Electronic Supplementary Information

Visualization of stable protein complexes with palladium, rhodium and iridium nanoparticles detected by catalytic activity in native polyacrylamide gels

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Experimental Section

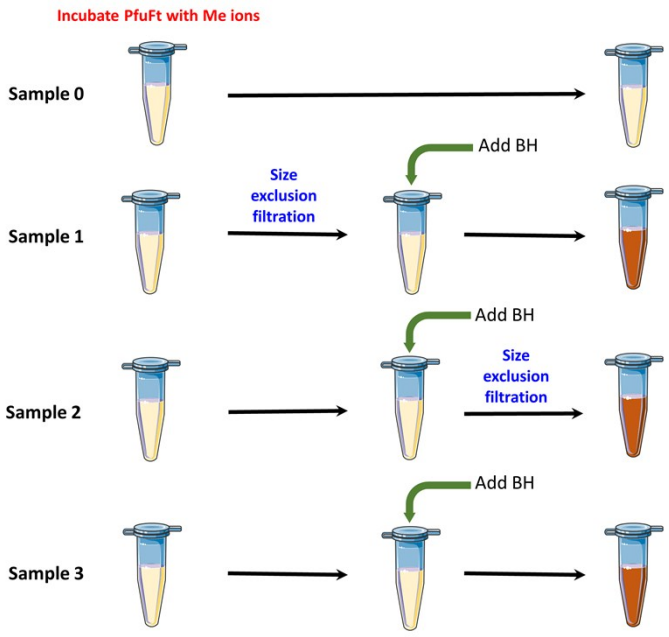
Cloning, expression, and preparation of Pyrococcus ferritin: The open reading frame (ORF) of *Pyrococcus furiosus* ferritin (PfuFt) gene DSM3638 strain was codon optimized for expression in *E. coli* K12 (GeneOptimizer, Thermo Fisher Scientific). The PfuFt ORF in the recombinant protein is preceded by Met-Ala in order to introduce NcoI site in-frame for cloning purposes. The gene synthesis and cloning into pSFT7 plasmid were carried out by Genewiz. The coding sequence was cloned into pET28(a) bacterial expression plasmid. The protein was produced in BL21-CodonPlus (DE3)-RIL (Agilent) *E. coli* strain induced with 0.5 mM IPTG. Induced bacteria were collected by centrifugation and the pellet resuspended in Ft lysis buffer (FLB) (25 mM 1/2Na.HEPES, 150 mM NaCl). Bacteria were lysed by sonication, centrifuged and the clarified lysate was heated for 15 min to 80 °C in order to denature mesophilic proteins that were not removed by centrifugation. Cleared supernatant was further treated with DNaseI (10 μg/ml) for 3 h at 37 °C in order to remove bacterial DNA. DNaseI was removed by second heat treatment (10 min at 90 °C). PfuFt was concentrated from clarified heat-treated samples by filtration through Amicon Ultra-2 (Ultracel – 100K membrane with exclusion limit 100 000 NMWL) (Millipore). Low molecular weight contaminants were removed by size exclusion chromatography with PD MiniTrap G-25 (GE Healthcare) equilibrated with FLB. The protein assembly and purity were analyzed by native and denaturing polyacrylamide gel electrophoresis.

Identification of expressed protein with MALDI-TOF peptide mass fingerprinting: The mass spectrometry experiments were performed on MALDI-TOF mass spectrometer Bruker ultrafleXtreme (Bruker Daltonik GmbH, Bremen, Germany). As a matrix, the α-cyano-4-hydroxycinnamic acid was used. The 20 mg/ml matrix solution was prepared in 50% acetonitrile and 0.1% trifluoroacetic acid. The mixture was thoroughly vortexed and ultrasonicated using Bandelin 152 Sonorex Digital 10P ultrasonic bath (Bandelin Electronic, Germany) for two minutes at 50 % of intensity at room temperature. Sample preparation method for MALDI-TOF was a dried-droplet method – solutions of digested proteins for analysis were mixed with matrix solution in a volume ratio of 1:1. After obtaining a homogeneous solution, 1 μl was applied on the MTP 384 polished steel target plate (Bruker Daltonik GmbH, Bremen, Germany) and dried under atmospheric pressure at 25 °C. All measurements were performed in the reflector positive mode in the *m/z* range 500-8000 Da. Mass spectra were typically acquired by averaging 1000 sub-spectra from a total of 1000 laser shots with a laser power set 5–10

% above the threshold. For digestion we used Trypsin Profile IGD Kit (Sigma-Aldrich, MO, USA), digestion was done according to the enclosed protocol. Tryptic digestion was done at 37 °C for 15 h. Peptide mass fingerprinting was done using MASCOT server (Matrix Science, MA, USA) for comparing mass spectra (excluding peaks of digested trypsin) with NCBI database. For database search the following parameters were used: trypsin was used as the enzyme, 1 missed cleavage was allowed, taxonomy was not specified, oxidation of methionine was added as variable modification, peptide tolerance was set to ± 0.3 Da, mass values were set as MH+ and were obtained from monoisotopic peaks. Good results had a significant statistical score ($p < 0.05$).

Ferroxidase assay: PfuFt was diluted in 10 mM Tris·HCl pH6.8. Immediately before measurement 40 mM $FeCl_2$ acidified with HCl was added to the reaction in a final concentration of 1 mM. The final concentration of Ft in reaction mix was 250 nM. Molar ratio between the Ft and Fe^{2+} ions was 1:4000. Absorbance at 315 nm was measured in 1 min intervals for one hour with Cytation 3 in UV transparent acrylic 96-well plate (Corning).

Preparation of Pd-, Rh-, Ir-PfuFt NPs complexes: PfuFt (2.8 µM) was diluted in FLB (in the case of Pd, Rh) or in 150 mM NaCl (in the case of Ir) and incubated 12 h at 25 °C with 1.6 mM Na_2PdCl_4, $RhCl_3$ and/or K_3IrCl_6. In none of the samples a visible protein precipitation after loading was apparent. The precursor loaded Ft was kept at 4 °C (sample 0) or the non-encapsulated precursor was removed by size exclusion filtration (Microspin™ G-25 Columns, GE Healthcare) before reacting with sodium borohydride (BH) (sample 1). Alternatively, the precursor loaded Ft was immediately reacted with BH (samples 2 & 3) and the product was used as produced (sample 3) or purified through the size exclusion chromatography (sample 2). Reaction with 10 mM BH at ambient temperature was used for reduction of the precursor of Pd and Rh NPs. Ir NPs were produced by reduction of the precursor with 50 mM BH at 60 °C. The reaction was carried out for 1 h and terminated by the addition of HEPES free acid (50 mM). The preparation of different samples is summarized in following scheme.



Scheme 1: Differences in preparation of Metal-PfuFt complexes.

Transmission electron microscopy (TEM): Individual samples were diluted with distilled water do concentration ~10 μg/ml and 4 μl of sample was loaded onto 400mesh TEM grid coated with 14 nm layer of the homemade continuous carbon layer. Subsequently, samples were stained with nanoW (methylamine tungstate, Nanoprobes) and air-dried for 1 h prior imaging. Data were collected on FEI Tecnai F20 (FEI, Eindhoven, The Netherlands) FEG TEM operating at 200kV. Individual images were collected at a nominal magnification of 50 000x (corresponding to the pixel size of 2.22 Å) on 16k CCD camera (FEI Eagle) under the low-dose conditions (~32e^-/Å^{2}s) and with the underfocus in the range of 2.0 – 6.0 μm.

Polyacrylamide gel electrophoresis (PAGE) analysis: Native PAGE analysis of Pd-, Rh-, and Ir-PfuFt complexes was performed on a Bio-Rad electrophoresis system (Mini-PROTEAN 3 Cell) using 1.5 mm thick 5 % resolving gel. After the electrophoresis, the gel was soaked for 30 – 60 min in 0.3 mM Congo red (CR) solution in water. After desired period of time, the gel was placed on the semitransparent plastic pad. The surface of the gel was overlaid with 40 mM solution of BH in water. We avoided full gel submersion in order to limit removal of CR by diffusion. After a few minutes hydrogen bubbles, that need to be regularly removed, formed under the gel (Fig. S6, ESI†). Not removing the bubbles results in staining inhomogeneity after the contrast enhancement. After several minutes, light bands corresponding to the catalytic activity of the NPs start to appear. The reaction can be carried out up to an hour. After the completion of the reaction, the gel is transferred into a plastic container and quickly rinsed with distilled water that is replaced with fixation solution (H_2O/MeOH/HAc - 50:40:10). Within 1 min, the gel becomes darker in a process resembling classical photography development enhancing the contrast between distained areas and the gel background. After image collection, the solution can be replaced with 20 μM solution of Coomassie blue in fixation solution and kept on the shaker over night to stain the proteins in the same gel. After image capture the blue CR form can be reverted into orange form by soaking the gel in 1 M NaOH solution in water gel (Fig. S8, ESI†). This step allows better visualization of Coomassie blue stained bands. However, extended treatment with NaOH might lead to distaining of the protein bands so the time has to be carefully jugged.

Dynamic light scaterring (DLS): Samples were centrifuged for 1 min at 14000 rpm in order to remove aggregated particles and DLS of 20 μl of protein-metal complexes dispersed in 1 ml of 150 mM NaCl was measured with Zetasizer Nano ZS (Malvern).

Spectral characterization of protein-metal complexes: Absorption spectra of 20 μl of protein-metal complexes diluted in final volume 200 μl of 150 mM NaCl and 25 mM hemisodium HEPES were measured with Cytation 3 Cell Imaging Multi-Mode Reader (Biotek) in instrument maximal range of 230 – 900 nm.

Size exclusion chromatography: Samples were centrifuged for 1 min at 14000 rpm in order to remove aggregated particles and 25 μl of sample was diluted in final volume 100 μl of chromatography buffer (300 mM NaCl, 10 mM hemisodium HEPES) and

loaded on Superdex 75 10/300 GL column connected to AKTA pure (GE Healthcare Life Sciences). The protein elution profile was recorded with UV detectors at 214 nm and 280 nm.

Catalytic discoloration of CR: The reaction was carried out in a 96-well plate with 200 µl of a sample containing 0.1 mM CR, 5 mM BH and 2 µl of Me-PfuFt. Absorption at 500 nm was recorded for three hours in 2 min intervals on Cytation 3 at 25 °C.

Catalytic discoloration of Methyl Orange: The reaction was carried out in a 96-well plate with 200 µl of a sample containing 0.2 mM Methyl orange, 5 mM BH and 2 µl of Me-PfuFt. Absorption at 465 nm was recorded for three hours in 2 min intervals on Cytation 3 at 25 °C.

Catalytic reduction of 4-nitrophenol: The reaction was carried out in a 96-well plate with 200 µl of a sample containing 0.2 mM 4-nitrophenol, 5 mM BH and 2 µl of Me-PfuFt. Alternatively, 5 mM hydrazine hydrate was used instead of BH. Absorption at 400 nm was recorded for three hours in 2 min intervals on Cytation 3 at 25 °C.

DNA and protein sequences:

ORF of PfuFt

Alignment of original *Pyrococcus furiosus* ferritin ORF (DSM3638) and codon optimized sequence (OPTIM).

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DSM3638      ------ATGTTGAGCGAAAGAATGCTCAAGGCTTTAAATGACCAGCTAAACAGGGAGCTT
OPTIM        ATGGCGATGCTGAGCGAACGTATGCTGAAAGCACTGAATGATCAGCTGAATCGTGAACTG
                   *** ******** * ***** ** **  * ***** ***** **  * ** **

DSM3638      TATTCTGCATATCTATACTTTGCCATGGCTGCCTACTTTGAAGATCTTGGCCTTGAAGGT
OPTIM        TATAGCGCCTATCTGTATTTTGCAATGGCAGCCTATTTTGAAGATCTGGGTCTGGAAGGT
             ***   ** ***** ** ***** ***** ***** ********** ** ** ******

DSM3638      TTCGCCAACTGGATGAAGGCTCAGGCTGAAGAAGAGATTGGGCATGCACTGAGGTTCTAC
OPTIM        TTTGCCAATTGGATGAAAGCACAGGCCGAAGAAGAAATTGGTCATGCACTGCGCTTTTAC
             ** ***** ******** ** ***** ******** ***** ********* * ** ***

DSM3638      AACTACATCTACGATCGCAATGGTAGGGTTGAGCTTGATGAAATTCCAAAGCCTCCAAAG
OPTIM        AACTATATCTATGATCGTAATGGTCGCGTGGAACTGGATGAAATTCCGAAACCGCCTAAA
             ***** ***** ***** ****** * ** ** ** ********** ** ** ** **

DSM3638      GAGTGGGAGAGCCCATTAAAAGCTTTTGAAGCTGCTTACGAGCATGAGAAATTCATAAGC
OPTIM        GAATGGGAAAGTCCGCTGAAAGCCTTTGAAGCAGCATATGAACATGAGAAATTCATCAGC
             ** ***** ** **  * ***** ******** ** ** ** ************** ***

DSM3638      AAGTCCATATATGAATTGGCAGCTTTAGCAGAGGAGGAAAAAGATTACTCGACGAGGGCA
OPTIM        AAAAGCATCTATGAACTGGCAGCACTGGCAGAAGAGGAAAAAGATTATAGCACCCGTGCA
             **   *** ****** *******  * ***** **************    **  * ***

DSM3638      TTCTTAGAGTGGTTTATCAACGAGCAGGTTGAGGAAGAGGCCAGCGTAAAGAAAATACTG
OPTIM        TTTCTGGAATGGTTTATTAACGAACAGGTTGAAGAAGAGGCCAGCGTCAAAAAAATCCTG
             **  * ** ******** ***** ******** ************** ** ***** ***

DSM3638      GACAAGTTAAAGTTTGCTAAGGACAGTCCTCAAATATTGTTCATGCTTGATAAGGAGTTG
OPTIM        GATAAACTGAAATTTGCCAAAGACAGTCCGCAGATTCTGTTCATGCTGGATAAAGAACTG
             ** **  * ** ***** ** ******** ** **  ********** ***** **  **

DSM3638      AGTGCGAGAGCTCCAAAGCTCCCAGGGCTCTTAATGCAGGGAGGAGAGTAA
OPTIM        AGCGCACGTGCACCGAAACTGCCTGGTCTGCTGATGCAGGGTGGTGAATAA
             ** **  * ** ** ** ** ** ** **  * ******** ** ** ***
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ATG – initiation codon
TAA – termination codon

Protein sequence of cloned PfuFt:

MAMLSERMLKALNDQLNRELYSAYLYFAMAAYFEDLGLEGFANWMKAQAEE
EIGHALRFYNYIYDRNGRVELDEIPKPPKEWESPLKAFEAAYEHEKFISKSIYEL
AALAEEEKDYSTRAFLEWFINEQVEEEASVKKILDKLKFAKDSPQILFMLDKEL
SARAPKLPGLLMQGGE*

176 amino acids
Theoretical pI/Mw: 4.79 / 20511.43 Da

MALDI-TOF identification of expressed protein. A list of peptides identified from a tryptic digest of protein excised from the SDS-PAGE followed peptide mass fingerprinting. Matched peptides are shown in red in the protein sequence of PfuFt.

MASCOT Search Results

Database: NCBInr
Protein sequence coverage: 70%

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1   MLSERMLKAL NDQLNRELYS AYLYFAMAAY FEDLGLEGFA NWMKAQAEEE
51  IGHALRFYNY IYDRNGRVEL DEIPKPPKEW ESPLKAFEAA YEHEKFISKS
101 IYELAALAEE EKDYSTRAFL EWFINEQVEE EASVKKILDK LKFAKDSPQI
151 LFMLDKELSA RAPKLPGLLM QGGE
```

Start – End	Observed	Mr(expt)	Mr(calc)	Delta	M	Peptide
9 – 16	943.3846	942.3773	942.4883	0.1110	0	K.ALNDQLNR.E
45 – 56	1323.5195	1322.5123	1322.6578	0.1456	0	K.AQAEEEIGHALR.F
57 – 64	1153.3936	1152.3864	1152.5240	0.1376	0	R.FYNYIYDR.N
68 – 85	2133.8801	2132.8728	2133.1357	0.2629	1	R.VELDEIPKPPKEWESPLK.A
86 – 95	1194.4137	1193.4065	1193.5353	0.1288	0	K.AFEAAYEHEK.F
86 – 99	1669.6348	1668.6275	1668.8147	0.1872	1	K.AFEAAYEHEKFISK.S
100 – 117	2087.7582	2086.7509	2087.0058	0.2549	1	K.SIYELAALAEEEKDYSTR.A
118 – 135	2167.7755	2166.7683	2167.0473	0.2790	0	R.AFLEWFINEQVEEEASVK.K
118 – 136	2295.8676	2294.8603	2295.1423	0.2820	1	R.AFLEWFINEQVEEEASVKK.I
146 – 161	1862.7409	1861.7336	1861.9608	0.2272	1	K.DSPQILFMLDKELSAR.A
146 – 161	1878.7368	1877.7296	1877.9557	0.2261	1	K.DSPQILFMLDKELSAR.A + Oxidation (M)
165 – 174	1014.4180	1013.4107	1013.5216	0.1109	0	K.LPGLLMQGGE.

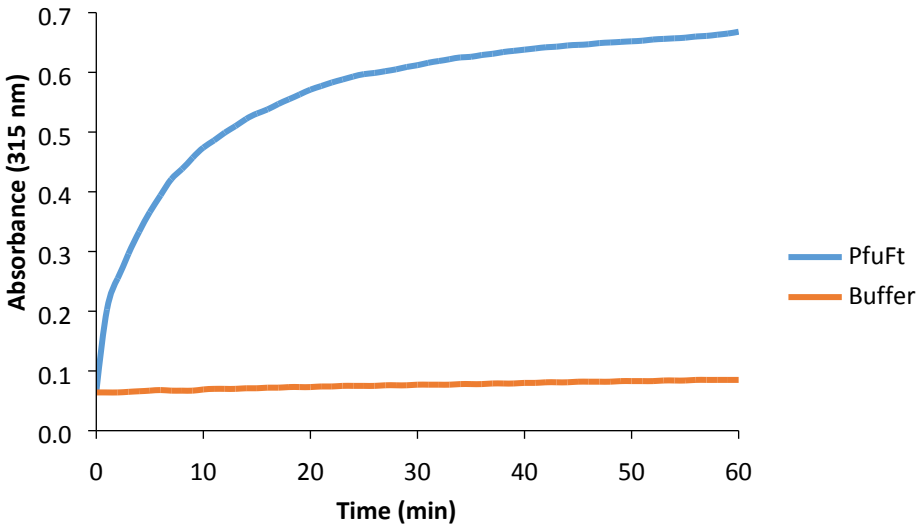

Fig. S1: Ferroxidase activity of recombinant PfuFt. The results of ferroxidase assay show, that isolated recombinant PfuFt catalyzes the oxidation of Fe^{2+} to Fe^{3+}.

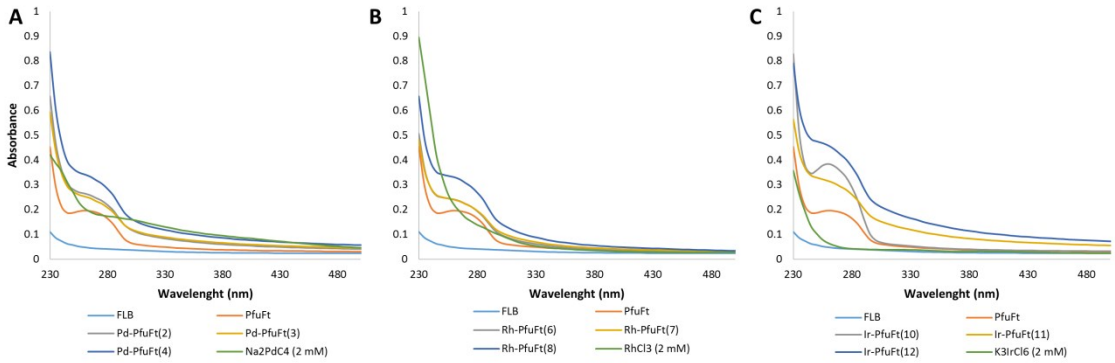

Fig. S2: Absorption spectra of buffer (FLB), PfuFt, Pd-PfuFt (A), Rh-PfuFt (B), and Ir-PfuFt (C). Samples numbering corresponds to Fig. 3.

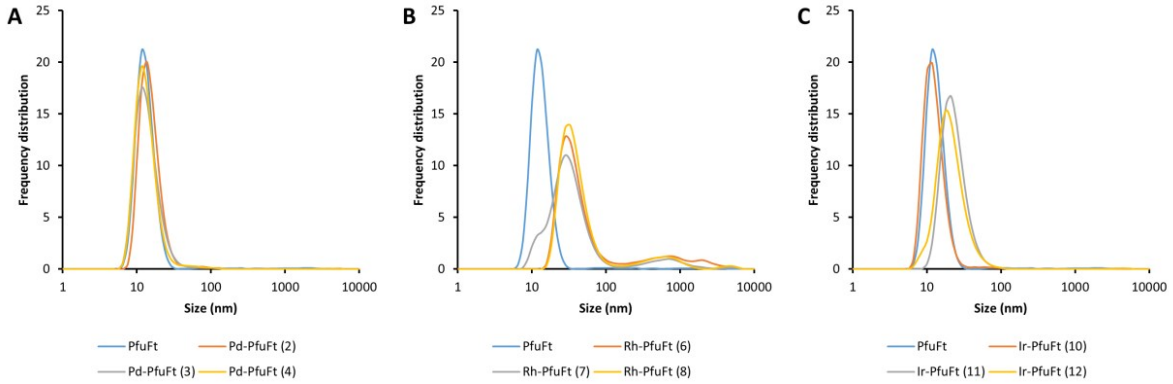

Fig. S3: Dynamic light scattering of protein-metal complexes. Pd-PfuFt (A), Rh-Pfu-Ft (B), and Ir-PfuFt (C). Samples numbering corresponds to Fig. 3.

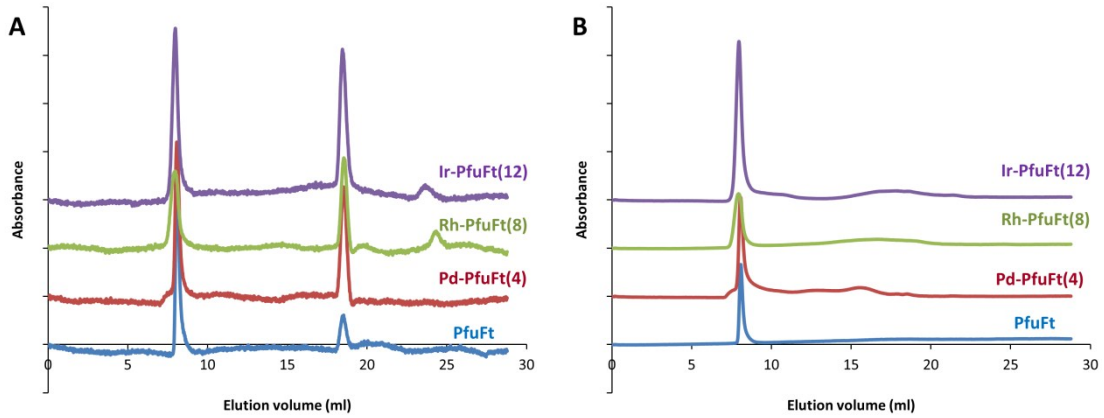

Fig. S4: Size exclusion chromatography of protein metal complexes. Samples were analyzed on Sephadex-75 column and absorption at 214 nm (A) and 280 nm (B) was recorded. Pure PfuFt and metal-PfuFt samples without any buffer exchange were analyzed (corresponding to sample 4, 8, 12 in Fig. 3). Chromatogram at 280 nm shows a single dominant peak corresponding to PfuFt (and corresponding to band Ac in Fig. 3). Interestingly, an additional peak appears on chromatogram acquired at 214 nm. We assume that it corresponds to the band Ad in Fig. 3 and due to its interaction with Coomassie blue is composed from short peptides. The absence of the second peak in figure B might suggest that present peptides lack tryptophan, which strongly absorbs at 280 nm. The presence of metal component increases absorbance but does not lead to any qualitative changes.

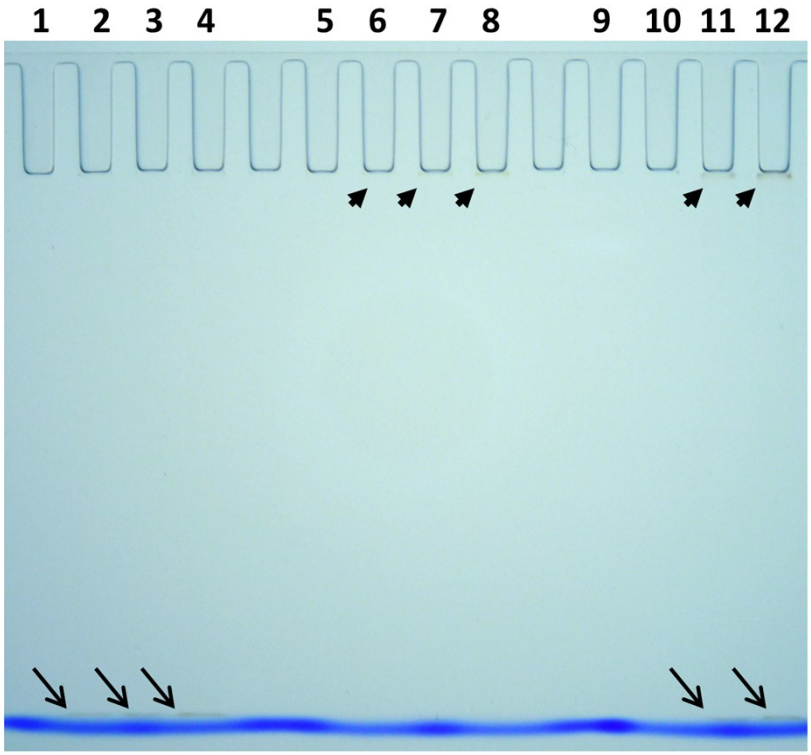

Fig. S5: Non-stained nPAG immediately after the electrophoresis of PfuFt loaded with Na_2PdCl_4 (Line 1, 2, 3, 4), $RhCl_3$ (line 5, 6, 7, 8), and K_3IrCl_6 (line 9, 10, 11, 12). Samples without BH treatment (1, 5, 9), samples where the precursor was removed before BH treatment (2, 6, 10), samples where the low molecular weight compounds were removed after BH treatment (3, 7, 11) and crude reaction products (4, 8, 12) were separated in 5 % PAG. Faint brown aggregates of metal NPs can be detected in the proximity of the wells (arrowheads). Faint sharp bands corresponding to external atomic nanoclusters or peptide complexed atomic metals can be seen migrating close to bromophenol blue used in protein loading buffer (arrows).

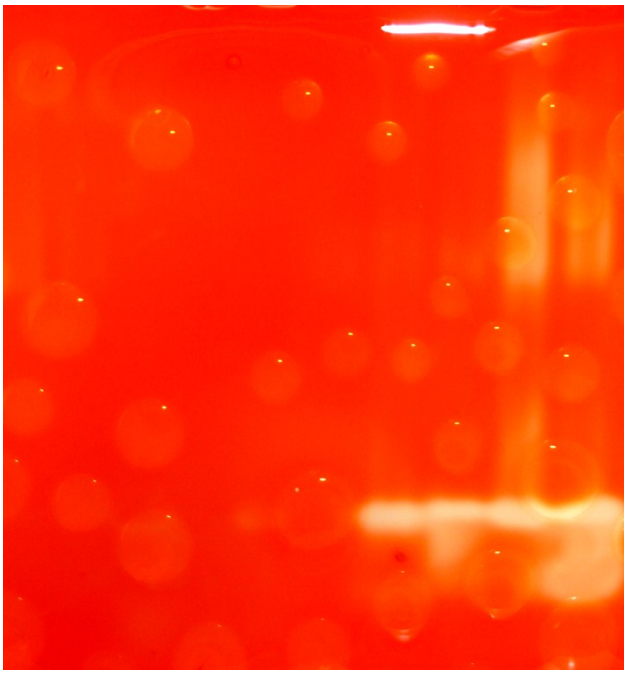

Fig. S6: Formation of hydrogen bubbles under the CR saturated gel during reaction with BH. The H_2 release is due to BH decomposition. The bubbles need to be regularly squeezed out by applying gentle pressure to the gel.

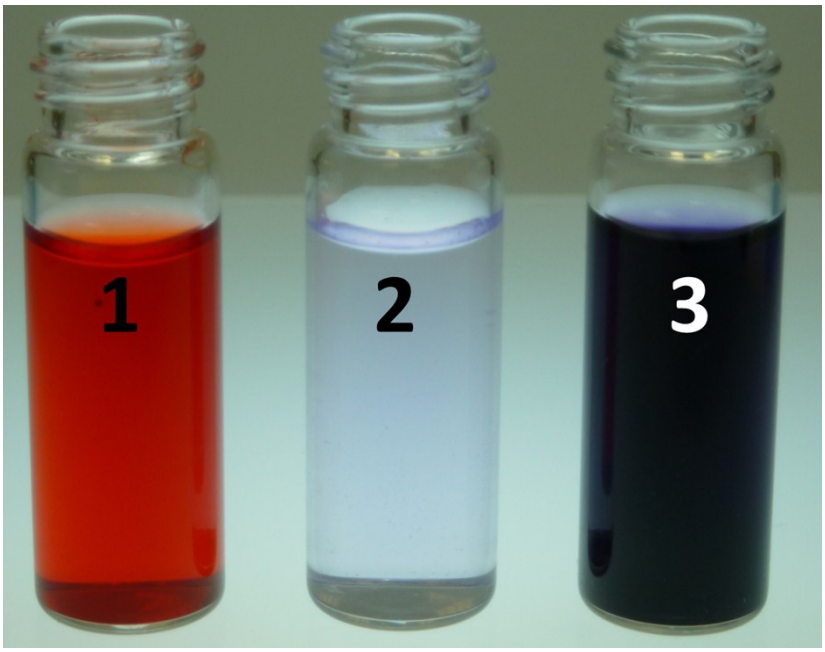

Fig. S7: Color change of Congo red after addition of gel fixation solution (10 % acetic acid, 40 % methanol, 50 % H_2O. 125 µM solution of CR (1), 1.25 µM solution of Coomassie blue (2) in fixation solution, and a mix of 125 µM CR with fixation solution yielding dark purple-violet solution (3).

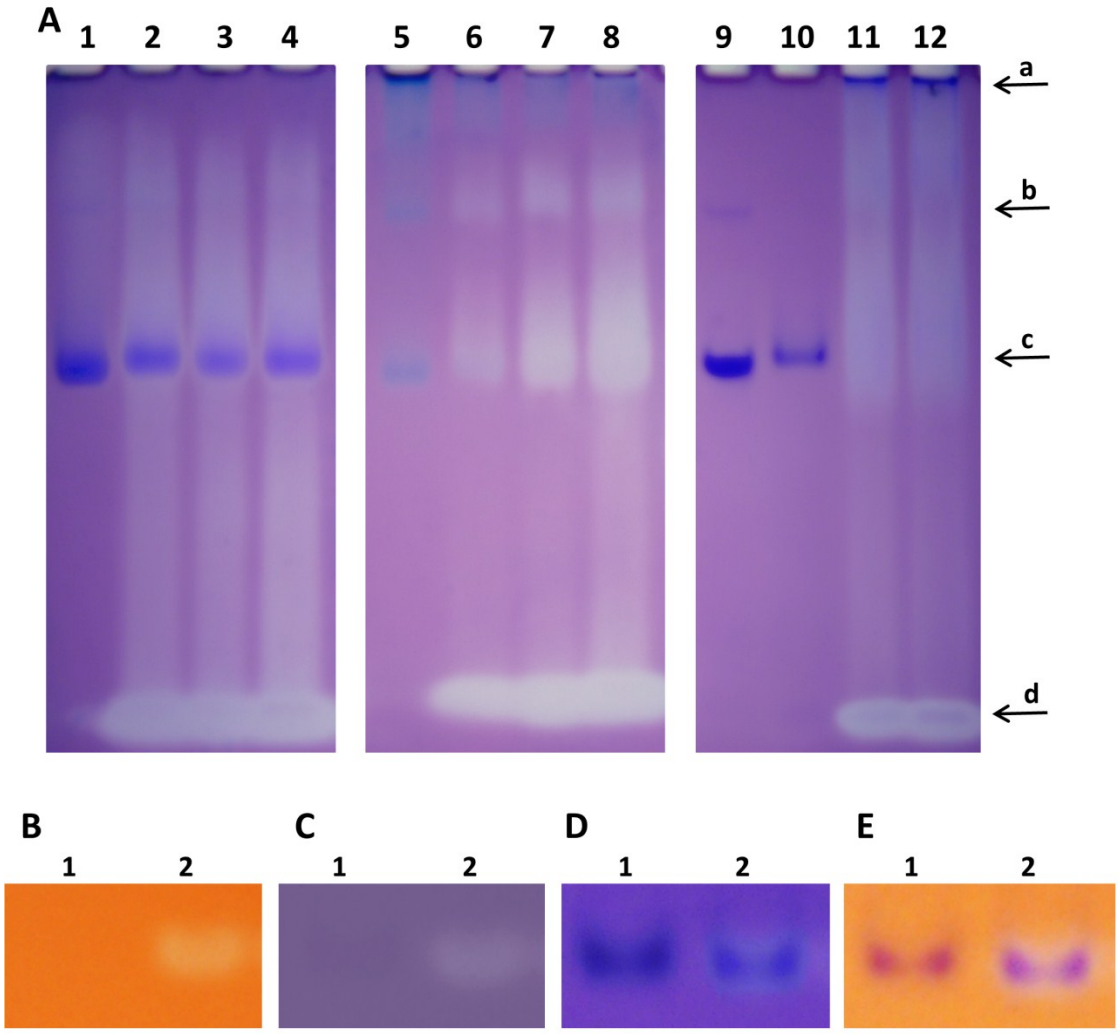

Fig. S8: Appearance of blue protein bands in gels stained with Coomassie blue after treatment with CR and BH demonstrates that in some cases the proteins can be visualized in the same gel as the metal NPs. Image of the same gel as shown in Fig. 3C stained for 12 hours with 20 μM Coomassie blue (A). The presence of Pd, Rh, and Ir does not interfere with the staining in samples after metal loading (lines 1, 5, 9) without BH treatment.

Pd does not interfere with Coomassie blue staining even after the NPs were produced (lines 2, 3, 4) while Rh-PfuFt and Ir-PfuFt nanoparticle synthesis interfere with the staining. Interestingly, if CR and BH treatment is omitted, the proteins can be stained by Coomassie blue in all samples (Fig 3A). The reason why CR and BH treatment in some cases interfere with Coomassie blue staining is at the moment unclear.

The protein staining can be further enhanced by reverting Congo red to its orange form by gel treatment with 1 M NaOH solution. PfuFt (1) and Pd-PfuFt (2) samples were separated by native PAGE and the gel was treated by $NaBH_4$ (B). Addition of fixation solution containing 10 % of acetic acid causes color change of Congo red from orange to blue (C). Staining with Coomassie blue leads to the appearance of blue bands corresponding to proteins (D). The visibility of blue protein bands can be further improved by soaking the gel in 1 M NaOH solution (E) leading to neutralization of acetic acid and restoring the orange color of Congo red.

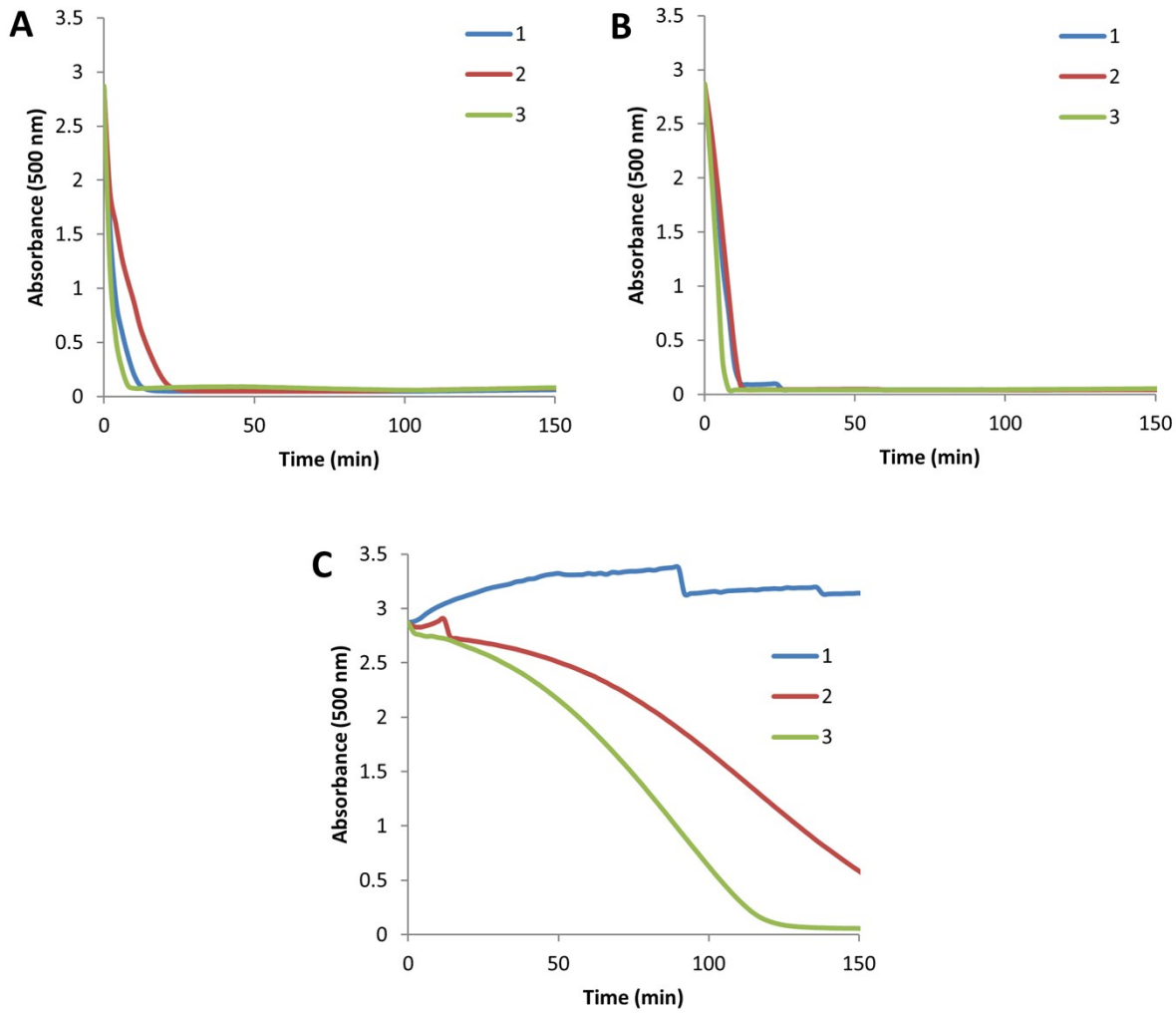

Fig. S9: Catalytic activity of Pd-PfuFt (A), Rh-PfuFt (B), and Ir-PfuFt (C) in BH mediated reduction of CR. Apparent discontinuities in reaction curves (C) are caused by the formation of H_2 bubbles in the wells of a 96-well plate due to decomposition of BH obscuring the optical path. Samples where the excess of precursors was removed before NPs synthesis (1), after NPs synthesis (2), and crude reaction products (3) were analyzed.

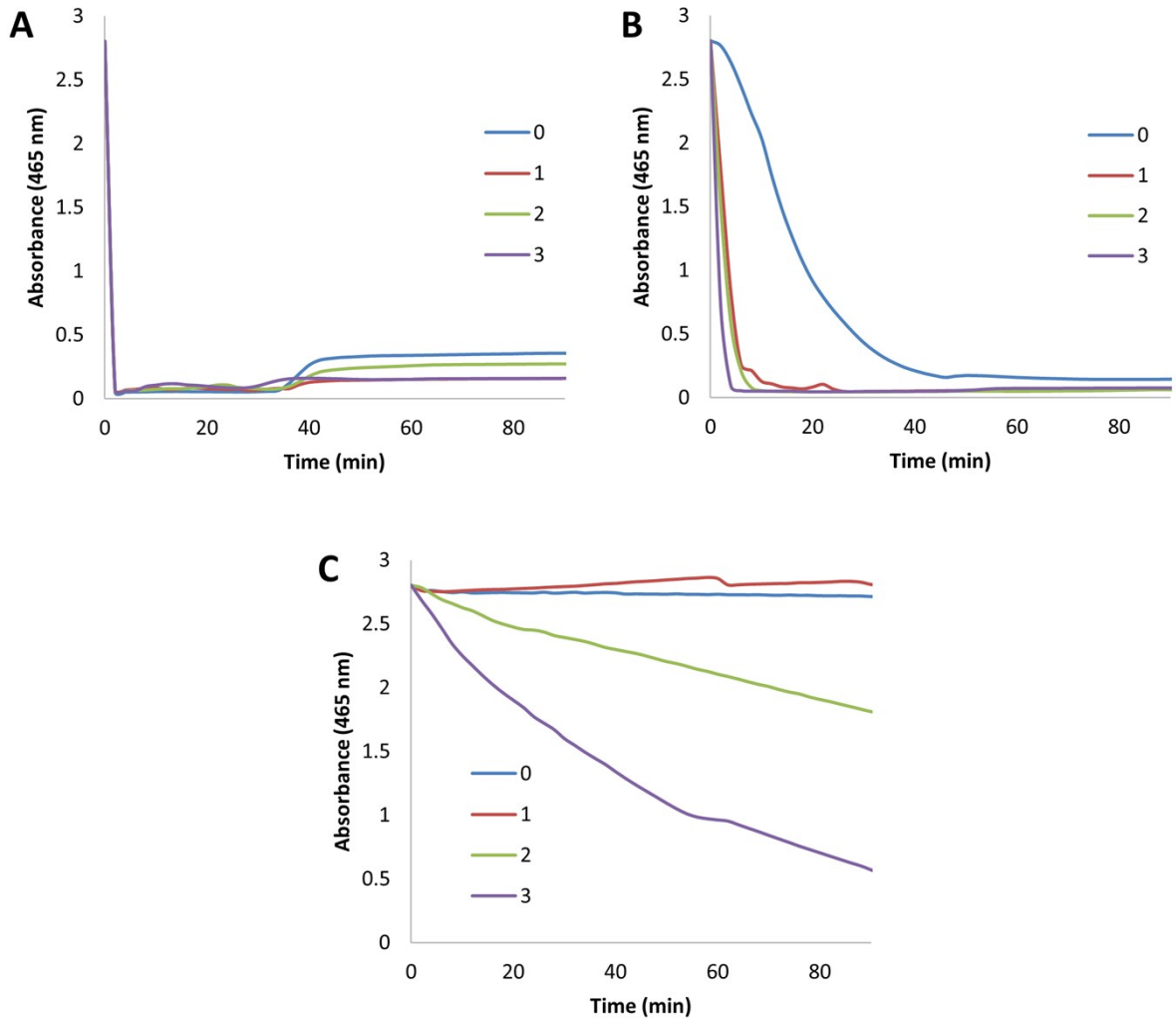

Fig. S10: Catalytic discoloration of methyl orange. Samples of Pd-PfuFt (A), Rh-PfuFt (B), and Ir-PfuFt (C) were reduced with BH.
(Legend: 0 - metal precursor loaded Ft without BH reduction, 1 - PfuFt loaded with metal precursor reduced by BH immediately after size exclusion chromatography removal of external precursor, 2 - PfuFt loaded with metal precursor reduced by BH in presence of external precursor with removal of low molecular weight compounds by size exclusion chromatography after reduction with BH, 3 - PfuFt loaded with metal precursor reduced by BH in presence of external precursor).

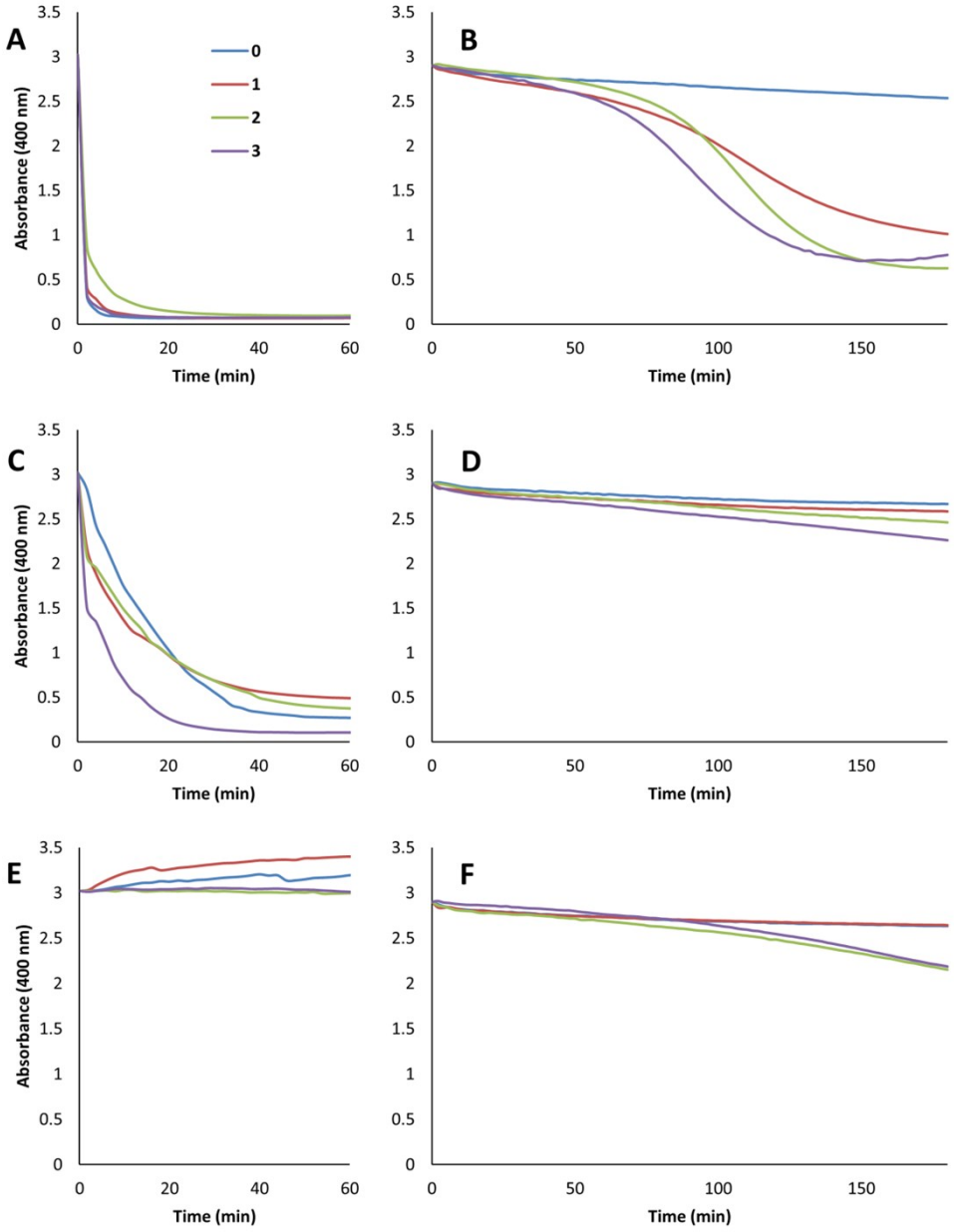

Fig. S11: Nitrophenol hydrogenation to aminophenol catalyzed by Pd-PfuFt (A, B), Rh-PfuFt (C, D), and Ir-PfuFt (E, F) by BH (A, C, E) or hydrazine (B, D, F) was analyzed. (Legend: 0 - metal precursor loaded ferritin without BH reduction, 1 - PfuFt loaded with metal precursor reduced by BH immediately after size exclusion chromatography removal of external precursor, 2 - PfuFt loaded with metal precursor reduced by BH in presence of external precursor with removal of low molecular weight compounds by size exclusion chromatography after reduction with BH, 3 - PfuFt loaded with metal precursor reduced by BH in presence of external precursor). The irregularities in the curves (A, C, E) are a consequence of H_2 bubbles formation due to BH decomposition in the 96-well plates during the measurement. The ability of Pd-, Rh-PfuFt (sample 0 – no BH treatment) to catalyze the reduction of nitrophenol can be explained by the formation of Pd^0 and Rh^0 from the precursor present in Ft sample and BH present in nitrophenol reaction mix (A, C). The inability of Pd-PfuFt (sample 0 – no BH treatment) to catalyze nitrophenol hydrogenation by hydrazine (B) is due to the inability of hydrazine present in nitrophenol reaction mix to reduce Pd^{2+} precursor to metallic Pd.

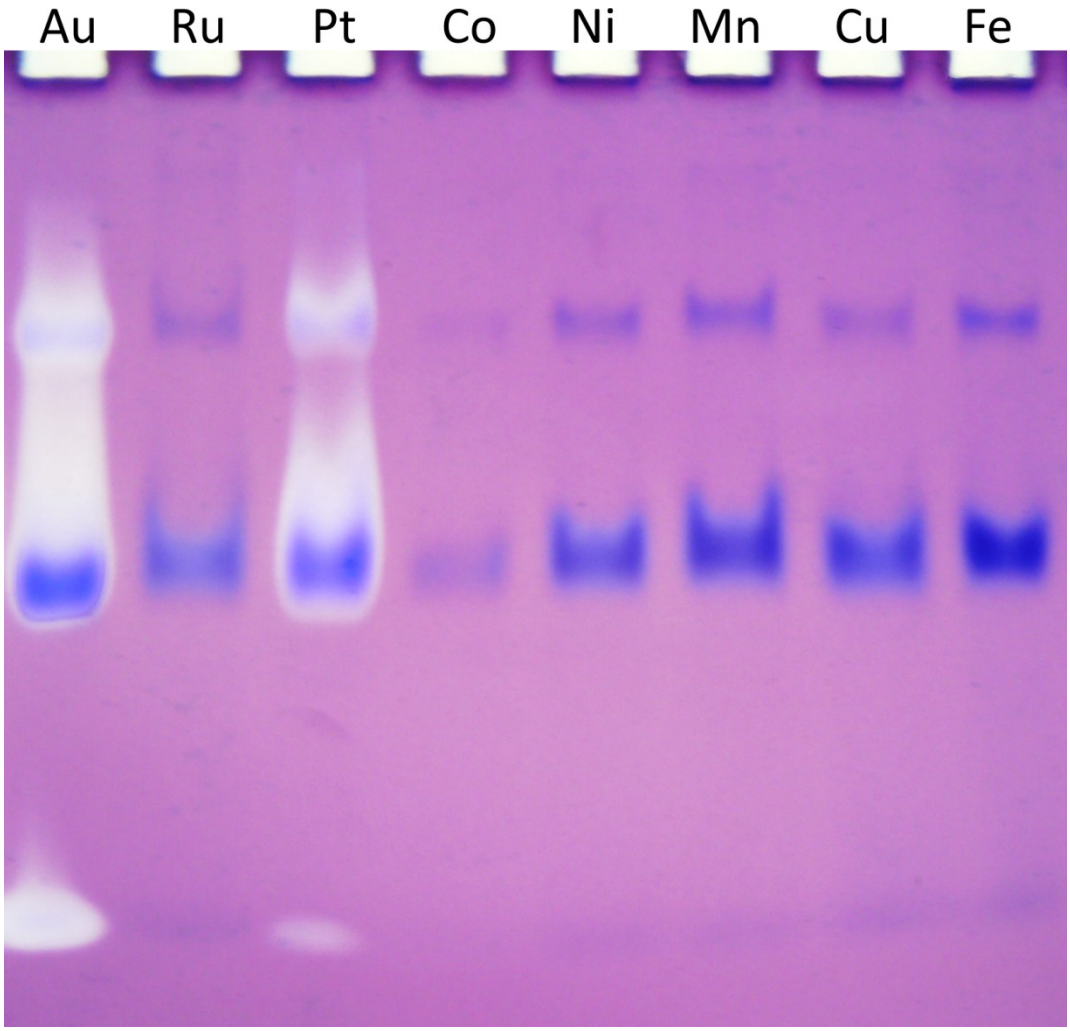

Fig. S12: Visualization of PfuFt loaded with various metals. 0.5 µM PfuFt was incubated in 0.25 mM solution of $AuCl_3$, $RuCl_3$, K_2PtCl_4, $CoCl_2$, $Ni(Ac)_2$, $MnCl_2$, $CuSO_4$, $FeCl_2$ for 12 h and then 5 mM BH was added and reduction of precursor was allowed to proceed for 30 min at 25 °C with the exception of K_2PtCl_4 where the temperature was raised to 60 °C. The reaction mix was separated in 5 % nPAG in Tris-Glycine buffer. The gel was soaked in CR and treated with BH as described previously for 1 h. The gel was then incubated in Coomassie Blue solution for 12 h allowing contrast enhancement of distained areas and parallel detection of protein location in the gel.