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Variability in the Immunodetection of His-tagged Recombinant Proteins

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Abstract

Labeling of recombinant proteins with polypeptide fusion partners, or affinity tagging, is a useful method to facilitate subsequent protein purification and detection. Poly-histidine tags (His-tags) are among the most commonly used affinity tags. We report strikingly variable immunodetection of two His-tagged recombinant human erythropoietins (Epo), wild type Epo (Epo^{wt}) and Epo containing an R103A mutation (Epo^{R103A}). Both were engineered to contain a C-terminal six residue His-tag. The cDNA constructs were stably transfected into CHO cells and COS-7 cells. Clones from the CHO cell transfections were selected for further characterization and larger-scale protein expression. Three chromatographic steps were utilized to achieve pharmacologically pure Epo. Conditioned media from the Epo-expressing cell lines and protein-containing samples from each step of purification were analyzed by SDS-PAGE and dot blot, using both monoclonal anti-human Epo antibody (AE7A5) and anti-His antibodies. While the successful incorporation of the His-tag into our constructs was confirmed by Epo binding to Ni²⁺-NTA resin and by μ LC/MS/MS amino acid sequencing, the levels of immunodetection of His-tagged protein varied markedly depending on the particular anti His-tag antibody used. Such variability in His-tag immunorecognition can lead to critical adverse effects on several analytical methods.

Keywords

Epo; erythropoietin; His-tag; protein purification; His-tag specific antibodies; immunodetection

Recombinant DNA technology enables the production of large quantities of highly purified and well-characterized proteins. Methods that will facilitate subsequent protein analysis and purification are of major interest during the initial design of the recombinant protein. Both detection and purification are greatly simplified by engineering the DNA construct so that the encoded protein is fused to a readily detectable affinant peptide partner, a method designated “affinity tagging”. There is often a preference for selection of a short affinity tag that can be fused to the target gene more easily using the polymerase chain reaction (PCR) [1,2]. Examples

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of commonly used small peptide affinity tags are poly-arginine (Arg), poly-histidine (His), myc, FLAG and Strep (reviewed in [3]).

Poly-histidine tags (His-tags), containing between two (His₂) and ten (His₁₀) histidine residues, are among the most common affinity tags used for protein purification. His-tagging involves the addition of a chosen number of histidines to either the N- or C-terminus of the recombinant protein. The chosen location of the His-tag is protein specific and is dependent upon post-translational modifications of the protein (e.g., removal of N-terminal signal peptide, partial C-terminal digestion). The His-tag allows subsequent interaction of the tagged protein with an immobilized metal-chelate resin, such as Ni²⁺-NTA. Imidazole side chains of the His residues bind to free coordination sites of Ni²⁺ metal ions in a strong and selective way and can be eluted by an imidazole gradient, usually from 20–250 mM [4]. Immobilized metal-affinity chromatography (IMAC) was described in 1975 [5] and was first used for protein purification in 1987 [6,7]. Since then it has been applied to purify many proteins from several expression systems including bacteria [8], yeast [9], mammalian cells [10] and insect cells [11]. Selection of the appropriate expression system is important when correct protein folding and post-translational modification (e.g., glycosylation and phosphorylation) are crucial for proper functioning of the resultant protein.

Detection of His-tagged proteins may employ antibodies directed against the His-tag itself or against other epitopes. Several monoclonal antibodies that bind to His-tags have been reported [11–15], and some are available commercially. There have been further technical developments, such as a His-tag specific BIACORE™, enabling analysis of His-tagged proteins using the Sensor chip NTA. [16]. Recently, an electrochemical technique using a His-tag specific antibody (anti-His₆)-modified electrode was developed [17]. For the most part, the reliability and consistency of His-tagged protein detection using these antibodies and various techniques has not been called into question.

In the present study, we generated two cDNAs encoding C-terminal His-tagged variants of recombinant human erythropoietin (Epo) and expressed them in both Chinese hamster ovary (CHO) and COS-7 cells. We observed consistent binding of these His-tagged Epo proteins to Ni²⁺-NTA resin and confirmed the presence of the His₆ C-terminal tag by μ LC/MS/MS peptide sequence analysis. Surprisingly, however, we found that detection of these His-tagged Epos using four different anti-His-tag antibodies was strikingly variable and, in some cases, failed completely.

Materials and Methods

Vector Design

Two different Epo cDNA constructs, which included the 579-bp coding sequence of the human erythropoietin cDNA, were designed in pcDNA3.1(+) (Invitrogen). Using pcDNA3.1-Epo^{wt} containing wild-type human Epo cDNA as starting material and PCR based *in vitro* mutagenesis, His₆-tagged Epowt (pcDNA3.1-Epo^{wt}-His₆) was produced by amplifying pcDNA3.1-Epo^{wt} (using primers EPO5'ATG-*Hind*III and EPO3'-1-*Xho*I), introducing an *Hind*III restriction site and a Kozak sequence before the start codon and an *Xho*I site after the stop codon, respectively (see Fig. 1 for these and all other primers described below). The EPO3'-1-*Xho*I end was extended by amplifying with EPO3'-2-*Xho*I, thereby deleting the C-terminal arginine (R166) and adding 6 histidines (His₆) to form the C-terminal His₆-tag.

His₆-tagged Epo^{R103A} (pcDNA3.1-Epo^{R103A}-His₆), an Epo mutant with arginine 103 replaced by alanine [18,19], was produced by amplifying pcDNA3.1-Epo^{wt} using three-step PCR. In the first step, the R103A mutation (CGC to GCC) was introduced by amplifying the 5' fragment with primers EPO5'ATG-*Hind*III / R103A anti-sense and the 3' fragment with primers R103A

sense / EPO3'-1-*XhoI*. In the second step, the PCR mixture of 5' and 3' fragments was amplified using primers EPO5'ATG-*HindIII* / EPO3'-1-*XhoI*. Finally, the third step extended the EPO3'-1-*XhoI* end by amplifying with EPO3'-2-*XhoI*.

Final PCR fragments of Epo^{wt}-His₆ and Epo^{R103A}-His₆ cDNA were digested with *HindIII* and *XhoI*, gel-purified, and ligated into the *HindIII*- and *XhoI*-digested expression vector pcDNA3.1(+). Ligation was performed with T4 DNA ligase (New England Biolabs) following the manufacturer's recommendations. The ligation reaction contained a vector:insert ratio of 1:6 and was carried out overnight at 16°C. 10 µl of Electromax DH5α *E. coli* competent cells (Invitrogen) were transformed with 2 µl of 10x diluted ligation mixture by electroporation, and colonies were selected following overnight growth (37°C) on LB agar plates containing 50 µg ampicillin/mL. Following expansion of selected bacterial colonies and subsequent plasmid purification, restriction digestion with *HindIII* and *XhoI* was used to verify the presence and size of the Epo cDNA insert. Positive constructs were sequenced using primers T7 and pcDNA3.1rev to confirm the identity of the insert in each vector. The pcDNA3.1-Epo^{wt}-His₆ and pcDNA3.1-Epo^{R103A}-His₆ expression plasmids were amplified using Maxiprep (Qiagen).

Polymerase Chain Reaction

High fidelity PCR amplification was performed using Vent polymerase, in a supplied Mg²⁺ containing buffer (New England Biolabs). The 50 µl reaction contained 0.5 µM 5' and 3' primer; 10 ng of DNA; 200 µM dATP, dUTP, dGTP, and dTTP. PCR conditions were: 5-min at 95 °C, following by 25 cycles of 30-sec at 95 °C, 30-sec at 68 °C and 1-min at 72 °C, finishing with 8-min at 72 °C.

Cell transfection and single-cell cloning

CHO cells and COS-7 cells were maintained in F12K medium (Mediatech) containing 10% fetal bovine serum (FBS) (HyClone) and 1% penicillin/streptomycin (Gibco), at 37°C, 5% CO₂. Cells were grown to 40–50% confluency and transfected with 2.5 µg of either pcDNA3.1-Epo^{wt}-His₆ or pcDNA3.1-Epo^{R103A}-His₆ recombinant plasmid DNA per 3-cm dish, using DOTAP liposomal transfection reagent (Roche), for 6 h. Stable transfectants were selected by replacing media with fresh media containing 400 µg G418/mL (Sigma) for one week. For the CHO cell transfectants, stable transfectants were subjected to two rounds of single-cell cloning, and ten clones per transfection condition were selected for expansion. Media from the selected clonal populations were collected and analyzed for Epo protein expression by western blotting (see below).

Larger scale protein expression and purification

One clone each from pcDNA3.1-Epo^{wt}-His₆ and pcDNA3.1-Epo^{R103A}-His₆ transfected CHO cells was selected for larger scale expansion and protein expression. Cells were expanded and plated in a Cell Factory 4-layer for Active Gassing vessel (Nunc) using F12K medium. When the cells reached 80% confluency, the medium was discarded. The adherent cells in the Cell Factory were washed twice with phosphate-buffered saline (PBS) and incubated with Opti-MEM I reduced serum medium (Gibco) containing 1.5g/l glucose solution (45%) (Sigma) and 1% Antibiotic-Antimycotic (Gibco). Spent (conditioned) medium was collected every second day and replaced with fresh medium, for a period of 40–60 days. A total of 20–30 L of conditioned medium (CM) was collected. CM was concentrated to 300 ml using an Amicon hollow fiber concentrator (an 80–100 fold concentration from starting CM) and stored at –80 °C.

For Epo purification, three chromatographic steps were employed: Ni²⁺-NTA resin, Cibacron blue agarose and DEAE Sephacel anion exchange chromatography. The presence and purity

of Epo protein throughout the purification/column elution steps were verified by SDS-PAGE and western blotting employing the monoclonal anti-Epo antibody AE7A5 produced in our laboratory [20](available commercially from R&D Systems) and by direct silver- and/or Coomassie staining of SDS-PAGE gels (see below). AE7A5 has been adopted by the World Anti-Doping Agency as the standard anti-Epo antibody used in the detection of epoetin and darbepoetin by IEF double blotting [21].

Concentrated CM of His₆-tagged proteins, equilibrated against 50 mM Na phosphate, 300 mM NaCl, 5 mM imidazole, pH 8.0, was applied onto a Ni²⁺-NTA column (Qiagen) for affinity purification. The column was washed with equilibration buffer (50 mM Na phosphate, 300 mM NaCl, 10 mM imidazole, pH 8.0) and proteins were eluted stepwise with equilibration buffer containing 20, 50, 80, 100 and 250 mM imidazole. Material from the 50 mM imidazole elution step was dialyzed towards PBS and applied onto a Cibacron blue agarose column (CM Affigel blue, BioRad). The agarose was washed with PBS, pH 7.2, and protein was eluted with PBS containing 1 M NaCl. For the third step, Epo-containing fractions from the Cibacron blue column were dialyzed towards equilibration buffer (10 mM Na phosphate, pH 7.2), applied onto a DEAE Sephacel column (Sigma), washed with equilibration buffer, and eluted in subsequent steps with equilibration buffer containing 18, 30, 50, 70, 100, 200 and 1150 mM NaCl.

μLC/MS/MS peptide sequencing

Peptide sequence analysis was performed at the Harvard Microchemistry and Proteomics Analysis Facility, William S. Lane, Ph.D., Director, by microcapillary reverse-phase HPLC nano-electrospray tandem mass spectrometry (μLC/MS/MS) on a Finnigan LCQ DECA XP Plus quadrupole ion trap mass spectrometer.

Protein analysis

Conditioned medium from Epo-expressing cell lines and fractions from all column elution steps were characterized by western blotting, using anti-human Epo monoclonal antibody AE7A5 [20]. Thirty to fifty μl of sample per lane were subjected to denaturing gel electrophoresis, followed by electrophoretic transfer [22] to PVDF membrane (Millipore). Highly purified recombinant human Epo (non His-tagged Epo^{wt}, rhEpo; gift of Elanex Pharmaceuticals, Bothell, WA) was used as a positive control. Blots were incubated in the presence of 0.25 μg AE7A5/ml, and horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (Santa Cruz Biotechnology) was employed as second antibody. Chemiluminescent detection was performed using Super Signal (Pierce).

The presence of His₆-tagged Epo in COS-7 CM, in CHO CM, and in samples at each step of the purification process was monitored using an anti-His tag antibody. Both standard SDS-PAGE/western blotting (to detect denatured protein) and dot blotting were used. For dot blot analysis, samples were applied directly to nitrocellulose membrane, dried, and fixed by baking at 80°C. Murine dihydrofolate reductase (DHFR) with a C-terminal His₆-tag (vector pQE-16, Qiagen) and human selenium binding protein (hSP56) with an N-terminal His₆-tag prepared in our laboratory, both purified on Ni²⁺-NTA resin, were used as positive controls. Following appropriate blocking, blots were incubated in the presence of one of the following anti-His monoclonal antibodies: anti-polyHistidine (Sigma), penta-His antibody, tetra-His antibody or RSG-His antibody (all from Qiagen), using the antibody concentrations recommended by the manufacturers (Table 1). HRP-conjugated goat anti-mouse IgG (Santa Cruz Biotechnology) was employed as second antibody, and chemiluminescent detection was performed using Super Signal (Pierce).

Commassie staining was performed with GelCode Blue Stain reagent (Pierce) following the manufacturer's protocol. Silver staining was performed using SilverQuest (Invitrogen).

Results

Recombinant Epo Proteins

pcDNA3.1-Epo^{wt}-His₆ and pcDNA3.1-Epo^{R103A}-His₆ cDNAs, containing the entire Epo protein coding region and appropriate start and stop codons, were expressed in two mammalian expression systems - Chinese hamster ovary (CHO) and African green monkey kidney (COS-7) cells - to enable glycosylation of the resultant proteins. To enhance subsequent protein analysis and purification, a His₆-tag was engineered at the C terminus by means of PCR (Fig. 1A). The His-tag was attached to aspartic acid (D165). Since Epo's C-terminal arginine (R166) is cleaved following protein maturation in CHO (and some other) cells, it was suggested that this cleavage would result in removal of any His-tag attached to R166 [23]. We selected C-terminal His-tagging, since the N-terminus of Epo contains a 27 amino acid signal peptide that also is removed during protein maturation (Fig. 1B).

Amino acid sequencing using ion trap μ LC/MS/MS confirmed the appropriate modifications of Epo^{wt}-His₆ and Epo^{R103A}-His₆ proteins, as expressed in CHO cells. By this analysis, both Epo^{wt}-His₆ and Epo^{R103A}-His₆ lack R166 and contain the C-terminal His₆-tag. The rest of Epo^{wt}-His₆ is unmodified (Fig. 2). The R103A mutation was confirmed in Epo^{R103A}-His₆ (not shown).

Purification of Epo^{wt}-His₆ and Epo^{R103A}-His₆

We purified the CHO-cell expressed Epos. In the first purification step using Ni²⁺-NTA affinity chromatography, His-tagged Epo bound to the resin, while the majority of the other proteins present in the CM did not bind and were found in the flow-through and subsequent wash (Fig. 3A). The 50 mM imidazole elution, containing the majority of the Epo protein, was selected for further purification. The second purification step, a Cibacron blue column, effectively separated Epo, which bound to the agarose, from a prominent 60 kDa contaminant, which did not bind (not shown). Final purification was effected using DEAE Sephacel chromatography (Fig. 3B).

Immunodetection

Four different anti-His-tag antibodies (Table 1) and anti-Epo antibody (AE7A5) were used for detection of native and denatured Epo samples, in comparison with appropriate controls. Dot blot samples containing unpurified CM, either CHO cell-derived CM concentrate or COS-7 cell-derived CM concentrate, were not detected with any of the antibodies (not shown). Dot blot of purified CHO cell-derived His-tagged Epo proteins were recognized only by the Tetra-His antibody and by the anti-Epo antibody, while the His-tagged DHFR and the His-tagged hSP56 used as positive controls were not detected by any of the antibodies. The control non-His tagged Epo^{wt} was detected only with the anti-Epo antibody, as expected (Fig. 4).

Epo samples from both CHO and COS-7 CMs were evaluated on denaturing SDS-PAGE and western blots (Fig. 5). Surprisingly, of the four anti-His antibodies tested, the His-tagged Epo samples were recognized specifically only by Tetra-His antibody and by the anti-Epo antibody. Conversely, all four anti-His antibodies successfully detected both of the positive controls, His-tagged DHFR and His-tagged hSP56, albeit with differing sensitivities. The RGS-His antibody appeared significantly less specific, resulting in the detection of several non-Epo bands.

Discussion

In the present study, we have prepared two His₆-tagged recombinant erythropoietin proteins and followed each step of protein purification by immunodetection, using an anti-Epo monoclonal antibody prepared originally in our laboratory [20] as well as with four commercially-available anti-His-tag antibodies. We show that there is a remarkable variability in the efficiency of detection of the His-tag on these proteins by each of the different anti-His-tag antibodies. Successful incorporation of the His₆-tag into the cDNA constructs for each of our recombinant proteins was confirmed both by the ability of the proteins to bind to Ni²⁺-NTA resin and by μ LC/MS/MS sequence analysis; however, the incorporated His-tag was not detected immunologically in a consistent and reliable manner.

From among the four anti-His-tag antibodies employed, only Tetra-His reliably detected His-tagged Epo proteins on SDS-PAGE western blots of concentrated but unpurified CM. In contrast, partially-purified C-terminal His₆-tagged DHFR and N-terminal His₆-tagged hSP56, used as positive controls, were recognized by each of the anti-His antibodies. Anti-Epo monoclonal antibody AE7A5 successfully detected all Epo proteins (Fig. 5).

Purity of the Epo-containing protein samples appeared to be one factor that affected His-tag immunodetection when applying native samples. Immunodetection with all four anti-His and anti-Epo antibodies was unsuccessful when concentrated but unpurified CM samples were applied to a dot blot under nondenaturing conditions (not shown). The detection was successful, however, with one anti-His-tag antibody (Tetra-His) and anti-Epo antibody when purified Epo samples were used. However, detection of Ni²⁺-NTA purified His-tagged DHFR and hSP56 on dot blots was not successful (Fig. 4). While our three-step chromatographic purification of Epo proteins enabled immunodetection by tetra-His antibody, the Ni²⁺-NTA purification step alone was not sufficient for DHFR and hSP56 detection under these (nondenaturing) conditions. Our chromatographically purified Epo proteins are > 98% pure, suggesting that sample purity plays an important role in the efficiency of dot blot detection. One of the reasons for a low signal in unpurified samples is the presence of albumin in the concentrate that prevents binding of other proteins to the membrane.

In addition to differences in protein purity, there is another difference between protein immunodetection on dot blots and on western blots. Dot blots enable immunodetection of protein(s) in a primarily native conformation, while western blots rely on detection of SDS-denatured and size-fractionated proteins. Therefore, the variability we observed between detection of DHFR and hSP56 on dot blots and on western blots may have resulted not only from protein purity but also from differential exposure of the His-tag epitopes in the native and denatured proteins. That is, the His-tag may not be exposed sufficiently for antibody binding in the native protein conformation, while it becomes more accessible after protein denaturation by SDS. However, differential accessibility of the His-tag does not explain the variability we observed in His-tag detection of Epo proteins, where the results were consistent between the dot blots and the western blots. The Tetra-His antibody reliably detected His-tagged Epo under both native and denaturing conditions, while the other three anti-His antibodies failed to do so.

Differential levels of detection of His-tagged ligands bound to their cell surface receptors using different monoclonal anti-His antibodies and immunofluorescence has been described previously [13], but the basis for the apparent differential sensitivity of the antibodies was not explained. Each anti-His antibody that we tested in the present study was successful in detection of some kind of His-tagged protein (i.e., the control proteins used in our SDS-PAGE western blots). This suggests that the position of His-tag on C-terminus *versus* the N-terminus of the protein is not itself the basis for the differential detection by the different antibodies. The N-terminus of Epo does not lend itself to His-tag modification, due to the removal of the 27 amino

acid signal peptide. Therefore we did not determine if N-terminal labeling of Epo would result in more efficient His-tag immunodetection. There have been previous reports of successful detection of His-tagged Epo. A Penta-His antibody (Qiagen) was used on TALON purified N-terminal His-tagged mature Epo, separated on SDS-PAGE gel [24], and an anti poly-His-tag monoclonal antibody (source unspecified) was reported to detect Ni²⁺-NTA purified C-terminal tagged Epo on SDS-PAGE [25].

It is interesting to note that a single amino acid difference in His antibody recognition sequence (linear epitope) drastically changes the efficiency of Epo His-tag recognition. While the sequence/epitope that is recognized by the Tetra-His antibody may be as small as His₄, these same four amino acid residues may not be recognized by other antibodies when additional amino acids are linked to His₄. An Epo protein tagged at the C-terminus by GDHHHHHH (i.e., His₆) should, in theory, be recognized by each of the selected anti His-tag antibodies (i.e. anti-His₄, anti-His₅, and anti-His₆), however in our case the GDHHHHHH-tagged Epo was recognized only by anti-His₄ antibody. We addressed the possibility that we had an "incomplete" His₆-tagged Epo, since binding to the Ni²⁺-NTA resin may be successful if as few as two His residues are involved. However, μ LC/MS/MS peptide sequencing of the Epo His-tag confirmed the presence of six histidines in both the Epo^{wt}-His₆ and Epo^{R103A}-His₆ proteins. The particular folding of the His-tag during formation of the SDS micelle may provide another possible explanation, making a portion of the His-tag inaccessible to antibody and, therefore, detectable only by Tetra-His antibody.

There are several factors that may lead to variability of detection among different His-tagged recombinant proteins. These include the availability of the His-tag to the antibody (due to both primary and higher-order protein structure), the location of the His-tag on the individual protein, protein purity, antibody dissociation constant, and the length of the His-tag. The observation that the detection of the same His-tag on different proteins (DHFR, hSP56, Epo) is not consistent is important for further characterization and analysis of His-tagged proteins and antibody selection. Furthermore, such variability in His-tag immunorecognition can lead to critical adverse effects on several analytical methods.

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Abbreviations used

Epo
erythropoietin

His

	histidine
Ab	antibody
NTA	nitrilotriacetic acid
DEAE	diethylaminoethyl
CHO	Chinese hamster ovary cells
COS-7	African green monkey kidney cells
CM	conditioned medium
DHFR	dihydrofolate reductase
SP56	selenium binding protein
μLC/MS/MS	microcapillary reverse-phase HPLC nano-electrospray tandem mass spectrometry
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis

Fig 1.

Epo pcDNA3.1-Epo^{wt/R103A}-His₆ expression vector and His-tagged Epo protein. **Panel A.** Epo^{wt} cDNA region is shown as a black rectangle. The white section is the site of the R103A mutation. The grey rectangle represents the His₆-tag, while the plasmid backbone is shown as a grey line. The locations of the primers used in PCR-based mutagenesis are identified with arrows and marked with capital letters A–E. The primer names and sequences are given below the figure. Non-Epo sequence is in grey, *HindIII* and *XhoI* restriction sites are in bold, and the His-tag sequence is in italics. The start and stop codons are marked with ATG and TGA and boxed. The site of the R103A mutation is underlined.

Panel B. Recombinant His-tagged Epo protein is shown as black rectangle with the signal peptide and the His-tag shown in grey and the site of the R103A mutation shown in white. The differences in amino acid sequences among Epo^{wt}, Epo^{wt}-His₆ and Epo^{R103A}-His₆ are given below. The His-tag sequence is shown in italics. The site of R103A mutation is underlined.

Fig. 2.

Amino acid sequence of Epo^{wt}-His₆ determined by μLC/MS/MS. Multiple peptide sequences (dark grey lines) were obtained for good protein coverage (highlighted in grey). The regions that were not sequenced contain glycosylation sites. The sites of the R103A mutation and the His-tag are boxed.

Fig. 3. Western blot and SDS-PAGE analysis of the purification of Epo^{R103A}-His₆. **Panel A.** Ni²⁺-NTA resin. **Panel B.** DEAE Sephacel anion exchange chromatography. The purification steps were analyzed using anti-Epo antibody (AE7A5) and Commassie blue staining. Epo^{R103A}-His₆ is identified by the arrows. Abbreviations: sample (S), load (L), wash (W), elutions (E or numbered), molecular weight markers (M). Transfer marker (T), Epo standard (+).

Fig. 5.

Western blot immunodetection of Epo^{wt}-His₆ and Epo^{R103A}-His₆ from CHO concentrate and COS-7 conditioned media by Tetra-His antibody, Penta-His antibody, RGS-His antibody, poly-His antibody and anti-human Epo antibody. Note that the His-tagged Epos are recognized only by Tetra-His antibody, even though all four anti-His antibodies detected both His-tagged positive controls successfully, DHFR and hSP56. Anti-human Epo antibody (AE7A5) recognized all Epos at various concentrations. The binding affinity of Penta-His antibody is lower than those of the other antibodies, as shown in Table 1. The RGS-His antibody specificity is rather poor, resulting in several nonspecific bands. Abbreviations: CHO concentrate (CHO), COS-7 condition media (COS-7), size marker (M), positive His-tagged controls (DHFR and hSP56).

TABLE 1

Characteristics of Anti His-tag and Anti-Epo Antibodies.

Antibody	Epitope	Working concentration	Dissociation constant K_d (M)
Monoclonal Tetra-His (Qiagen)	HHHH	0.1 μ g/ml	1×10^{-8} – 5×10^{-8}
Monoclonal Penta-His (Qiagen)	HHHHH	0.1 μ g/ml	5×10^{-8} – 1×10^{-9}
Monoclonal RGS-His (Qiagen)	RGSHHHH	0.1 μ g/ml	1×10^{-8} – 5×10^{-8}
Monoclonal anti-polyHistidine (Sigma)	polyHistidine	1:10000 [*]	Not specified
Monoclonal anti-human Epo AE7A5 (R&D)	N-terminal 26 aa Epo	0.25 μ g/ml	6.7×10^{-9}

^{*} The concentration of the Sigma antibody is not specified.