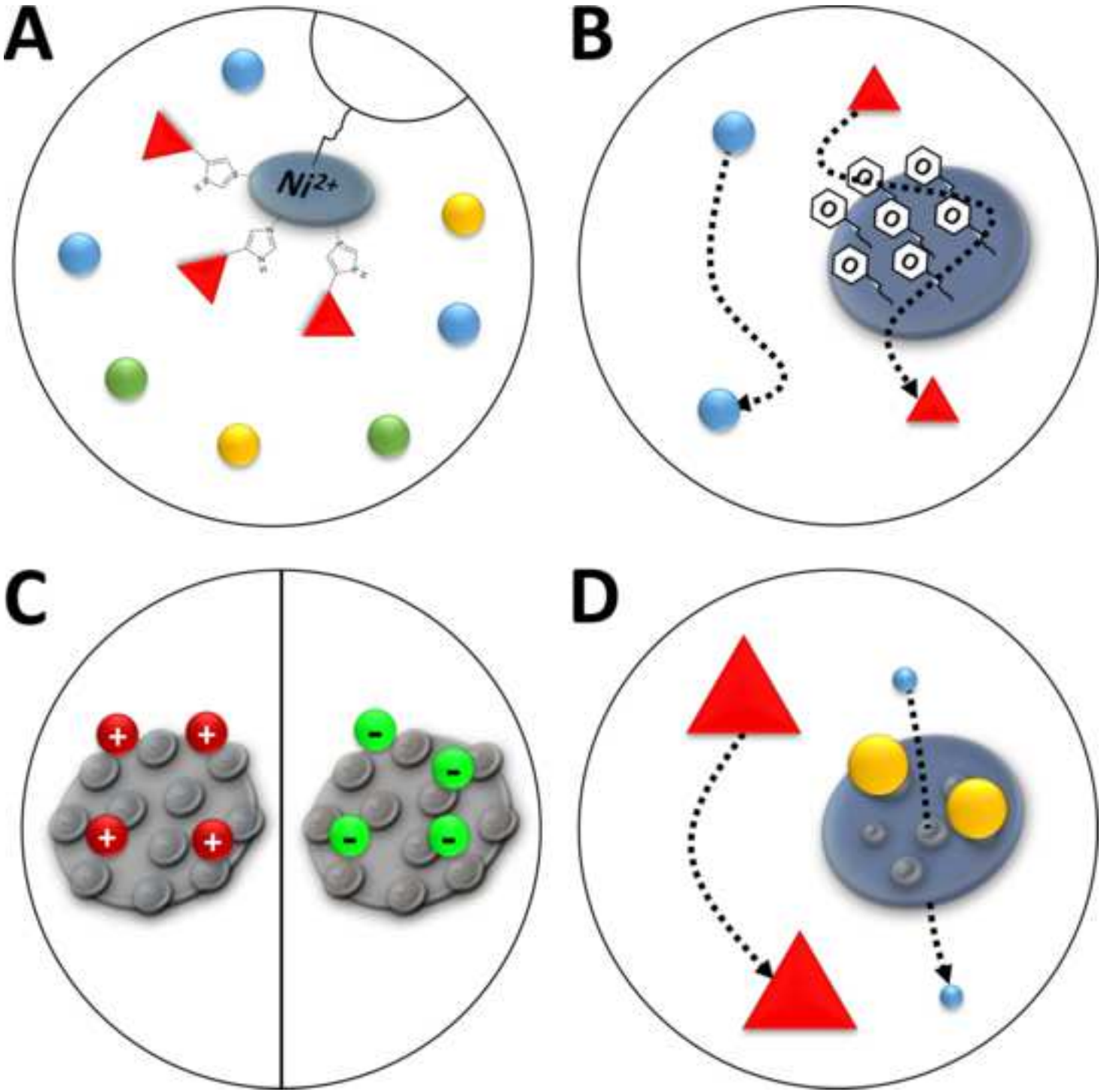

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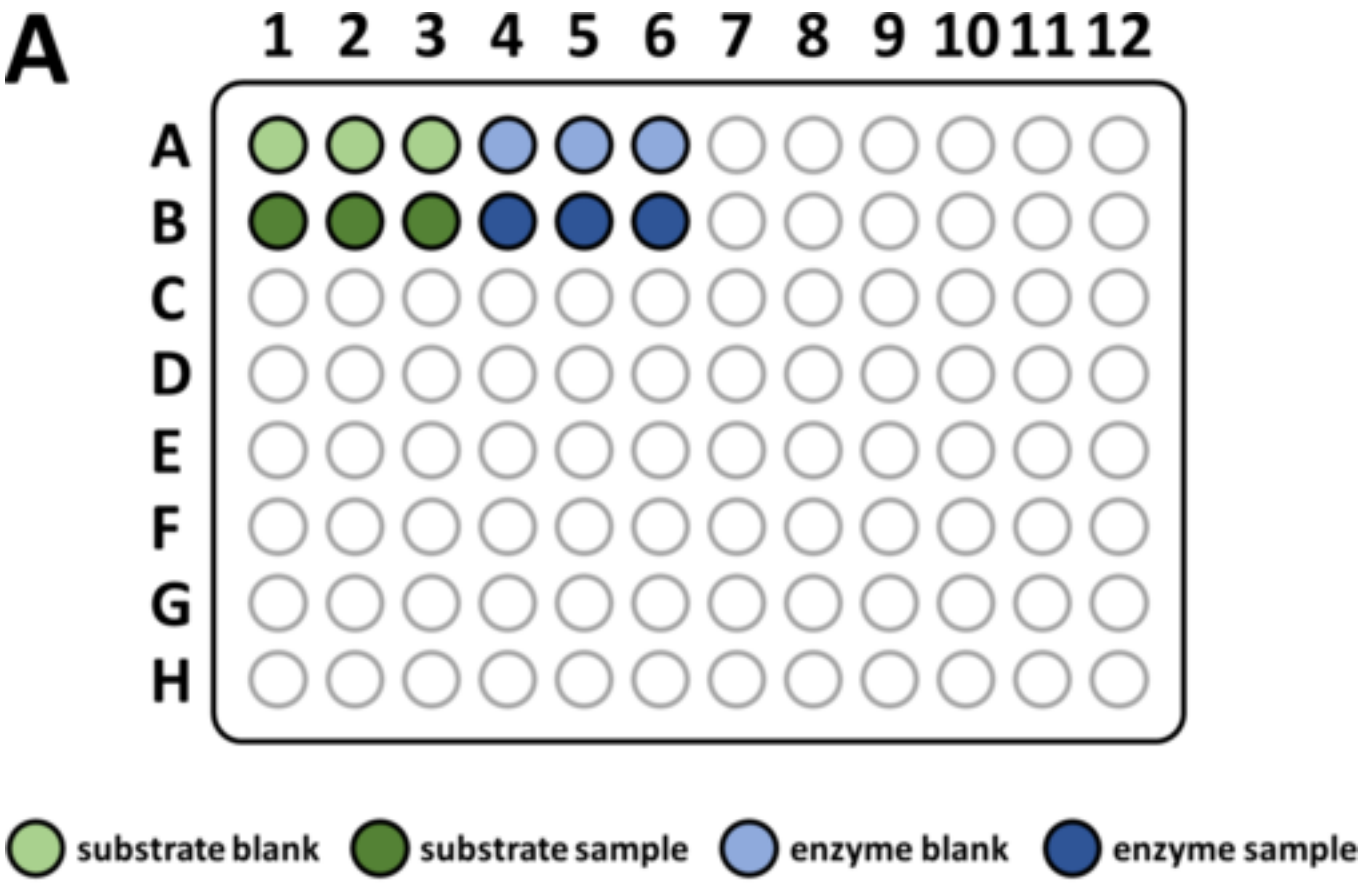

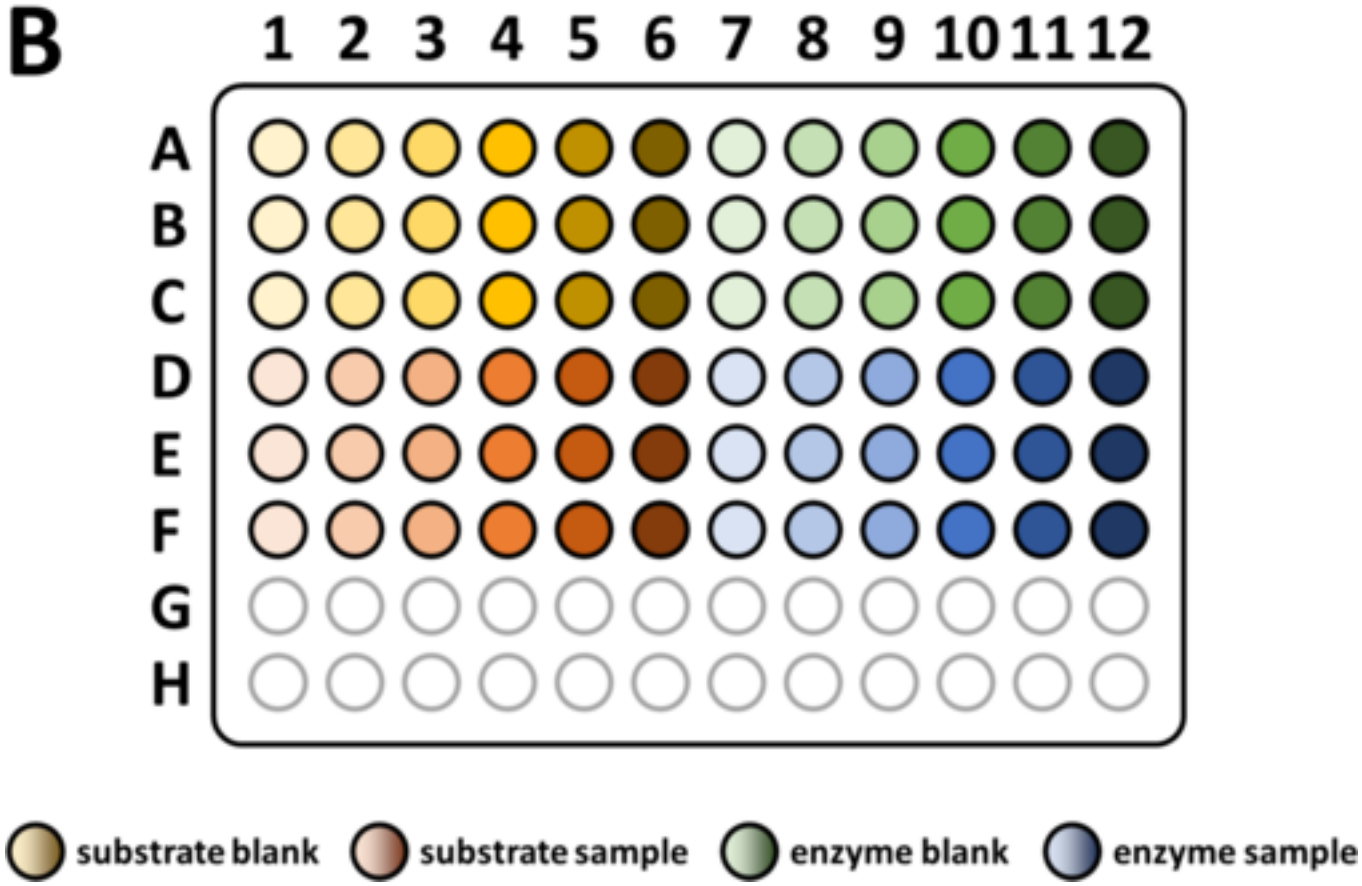

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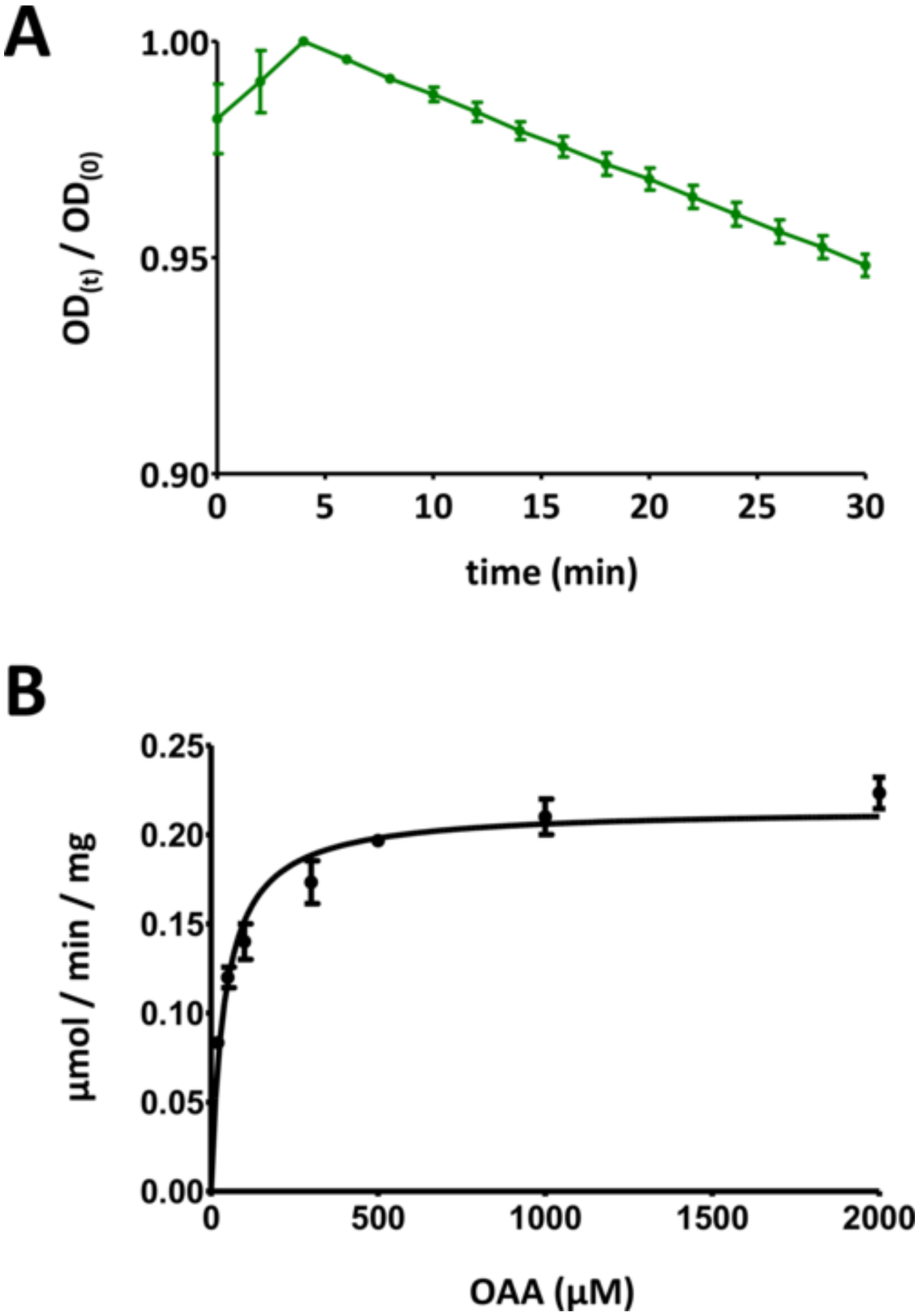

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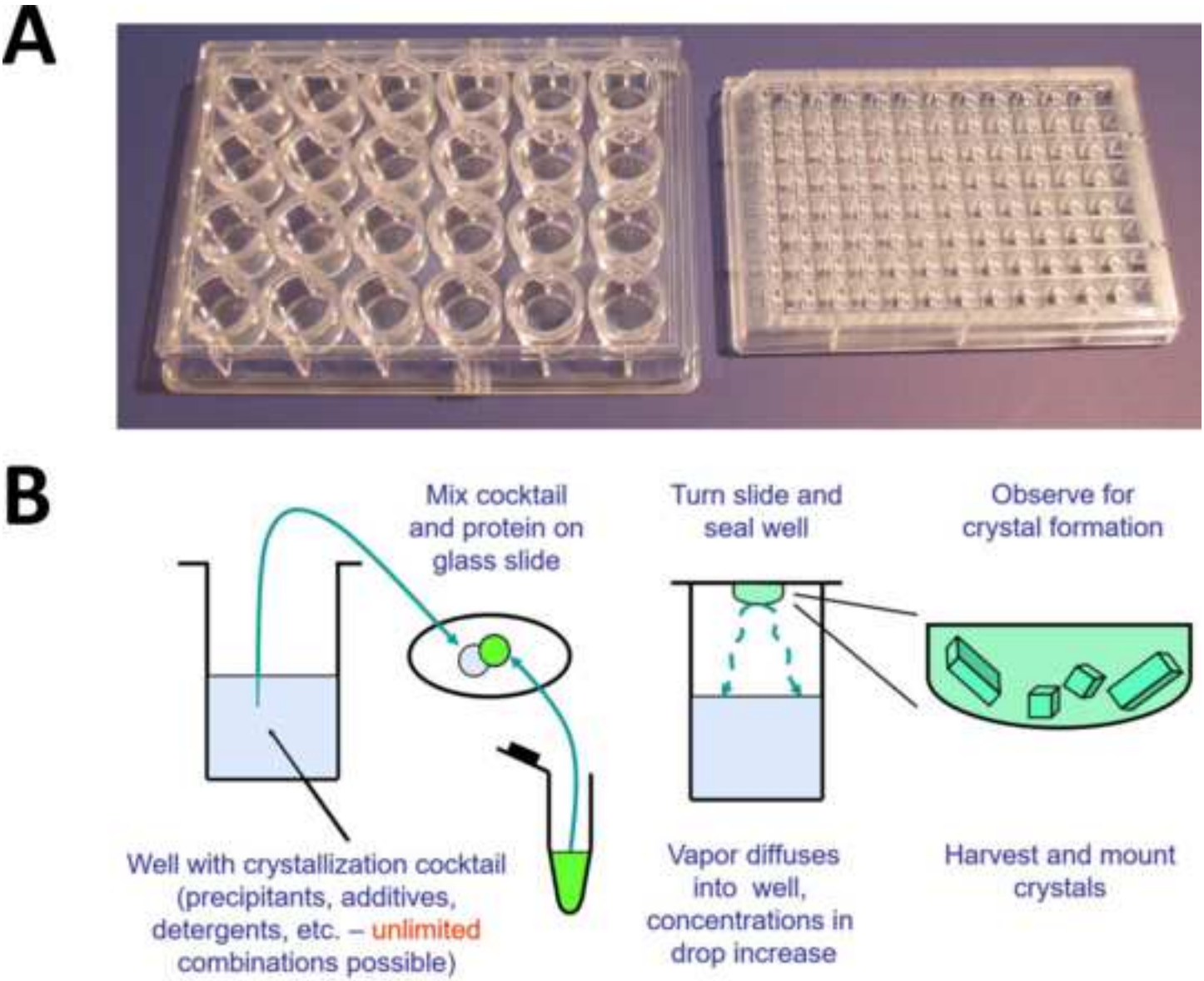

Figure 7 revised 300 dpi

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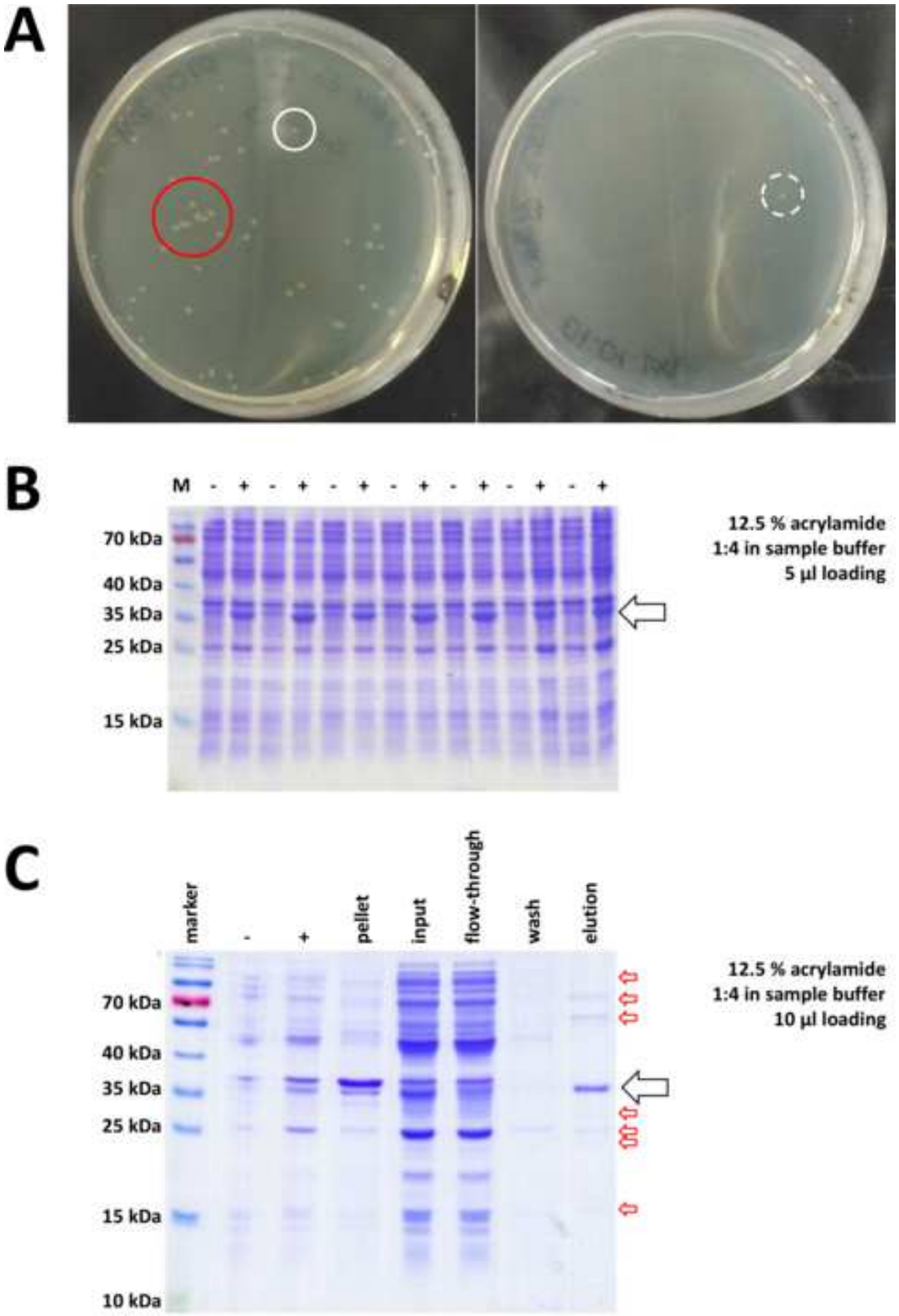

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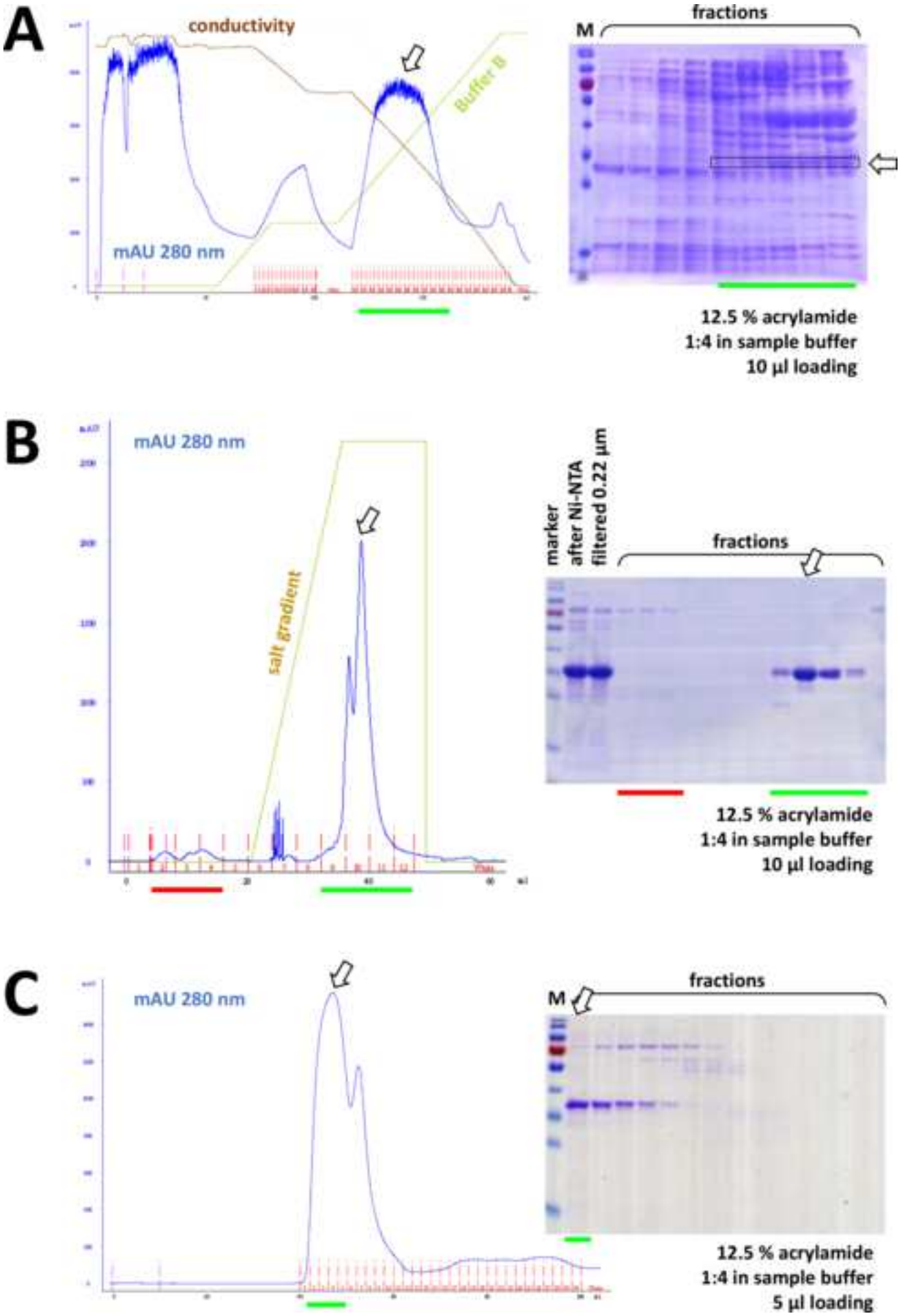

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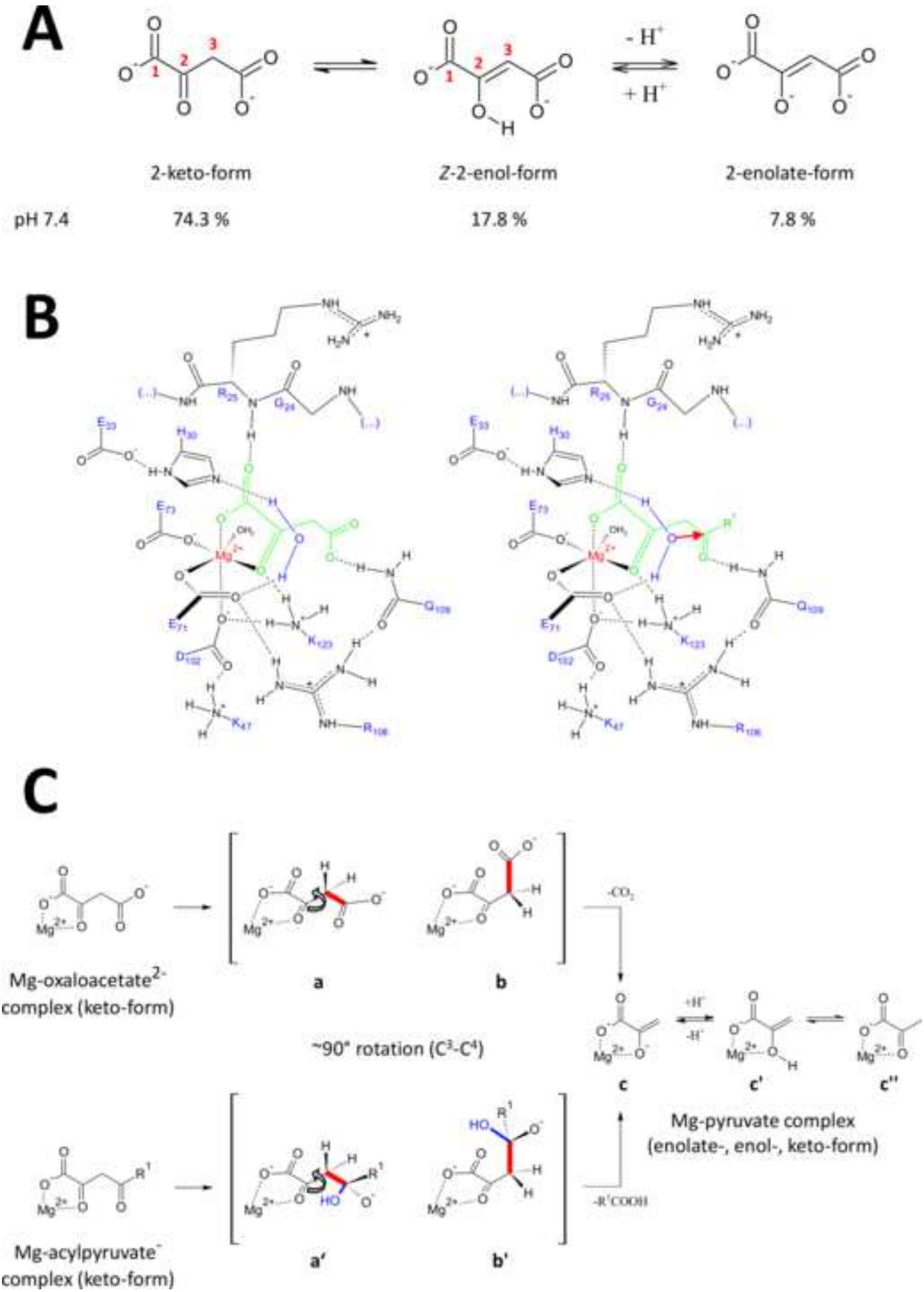

Table of Materials

Click here to access/download;Table of Materials;59729_R1-EDIT - 16032019 materials.xlsx

Name of Material/Equipment	Company	Catalog Number	Comments/Description
BL21(DE3) pLysS competent E. coli	Promega	L1195	High-efficiency protein expression from gene with T7 promoter and ribosome binding site
pET E. coli T7 Expression Vectors	MERCK	-	http://www.merckmillipore.com/AT/de/life-science-research/genomic-analysis/dna-preparation-cloning/pet-expression-vectors/qFSb.qB.mLQAAAFA6.VkiQ0G,nav
0.45 µm filter units	MERCK	SLHP033NS	Millex-HP, 0.45 µm, PES 33 mm, not steril
0.22 µm filter units	MERCK	SLGP033RS	Millex-HP, 0.22 µm, PES 33 mm, not steril
Eppendof tubes 1.5 mL	VWR	525-1042	microcentrifugal tubes; autoclaved
15 mL Falcon	VWR	734-0451	centrifugal tubes
50 mL Falcon	VWR	734-0448	centrifugal tubes
PS Cuvettes Spectrophotometer Semi-Micro	VWR	30622-758	VIS transparent cuvettes
UV Cuvettes Spectrophotometer Semi-Micro	VWR	47727-024	UV/VIS transparent cuvettes
isopropyl-β-D-thiogalactopyranosid (IPTG)	ROTH	2316	chemical used for induction of protein expression with the DE3/pET system
imidazole	ROTH	X998	chemical used for elution of polyhistidine (6xHis) sequences from a nickel-charged affinity resin
Glass Econo-Column Columns	Bio-Rad	-	http://www.bio-rad.com/de-at/product/glass-econo-column-columns?ID=2cfb1c6e-32e8-4c72-b532-dd39013d707d&pcp_loc=catprod
chloramphenicol	Sigma-Aldrich	C0378	antibiotic for bacterial growth selection; resistance endióded in pLysS plasmid of BL21(DE3) E. coli; 25 µg/mL final concentration
kanamycin	Sigma-Aldrich	60615	antibiotic for bacterial growth selection; to be used if this resistance is encoded in the employed pET vector; 50 µg/mL final concentration
ampicillin	Sigma-Aldrich	A1593	antibiotic for bacterial growth selection; to be used if this resistance is encoded in the employed pET vector; 100 µg/mL final concentration
Ultra-15, MWCO 10 kDa	Sigma-Aldrich	Z706345	centrifigal filters for protein enrichment; https://www.sigmaaldrich.com/catalog/product/sigma/z706345?lang=de&region=AT
Ultra-0.5 Centrifugal Filter Units	Sigma-Aldrich	Z677108	centrifigal filters for protein enrichment; https://www.sigmaaldrich.com/catalog/product/ALDRICH/Z677108?lang=de&region=AT&cm_sp=Insite-_-prodRecCold_xviews-_-prodRecCold5-2
oxaloacetic acid	Sigma-Aldrich	O4126	TCA metabolite
sodium oxlalate	Sigma-Aldrich	71800	a competitive inhibitor of FAH superfamily enzymes
Dialysis tubing cellulose membrane	Sigma-Aldrich	D9277	https://www.sigmaaldrich.com/catalog/product/sigma/d9277; or comparable
Ni-NTA agarose	Thermo-Fischer	R90101	a nickel-charged affinity resin that can be used to purify recombinant proteins containing a polyhistidine (6xHis) sequence
96-Well UV Microplate	Thermo-Fischer	8404	UV/VIS transparent flat-bottom 96 well plates
PageRuler Prestained Protein Ladder, 10 to 180 kDa	Thermo-Fischer	26616	https://www.thermofisher.com/order/catalog/product/26616?SID=srch-hj-26616
ÄKTA FPLC system	GE Healthcare Life Sciences	-	using the FPLC system by GE Healthcare; different custom versions exist; this work used the "ÄKTA pure" system
HiTrap Phenyl HP column	GE Healthcare Life Sciences	-	https://www.gelifesciences.com/en/it/shop/chromatography/prepacked-columns/hydrophobic-interaction/hitrap-phenyl-hp-p-05630
Mono S 10/100 GL	GE Healthcare Life Sciences	-	https://www.gelifesciences.com/en/ch/shop/chromatography/prepacked-columns/ion-exchange/mono-s-cation-exchange-chromatography-column-p-00723
Mono Q 10/100 GL	GE Healthcare Life Sciences	-	https://www.gelifesciences.com/en/ch/shop/chromatography/prepacked-columns/ion-exchange/mono-q-anion-exchange-chromatography-column-p-00608
HiLoad Superdex column 75 pg (G75)	GE Healthcare Life Sciences	-	https://www.gelifesciences.com/en/ch/shop/chromatography/prepacked-columns/size-exclusion/hiload-superdex-75-pg-preparative-size-exclusion-chromatography-columns-p-05800
HiLoad Superdex column 200 pg (G200)	GE Healthcare Life Sciences	-	https://www.gelifesciences.com/en/ch/shop/chromatography/prepacked-columns/size-exclusion/hiload-superdex-200-pg-preparative-size-exclusion-chromatography-columns-p-06283
TECAN microplate reader	TECAN Life Sciences	-	https://lifesciences.tecan.com/microplate-readers
acetylpyruvate	MoleculeCrafting.HuGs e.U.	-	custom synthesis
benzoylpyruvate	MoleculeCrafting.HuGs e.U.	-	custom synthesis
VDX™ plate (24 wells)	Hampton	HR3-142	24 well plates used for crystallization via Hanging Drop Vapor Diffusion
paraffin oil	Hampton	HR3-411	used for crystallization via Hanging Drop Vapor Diffusion
coverslips (22 mm)	Karl Hecht KG	14043	coverslips used for crystallization via Hanging Drop Vapor Diffusion
Luria broth (LB) medium	self-prepared	-	a general growth medium for E. coli: 5 g/L yeast extract; 10 g/L peptone from casein; 10 g/L sodium chloride; 12 g/L agar-agar
NZCYM medium	self-prepared	-	a better growth medium for E. coli, used for amplification: 10 g/L NZ amine; 5 g/L NaCl; 5 g/L yeast extract; 1 g/L casamino acids; 2 g/L MgSO4; adjust pH to 7.4
Luria broth (LB) agarose plates	self-prepared	-	autoclaved agarose plates containing LB-medium and antibiotics for bacterial groth selection; https://www.addgene.org/protocols/pouring-lb-agar-plates/
Ni-NTA running buffer	self-prepared	-	20 mM Tris-HCl pH 7,4; 50-300 mM NaCl; 10-200 mM imidazole; ranges: optimal value varies among FAHD proteins
Ni-NTA elution buffer	self-prepared	-	20 mM Tris-HCl pH 7,4; 50-300 mM NaCl; 200-500 mM imidazole; ranges: optimal value varies among FAHD proteins
HIC running buffer	self-prepared	-	44 mM NaH_2PO_4; 6 mM Na_2HPO_4; 100 mM NaCl; 20 mM DTT; adjust to pH 7
HIC running buffer AS	self-prepared	-	HIC running buffer saturated with ammonium sulfate (AS); adjust to pH 7: 70 g ammonium sulfate + 90 mL buffer, stirred overnight in the cold room; adjust to pH 7.0
Mono S low salt buffer	self-prepared	-	44 mM NaH_2PO_4; 6 mM Na_2HPO_4; 10-300 mM NaCl; ranges: optimal value varies among FAHD proteins
Mono S high salt buffer	self-prepared	-	44 mM NaH_2PO_4; 6 mM Na_2HPO_4; 1-2 M NaCl; ranges: optimal value varies among FAHD proteins
Mono Q low salt buffer	self-prepared	-	20 mM Tris-HCl; 15 mM NaCl; adjust to pH 8.0
Mono Q high salt buffer	self-prepared	-	20 mM Tris-HCl; 1 M NaCl; 10 % glycerol; adjust to pH 8.0
G75 / G200 running buffer	self-prepared	-	15 mM Tris-HCl; 300 mM NaCl; adjust to pH 7.4
enzyme assay buffer	self-prepared	-	50 mM Tris-HCl pH7.4; 100 mM KCl; 1 mM $MgCl_2$
protein crystallization buffer	self-prepared	-	G75 / G200 running buffer with 1 mM DTT
reservoir solution for crystallization	self-prepared	-	100 mM Na-HEPES pH 7.5; 5-20 % (w/v) PEG4k; 10 mM-200 mM $MgCl_2$

Type	Protocol step	Name of Material/Equipment	Company	Catalog Number
General supply	1, 3, 4, 5, 6	Eppendorf tubes 1.5 mL	VWR	525-1042
	3	15 mL Falcon	VWR	734-0451
	2, 3	50 mL Falcon	VWR	734-0448
	2	0.45 um filter units	MERCK	SLHP033NS
	2	0.22 um filter units	MERCK	SLGP033RS
	3, 4, 5, 6	Ultra-15. MWCO 10 kDa	Sigma-Aldrich	Z706345
	3, 4, 5, 6	Ultra-0.5 Centrifugal Filter Units	Sigma-Aldrich	Z677108
	1	PS Cuvettes Spectrophotometer Semi-Micro	VWR	30622-758
	3	UV Cuvettes Spectrophotometer Semi-Micro	VWR	47727-024
	7, 8	96-Well UV Microplate	Thermo-Fischer	8404
	2,3,4,5,6	PageRuler Prestained Protein Ladder, 10 to 180 kDa	Thermo-Fischer	26616
	3	Dialysis tubing cellulose membrane	Sigma-Aldrich	D9277
FPLC	4, 5, 6	ÄKTA FPLC system	GE Healthcare Life Sciences	-
	4	HiTrap Phenyl HP column	GE Healthcare Life Sciences	-
	5	Mono S 10/100 GL	GE Healthcare Life Sciences	-
	5	Mono Q 10/100 GL	GE Healthcare Life Sciences	-
	6	HiLoad Superdex column 75 pg (G75)	GE Healthcare Life Sciences	-
	6	HiLoad Superdex column 200 pg (G200)	GE Healthcare Life Sciences	-
FPLC supply	4	HIC running buffer	self-prepared	-
	4	HIC running buffer AS	self-prepared	-
	5	Mono S low salt buffer	self-prepared	-
	5	Mono S high salt buffer	self-prepared	-
	5	Mono Q low salt buffer	self-prepared	-
	5	Mono Q high salt buffer	self-prepared	-
	6	G75 / G200 running buffer	self-prepared	-
Medium and related	1	Luria broth (LB) medium	self-prepared	-
	1	NZCYM medium	self-prepared	-
	1	Luria broth (LB) agarose plates	self-prepared	-
Chemicals	1	chloramphenicol	Sigma-Aldrich	C0378
	1	kanamycin	Sigma-Aldrich	60615
	1	amoicillin	Sigma-Aldrich	A1593
	1	isopropyl-β-D-thiogalactopyranosid (IPTG)	ROTH	2316
	3	imidazole	ROTH	X998
	7, 8	oxaloacetic acid	Sigma-Aldrich	O4126
	7, 8	acetylpyruvate	MoleculeCrafting.HuGs e.U.	-
	7, 8	benzoylpyruvate	MoleculeCrafting.HuGs e.U.	-
Ni-NTA chromatography	3	Glass Econo-Column Columns	Bio-Rad	-
	3	Ni-NTA agarose	Thermo-Fischer	R90101
	3	Ni-NTA running buffer	self-prepared	-
	3	Ni-NTA elution buffer	self-prepared	-
Protein expression	1	BL21(DE3) pLysS competent E. coli	Promega	L1195
	1	pET E. coli T7 Expression Vectors	MERCK	-
Enzyme assays	7, 8	TECAN microplate reader	TECAN Life Sciences	-
	7, 8	enzyme assay buffer	self-prepared	-
Crystallization	9	VDX™ plate (24 wells)	Hamoton	HR3-142
	9	coverslips (22 mm)	Karl Hecht KG	14043
	9	paraffin oil	Hampton	HR3-411
	9	protein crystallization buffer	self-prepared	-
	9	reservoir solution for crystallization	self-prepared	-
	9	sodium oxlalate	Sigma-Aldrich	71800

Comments/Description

microcentrifuaal tubes: autoclaved
centrifugal tubes
centrifuaal tubes
Millex-HP, 0.45 um, PES 33 mm, not steril
Millex-HP, 0.22 um, PES 33 mm, not steril
centrifiaal filters for protein enrichment: https://www.sigmaaldrich.com/cataloa/product/sigma/z7063457lang=de&reaion=AT
centrifiaal filters for protein enrichment; https://www.sigmaaldrich.com/catalog/product/ALDRICH/Z677108?lang=de&region=AT&cm_sp=Insite-_-prodRecCold_xviews-_-prodRecCold5-2
VIS transparent cuvettes
UV/VIS transparent cuvettes
UV/VIS transparent flat-bottom 96 well plates
https://www.thermofisher.com/order/cataloa/product/266167SID=srch-hi-26616
https://www.sigmaaldrich.com/catalog/product/sigma/z0277; or comparable
using the FPLC system by GE Healthcare; different custom versions exist; this work used the "ÄKTA pure" system
https://www.aelifesciences.com/en/t/shop/chromatoaraphy/prepacked-columns/hydrophobic-interaction/hitrap-phenyl-hp-o-05630
https://www.aelifesciences.com/en/ch/shop/chromatography/prepacked-columns/ion-exchange/mono-s-cation-exchange-chromatography-columns-p-00723
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https://www.aelifesciences.com/en/ch/shop/chromatography/prepacked-columns/size-exclusion/hiload-superdex-75-pg-preparative-size-exclusion-chromatography-columns-p-05800
https://www.aelifesciences.com/en/ch/shop/chromatography/prepacked-columns/size-exclusion/hiload-superdex-200-pg-preparative-size-exclusion-chromatography-columns-p-06283
44 mM NaH_2PO_4; 6 mM Na_2HPO_4; 100 mM NaCl; 20 mM DTT; adjust to pH 7
HIC running buffer saturated with ammonium sulfate (AS); adjust to pH 7: 70 g ammonium sulfate + 90 mL buffer, stirred overnight in the cold room; adjust to pH 7.0
44 mM NaH_2PO_4; 6 mM Na_2HPO_4; 10-300 mM NaCl; ranges: optimal value varies among FAHD proteins
44 mM NaH_2PO_4; 6 mM Na_2HPO_4; 1-2 M NaCl; ranges: optimal value varies among FAHD proteins
20 mM Tris-HCl: 15 mM NaCl: adiust to pH 8.0
20 mM Tris-HCl; 1 M NaCl; 10 % glycerol; adjust to pH 8.0
15 mM Tris-HCl; 300 mM NaCl; adjust to pH 7.4
a aeneral arowth medium for E. coli: 5 a/L veast extract: 10 a/L peotone from casein: 10 a/L sodium chloride: 12 a/L aear-aear
a better growth medium for E. coli, used for amplification: 10 g/L NZ amine; 5 g/L NaCl; 5 g/L yeast extract; 1 g/L casamino acids; 2 g/L $MgSO_4$; adjust pH to 7.4
autoclaved aaarose plates containine LB-medium and antibiotics for bacterial arath selection: https://www.addaene.ora/protocols/bourine-lb-aaar-plates/
antibiotic for bacterial growth selection; resistance endided in pLysS plasmid of BL21(DE3) E. coli; 25 µg/mL final concentration
antibiotic for bacterial growth selection; to be used if this resistance is encoded in the employed pET vector; 50 µg/mL final concentration
antibiotic for bacterial arowth selection: to be used if this resistance is encoded in the emploved pET vector: 100 ua/mL final concentration
chemical used for induction of protein expression with the DE3/pET system
chemical used for elution of polyhistidine (6xHis) sequences from a nickel-charged affinity resin
TCA metabolite
custom synthesis
custom synthesis
http://www.bio-rad.com/de-at/product/glass-econo-column-columns?ID=2cfb1c6e-32e8-4c72-b532-dd39013d707d&pcp_loc=catprod
a nickel-charaed affinity resin that can be used to purify recombinant proteins containine a polyhistidine (6xHis) sequence
20 mM Tris-HCl pH 7,4; 50-300 mM NaCl; 10-200 mM imidazole; ranges: optimal value varies among FAHD proteins
20 mM Tris-HCl pH 7,4; 50-300 mM NaCl; 200-500 mM imidazole; ranges: optimal value varies among FAHD proteins
Hiah-efficiency protein expression from aene with T7 promoter and ribosome bindine site
http://www.merckmillipore.com/AT/de/life-science-research/genomic-analysis/dna-preparation-cloning/pet-expression-vectors/qF5b.qB.mLQAAAFA6.VkiQ0G,nav
https://lifesciences.tecan.com/microplate-readers
50 mM Tris-HCl pH7.4; 100 mM KCl; 1 mM $MgCl_2$
24 well plates used for crystallization via Hanaine Drop Vapor Diffusion
coverslips used for crystallization via Hanaine Drop Vapor Diffusion
used for crystallization via Hanging Drop Vapor Diffusion
G75 / G200 runnine buffer with 1 mM DTT
100 mM Na-HEPES pH 7.5; 5-20 % (w/v) PEG4k; 10 mM-200 mM $MgCl_2$
a competitive inhibitor of FAH superfamily enzymes

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Title of Article: EXPRESSION, PURIFICATION, CRYSTALLIZATION AND ENZYME ASSAYS OF FUMARYLACETOACETATE HYDROLASE DOMAIN CONTAINING PROTEINS (FAHD).

Author(s): WEISS AKH, HOLZKNECHT M, CAPPUCCIO E, DORIGATTI I, KREIDL K, KASCHGEGGER A, RUPP B, GSTACH H, JANSEN-DÜRR P.

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Rebuttal Letter for revision
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Manuscript number: **JoVE59729**
Date **10.03.2019**

Response to editor comments

Dear Dr. Jansen-Dürr,

Your manuscript, JoVE59729 "Expression, Purification, Crystallization and Enzyme Assays of Fumarylacetoacetate Hydrolase Domain containing Proteins (FAHD).," has been editorially and peer reviewed, and the following comments need to be addressed. Note that editorial comments address both requirements for video production and formatting of the article for publication. Please track the changes within the manuscript to identify all of the edits.

RESPONSE: We thank the editor and the reviewers for considering our manuscript. Gladly we incorporate the reviewer's suggestions, as outlined in the following. In addition to the changes made, we would like to state that the publication fees for this manuscript have partly been covered by the Austrian Science Fund (FWF): project number P 31582-B26. Work in PJD's lab has also been supported by this funding. We added this information to the Disclosures section of the revised manuscript.

After revising and uploading your submission, please also upload a separate rebuttal document that addresses each of the editorial and peer review comments individually. Please submit each figure as a vector image file to ensure high resolution throughout production: (.svg, .eps, .ai). If submitting as a .tif or .psd, please ensure that the image is 1920 x 1080 pixels or 300 dpi. Additionally, please upload tables as .xlsx files.

RESPONSE: We submitted the requested files accordingly.

Your revision is due by Mar 12, 2019.

To submit a revision, go to the JoVE submission site and log in as an author. You will find your submission under the heading "Submission Needing Revision".

Best,

Alisha DSouza, Ph.D.

Senior Review Editor

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Manuscript number: **JoVE59729**
Date **10.03.2019**

Response to Editorial Office comments

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NOTE: Please include a line-by-line response to each of the editorial and reviewer comments in the form of a letter along with the resubmission.

Editorial Comments:

• Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammatical errors.

RESPONSE: Amended.

• Protocol Detail: Please note that your protocol will be used to generate the script for the video, and must contain everything that you would like shown in the video. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol. Some examples of missing items:

1) 1.1.3: How are amplification and verification done? Mention primers used and cite references.

RESPONSE: Amended. Changes to the manuscript are highlighted in the submitted tracked version.

• Protocol Highlight: After you have made all of the recommended changes to your protocol (listed above), please re-evaluate the length of your protocol section. There is a 10-page limit for the protocol text, and a 2.75- page limit for filmable content. Please highlight ~2.5 pages or less of text (which includes headings and spaces) in yellow, to identify which steps should be visualized to tell the most cohesive story of your protocol steps.

1) The highlighting must include all relevant details that are required to perform the step. For example, if step 2.5 is highlighted for filming and the details of how to perform the step are given in steps 2.5.1 and 2.5.2, then the sub-steps where the details are provided must be included in the highlighting.

2) The highlighted steps should form a cohesive narrative, that is, there must be a logical flow from one highlighted step to the next.

3) Please highlight complete sentences (not parts of sentences). Include sub-headings and spaces when calculating the final highlighted length.

RESPONSE: We selected key steps of the protocol for filming and highlighted them in the revised manuscript, respectively.

4) Notes cannot be filmed and should be excluded from highlighting.

RESPONSE: We did not select any notes for filming.

• Figures: Please add a title to each figure legend.

RESPONSE: Amended.

• References: Please spell out journal names.

RESPONSE: Amended.

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RESPONSE: Amended.

2) Please remove the registered trademark symbols TM/R from the table of reagents/materials.

RESPONSE: Amended.

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RESPONSE: We used a figure prepared by Bernhard Rupp, who is co-authoring this work. He retains the right to use and modify parts of this artwork with publisher's permission in future work as long as the original publication source is referenced, as we do.

Manuscript number: **JoVE59729**
Date **10.03.2019**

Response to reviewer comments

Response to Reviewer #1 comments:

Comments from Peer-Reviewers:

Reviewer #1:

Manuscript Summary:

This manuscript describes a general method of expression, purification, crystallization and enzymatic assay using FAHD. The overall work covers wide range of experiments on proteins and was well-done. Important points in these methods are sufficiently referred, though they include a bit time-consuming steps. However, there are still a lot of minor points to revise.

RESPONSE: We thank the reviewer for the positive comments. We have revised the corresponding parts of the manuscript, as outlined in the following.

Minor Concerns:

-Line 145 1.2.1

LB medium would be referred along with NZCYM because LB is one of the major medium for culturing E. coli.

RESPONSE: Amended.

-Line 149 1.2.2

A volume (or rate) of seeding should be added.

RESPONSE: Amended.

-Line 150, 159 1.2.2, 1.2.4

"Take a sample for photometric detection and" seems not to be needed. OD of this sample is not measured and what to judge by the OD is not described. Otherwise, these points should be added.

RESPONSE: Amended.

-Line 158 1.2.4

Because induction was usually done at low temperature (down to 20°C overnight), this point should be added, though the authors described it in discussion section.

RESPONSE: Amended.

-Line 163 1.2.5

The first sentence is not understandable. Why could induction be controlled by centrifugation ?

RESPONSE: We rephrased this confusing statement. Please refer to tracked changes in the revised manuscript.

-Line 167

Low induction level is a common problem for production of proteins. However, this is enough for following steps in not a few cases. So, high induction level is quite preferable but not mandatory.

RESPONSE: We thank the reviewer for raising this point, and included this comment into the revised manuscript.

-Line 187 2.4

Either kind of filter units works, I think.

RESPONSE: Filtration should be done down to the low µm level, as the Ni-NTA purification is significantly faster and efficient if the sample is prepared that way. However, filtration directly at low µm is tedious and requires a pre-filtration at higher µm scale. We included this comment into the revised manuscript.

-Line 196 3.Note

The authors could introduce prepack columns of Ni-NTA since a prepack column of HiTrap phenyl HP column is used in the section 4.

RESPONSE: Amended.

-Line 210 Typo he to the

RESPONSE: Amended.

-Line 211 Grammatical error "sediment" may be noun. A verb should be used.

RESPONSE: Amended.

-Line 239, 289 3.1.10 4.2.3

"reach" may lead misunderstanding. "fall below" is more suitable in this case.

RESPONSE: Amended.

-Line242 3.1.11

In many case, most of target proteins bind to the column. Thus, this step should be optional. In the case of FAHD, is repetition needed ?

RESPONSE: As we use Ni-NTA agarose slurry, not prepacked Ni-NTA columns (which are indeed a viable alternative, as pointed out by the reviewer before; we included a comment in the revised manuscript), the column volume is rather low (a few mg). According to our protocol, mg quantities of protein can be obtained, for which this step should be repeated in order to harvest most of the protein. In addition, as outlined in the troubleshooting part of the discussion section, His-tagged proteins may bind insufficiently to the Ni^{2+}-resin. In

such cases, repetition or alternative methods (cationic exchange) are required. This statement was included in the revised manuscript (p. 6).

-Line262 4.note

Because the columns can be washed with NaOH, reuse for different proteins is possible in many case. Of course, one column for one kind of protein is ideal but it is usually not practical.

RESPONSE: The reviewer is correct. We included a remark about the possibility of regenerating hydrophobic exchange columns in the revised manuscript.

-Line 291 4.2.4

I cannot understand "a gradient flow from buffer A to buffer B: 5.7 % buffer B with 1 % B/ml slope, i.e., 1.5 ml buffer B in 3.8 min". Though it might be due to my comprehension, please change the expression to other ones.

RESPONSE: Amended (p. 8).

-Line 364 6.4

Proteins used for SEC are usually filtered by filter unit.

RESPONSE: The reviewer is correct. In our protocol, samples have already been filtered before the Ni-NTA column or cationic exchange column. However, it is always advised to filter proteins right before applying them to any FPLC column. We included a remark into the revised manuscript.

-Line 375 6.7

Generally, purified proteins are desalted for assay and crystallization. At least, this point should be referred even if desalting is not performed for FAHDs. In addition, for ion-exchange chromatography, buffers without NaCl are usually the first option for loading protein samples and washing the column.

RESPONSE: Amended.

-Line 431 7.11

The blank … is… to The blanks … are…

RESPONSE: Amended.

-Line 439 7.13

Necessity of normalization is quite obscure because specific activity (or reaction velocity) is calculated from the raw date.

RESPONSE: The problem with this assay is that we usually observe shifts in OD arising from both the substrate and the protein. In order to work with absolute OD values, adequate and concise blanking has to be applied. Of course this is possible, however, using internal blanking (the method described) is a simple and effective solution developed by everyday lab practice.

-Line 443 7.14

The OD is probably not appropriate. Time course decrease in OD is suitable in this case.

RESPONSE: Amended.

-Line 474 8.6

Do the authors mean that enzyme concentrations change corresponding to substrate concentrations? Or is assay performed for all the enzyme concentrations against each substrate concentration?

RESPONSE: The assay is performed with pairwise ("adjusted") enzyme / substrate concentrations. We rephrased this part of the protocol to clarify this point (p. 12).

-Line 837

The authors says OAA with C3-C4 bond rotated is stabilized in the crystal. However, the conformation of OAA in Fig9B look corresponding to the conformation before rotation in Fig9C middle (a). Please solve this inconsistency.

RESPONSE: In our model the C^3-C^4 bond rotation (Fig. 9C) happens after the nucleophilic attack by the formed hydroxyl indicated in Fig.9B, i.e., it prepares for the bond cleavage. We rephrased this part of the manuscript to clarify this point (p. 21).

-Fig .1C line 145

The volume of the medium does not look like 5 ml.

RESPONSE: Fig. 1 is rather sketchy on purpose. We modified the figure according to the reviewer's comment.

-Fig. 1C section 1

In the main text, E. coli is seeded to a plate, a preculture medium, and a culture medium in this order. Meanwhile, in the figure, plate, Eppendorf tube, Falcon tube, and a flask. Thus, the explanation in the main text does not correspond to the figure. (Preculture seems to be performed using a Falcon tube, but where is Eppendorf used?)

RESPONSE: Based on the reviewer's comment, we decided to remove the Eppendorf from the revised figure, to avoid any misunderstanding.

-Fig.2B legend line 656

E. coli. RNA Period should be removed after coli

RESPONSE: Amended.

-Fig.3A

Drawing histidine tags is visibly more understandable.

RESPONSE: Amended.

-Fig.3C

Because both resins and proteins are represented with circle, it is quite confusing.

RESPONSE: We changed the display style, according to the reviewer's comment.

-Fig.3D

Direction for movement could be added in the arrows. The triangle (large protein) does not look like migrate faster in this picture. The authors could improve that.

RESPONSE: Amended.

-Fig. 4

As an explanation of "substrate blank, substrate sample, enzyme blank, enzyme sample", attaching "-S/-E, +S/-E, -S/+E, +S/+E (S: substrate, E: enzyme)" or something is helpful for understanding.

RESPONSE: Amended.

-Fig.5

What do OD(t) and OD(0) mean? Please add the explanation on them somewhere in the manuscript.

RESPONSE: Amended.

-Fig. 6C

unligated, ligated to unliganded, liganded ?

RESPONSE: Amended. We replaced the word „ligated" by „liganded".

-Fig. 8A

Why does not the green line (AS gradient) correspond to the cyan line (% buffer B)?

RESPONSE: We thank the reviewer for this comment. To correct this flaw, we revised panel A of Fig. 8 and added additional information to the revised manuscript (p. 8).

-Fig. 8BC

Arrows and bars on the gels should be off at least the lane that you want to show.

RESPONSE: Amended.

-Fig. 8BC(legend)

"similar results, as described in …" seems not to be needed.

RESPONSE: Amended.

-Fig. 9A

74,3 % to 74.3% (comma to period)

17,8% and 7,8 %, too

RESPONSE: Amended.

Table of Materials

Bottom parts of the letters disappear in some lanes.

imidazole to imidazole

E. coli (italic)

Protein markers for SDS-PAGE and SEC could be added.

RESPONSE: Amended.

Response to Reviewer #2 comments:

Reviewer #2:

Manuscript Summary:

In their manuscript, Weiss et al describe a protocol for the expression, purification, crystallization and enzymatic characterization of FAHD containing proteins.

Major Concerns:

The protocol is very detailed for a protocol published in scientific literature, but it is not detailed enough for a novice to the above mentioned methods. This means that a decently experienced protein chemist will be deterred by the level of detail and that an inexperienced protein chemist will need some more guidance. I do concede that the breakdown of the protocol into simple tasks is needed for the production of the video, but it also makes the reading and the following of the protocol rather cumbersome.

RESPONSE: We agree to the reviewer's opinion that this manuscript is at the border of very in-depth technology and rather simple tasks. However, we feel that this is a general, and to some extent unavoidable, problem with this manuscript format.

Another major concern is that the literature is not adequately cited in the paper. The authors should do their best to assemble all relevant literature on their topic AND discuss them accordingly. There may be other ways to purify these proteins, and sometimes one may choose one and sometimes the other. One such missing example is Mizutani et al (2007) in Acta Crystallogr. F. And I am sure that there are others. Furthermore, instead of the rather sketchy figures, it would be preferable to work along an actual expression and purification experiment, and to display actual results.

RESPONSE: We are thankful to the reviewer for this comment. The work of Mizutani et al. has been referenced and alternatives to our method are outlined in the introduction of the revised manuscript (p. 3). As requested by the reviewer, Fig. 7 and Fig. 8 display actual results of representative experiments with the same protein expression, one panel for each protocol step outlined.

Minor Concerns:

In the discussion on the structural results, it was not mentioned which PDB Entries this is based on. It would be nice to add a few paragraphs on them. Also, a note of care should be added, in the sense that structures may contain errors. Nothing of that is in any ways reflected in the discussion.

RESPONSE: The data is based on PDB entries 6FOH and 6FOG (Weiss et al.), as well as the original data 1SAW (Manjasetty et al.). We added this information to the relevant parts in the revised manuscript (p. 3, p.20).

Response to Reviewer #3 comments:

Reviewer #3:

Major Concerns:

None

RESPONSE: We thank the reviewer for his/her positive assessment of our work.

Minor Concerns:

Fig. 1 and Fig. 2 needs to be shorted for clarity.

RESPONSE: As requested, we slightly shortened Fig. 1B. For clarity, we decided to revise and augment the caption of Fig. 1.

Response to Reviewer #4 comments:

Reviewer #4:

Manuscript Summary:

This manuscript describes protocols for the expression, purification, and analysis of fumarylacetoacetate hydrolase superfamily enzymes. The protocols provided are sufficiently in-depth enough to allow for their replication in other labs, and overall the authors provided sufficient details concerning quality control and troubleshooting, areas which are welcomed by others trying to reproduce these protocols.

RESPONSE: We thank the reviewer for his/her encouraging comments.

Major Concerns:

Overall, this manuscript provides a sufficient level of detail and clarity. The only major concern is the need to re-write the entire section on the catalytic activity of the enzymes, notably the paragraphs titled "Remarks to catalytic mechanisms of FAHD2" and The hydrolase activity of FAHD1.

RESPONSE: As suggested by the reviewer, we have revised the section on the catalytic activity of the enzyme in order to make the content easier to understand for a broader readership. For details, see below.

Several sentence fragments and confusing phrases should be reexamined and re-written. An example includes the phrases on pg20 paragraph 1,

"Remains the question how the observed end-product, namely pyruvate is formed, and released from the catalytic cavity? The bound pyruvate enol does not provide any hint why the closed lid should open. Up to this step the proposed mechanism looks like an enzyme inhibition by the product."

RESPONSE: We rephrased the critical parts in the Discussions section (p. 21), which now reads: "*At this step in our hypothesized model, experimental data does not provide any further hint why the closed lid should open to release the product. We may deduce, however, that the proposed mechanism looks like an enzyme inhibition by the product.*"

Minor Concerns:

There are several instances where the authors could expand and clarify and ellipsis that should not be present in scientific work. One example is found on pg. 18, paragraph 2

"Small volumes per well and high enzyme concentrations bias the result, because there is too much enzyme per well dimension (coating the wall, etc...)."

RESPONSE: As requested, we rephrased this sentence and resolved the ellipsis in the revised manuscript (p. 19).

Page 18 paragraph 3

"In such cases, protein expression in other model systems (insect cells, ...) is recommended."

RESPONSE: As requested, we rephrased this sentence and resolved the ellipsis in the revised manuscript (p. 19).

Page 18 paragraph 4

"Individual concentrations of key chemicals (NaCl, imidazole, ...) "

RESPONSE: As requested, we rephrased this sentence and resolved the ellipsis in the revised manuscript (p. 19).

In each of these instances, readers would benefit from an expansion of the items described in the ellipsis, or at least a description of the possible groups they could belong to. The authors should not assume that all readers will understand these different possibilities.

RESPONSE: We agree with the reviewer that submission to JoVE requires a detailed explanation of every precise step of the protocol, and incorporated the reviewer's suggestions in the manuscript.