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(54) Titre : CINETIQUE REACTIONNELLE RAPIDE D'ENZYMES PRESENTANT UNE ACTIVITE INFERIEURE DANS DES COUCHES CHIMIQUES SECHES

(54) Title: FAST REACTION KINETICS OF ENZYMES HAVING LOW ACTIVITY IN DRY CHEMISTRY LAYERS

(57) Abrégé/Abstract:
The present description concerns a method for determining an analyte as well as a diagnostic element suitable therefor, the method comprising the steps of contacting a sample containing the analyte with a diagnostic element comprising a dry reagent layer which contains a mutated glucose dehydrogenase (EC 1.1.1.47) which is specific for the analyte, and an artificial coenzyme; and determining at least one of the presence and the amount of the analyte.

Abstract

The present description concerns a method for determining an analyte as well as a diagnostic element suitable therefor, the method comprising the steps of contacting a sample containing the analyte with a diagnostic element comprising a dry reagent layer which contains a mutated glucose dehydrogenase (EC 1.1.1.47) which is specific for the analyte, and an artificial coenzyme; and determining at least one of the presence and the amount of the analyte.

Fast reaction kinetics of enzymes having low activity in dry chemistry layers

Description

The present invention concerns a method for determining an analyte and a suitable diagnostic element therefor.

Diagnostic elements are important components of clinically relevant analytical methods. In this connection, the primary focus is on the measurement of analytes, e.g. metabolites or substrates, which are for example determined directly or indirectly with the aid of an enzyme which is specific for the analyte. In this case, the analytes are converted with the aid of an enzyme-coenzyme complex and subsequently quantified. In this process, the analyte to be determined is brought into contact with a suitable enzyme, a coenzyme and optionally a mediator and the coenzyme is physicochemically changed by the enzymatic reaction, e.g. oxidized or reduced. If a mediator is additionally used, this mediator usually transfers the electrons released during the reaction of the analyte from the reduced coenzyme to an optical indicator or to the conductive components of an electrode so that the process can for example be detected photometrically or electrochemically. A calibration yields a direct relationship between the measured value and the concentration of the analyte to be determined.

Diagnostic elements known from the prior art are characterized by a limited shelf-life and by special requirements for the environment, e.g. cooling or dry storage, in order to achieve this shelf-life. Thus, in certain forms of application, e.g. in the case of tests which are carried out by the end user himself such as blood glucose self-monitoring, erroneous results may occur due to a false, unnoticed incorrect storage of the measurement system which can hardly be recognized by the consumer and may lead to an erroneous treatment of the respective disease. The erroneous results

are primarily due to the fact that the enzymes, coenzymes and mediators used in such diagnostic elements generally react sensitively to moisture and heat and are inactivated over time.

A known measure which is used to increase the stability of diagnostic elements is the use of stable enzymes, e.g. the use of enzymes from thermophilic organisms. Furthermore, enzymes can be stabilized by chemical modification, and in particular by cross-linking. Moreover, enzyme stabilizers such as e.g. trehalose, polyvinyl pyrrolidone and serum albumin can also be added, or the enzymes can be enclosed in polymer networks e.g. by photopolymerization.

Another method of stabilizing enzymes is by means of mutations that are introduced site-specifically or non-site-specifically. In this connection, the use of recombinant techniques which specifically influence the properties of the corresponding enzyme by means of a targeted change in the DNA coding for an enzyme, have proven to be particularly suitable.

Baik et al. (Appl. Environ. Microbiol (2005), 71, 3285) describe the isolation and characterization of three mutants of glucose dehydrogenase from Bacillus megaterium which contain the amino acid substitutions E170K, Q252L or E170K/Q252L. Whereas the mutants E170K and Q252L only have a low stability at low salt concentrations and high pH values, the double mutant exhibits a significantly increased stability under the test conditions due to an enhanced interaction at the dimer-dimer interface.

Vázquez-Figueroa et al. (ChemBioChem (2007), 8, 2295) disclose the development of a thermostable glucose dehydrogenase which comprises introducing amino acid substitutions at positions 155, 170 and 252 of the glucose dehydrogenase from Bacillus subtilis, Bacillus thuringiensis and Bacillus licheniformis. In this connection, it is stated that the mutations E170K and Q252L, individually as well as

in combination, result in a stabilization of glucose dehydrogenase from Bacillus subtilis.

However, when stabilized enzymes which are genetically modified compared to the wild-type variant are used, the problem arises that they usually have a considerably lower activity than the corresponding wild-type enzyme, and thus vause a lower substrate turnover per unit of time. If one takes into consideration the fact that enzymes having a high specific activity are preferably used in clinical and diagnostic chemistry such as in the detection of blood glucose, the use of stabilized enzymes is often an unacceptable alternative to the use of native enzymes.

Another difficulty is that high enzyme activities which correlate with a high substrate turnover per unit of time are usually only achieved with the respective native coenzyme in each case. If an artificial coenzyme is used instead of the native coenzyme, then the enzyme activity is usually drastically reduced and the rate of turnover with the substrate decreases accordingly.

The object which is the basis for the present invention was thus to provide a stable diagnostic element, in particular for determining glucose in which the disadvantages of the prior art are at least partially eliminated. In particular, the diagnostic element should ensure a high turnover rate of substrate while at the same time ensuring a high stability of the enzyme as well as of the coenzyme.

This object is achieved according to the invention by a method for determining an analyte, comprising the steps:

(a) contacting a sample containing the analyte with a diagnostic element comprising a dry reagent layer which contains

(i) a mutated dehydrogenase which is specific for the analyte and

(ii) an artificial coenzyme, and

(b) determining the presence or/and the amount of the analyte.

Surprisingly, it was found within the scope of the present invention that mutated dehydrogenases which have extremely low activities in the presence of an artificial coenzyme in the cuvette test, exhibit more rapid kinetics in diagnostic elements with dry reagent layers such as in test strips, and yield at least as much turnover as in the presence of the native coenzyme (wild type coenzyme). The reason for this is presumably due to the fact that at high concentrations of ingredients other factors than the activity of the enzyme decisively influence the turnover rate, and in this connection the state of complex formation between enzyme, coenzyme, reduced coenzyme, analyte and oxidized analyte appears to be particularly crucial.

In this respect, the method according to the invention provides in a preferred embodiment that the turnover rate of the analyte in the diagnostic element described herein is equal to or higher than the turnover rate of the analyte in a corresponding diagnostic element which comprises the corresponding wild type coenzyme instead of the artificial coenzyme. The turnover rate of the analyte in a diagnostic element used according to the invention is preferably increased by at least 20 %, more preferably by at least 50 % and most preferably by at least 100 %, for example by 100 % to 200 % compared to the turnover rate of the analyte in a diagnostic element comprising the wild type coenzyme.

The terms "mutated dehydrogenase" or "dehydrogenase mutant" as used in the present application refer to a genetically modified variant of a native dehydrogenase (wild type dehydrogenase) which has an amino acid sequence that is modified compared to the wild type dehydrogenase while having the same number of amino acids, i.e. which differs by at least one amino acid from the wild type dehydrogenase.

The mutated dehydrogenase can be obtained by mutation from a wild type dehydrogenase derived from any biological source, where the term "biological source" in the sense of this invention encompasses prokaryotes such as bacteria as

well as eukaryotes such as mammals and other animals. The introduction of the mutation(s) can take place site-specifically or non-site-specifically, preferably site-specifically using recombinant methods known in the art, resulting in at least one amino acid substitution within the amino acid sequence of the native dehydrogenase in accordance with the respective requirements and conditions.

A dehydrogenase mutant obtained in this manner and used in the method according to the invention preferably has an increased thermal or/and hydrolytic stability compared to the corresponding wild type dehydrogenase. Examples of such mutants are described among others in Baik (Appl. Environ. Microbiol. (2005), 71, 3285), Vázquez-Figueroa (ChemBioChem (2007), 8, 2295) as well as in WO 2005/045016 A2.

A mutated dehydrogenase in the sense of the present invention particularly preferably has a reduced specific enzyme activity compared to the corresponding wild type dehydrogenase. The term "specific enzyme activity" (stated in U/mg enzyme) as used in the present application refers to the amount of substrate which is converted under predefined conditions per minute and per milligram of enzyme. On the other hand, the term "lyophilisate activity" refers to the amount of substrate which is converted under predetermined conditions per minute and per milligram of lyophilisate comprising the enzyme in combination with auxiliary substances.

The mutated dehydrogenase used in the method according to the invention is preferably a nicotinamide adenine dinucleotide (NAD/NADH)-dependent or nicotinamide adenine dinucleotide phosphate (NADP/NADPH)-dependent mutated dehydrogenase which is preferably selected from a mutated alcohol dehydrogenase (EC 1.1.1.1; EC 1.1.1.2), a mutated L-amino acid dehydrogenase (1.4.1.5), a mutated glucose dehydrogenase (EC 1.1.1.47), a mutated glucose-6-phosphate dehydrogenase (EC 1.1.1.49), a mutated glycerol dehydrogenase (EC 1.1.1.6), a mutated 3-hydroxybutyrate dehydrogenase (EC 1.1.1.30), a mutated lactate

dehydrogenase (EC 1.1.1.27; EC1.1.1.28), a mutated malate dehydrogenase (EC 1.1.1.37) and a mutated sorbitol dehydrogenase. The mutated dehydrogenase is particularly preferably a mutated glucose dehydrogenase (EC 1.1.1.47).

If a mutated glucose dehydrogenase is used within the scope of the present invention, it can contain (a) modified amino acid(s) compared to the corresponding wild type glucose dehydrogenase basically at any position in its amino acid sequence. The mutated glucose dehydrogenase preferably comprises a mutation at at least one of the positions 170 and 252 of the amino acid sequence of the wild type glucose dehydrogenase, where mutants having mutations at position 170 and position 252 are particularly preferred. It has proven to be advantageous when the mutated glucose dehydrogenase contains no further mutations in addition to these mutations.

The mutation at positions 170 or/and 252 can basically comprise any amino acid substitution which results in a stabilization, e.g. in an increase in the thermal or/and hydrolytic stability of the wild type dehydrogenase. The mutation at position 170 preferably comprises an amino acid substitution of glutamic acid by arginine or lysine, in particular an amino acid substitution of glutamic acid by lysine, whereas with reference to position 252 an amino acid substitution of lysine by leucine is preferred.

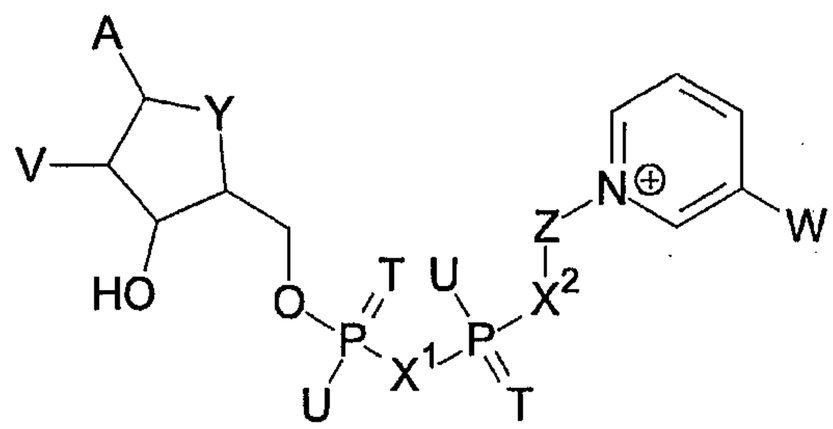
The wild type glucose dehydrogenases used to produce the above-mentioned mutants of glucose dehydrogenase are preferably derived from a bacterium, with a glucose dehydrogenase from Bacillus megaterium, Bacillus subtilis or Bacillus thuringiensis, in particular from Bacillus subtilis being particularly preferably used. In the most preferred embodiment, a mutated glucose dehydrogenase GlucDH_E170K_K252L having the amino acid sequence shown in SEQ ID NO:1 obtained by mutation of wild type glucose dehydrogenase from Bacillus subtilis is used within the scope of the method according to the invention.

It is provided a method for determining an analyte comprising, the steps of:

(a) contacting a sample containing the analyte with a diagnostic element comprising a dry reagent layer which contains;

(i) a mutated glucose dehydrogenase (EC 1.1.1.47) which is specific for the analyte and having an increased in at least one of thermal and hydrolytic stability compared to the corresponding wild-type glucose dehydrogenase, the mutated glucose dehydrogenase comprising a mutation at position 170, position 252, or at positions 170 and 252 of the amino acid sequence of the corresponding wild type glucose dehydrogenase, and

(ii) an artificial coenzyme, the artificial coenzyme being a compound of formula (II):



(II)

in which

A = adenine or an analogue thereof,

T = in each case independently denotes O, S,

U = in each case independently denotes OH, SH, BH_3^-, $BCNH_2^-$,

V = in each case independently denotes OH or a phosphate group, or two groups which form a cyclic phosphate group;

W = COOR, $CON(R)_2$, COR, $CSN(R)_2$ where R in each case independently denotes H or C_1-C_2 alkyl,

X^1,X^2 = in each case independently denote O, CH_2, $CHCH_3$, $C(CH_3)_2$, NH, NCH_3,

Y = NH, S, O, CH_2,

Z = is a linear or cyclic organic residue,

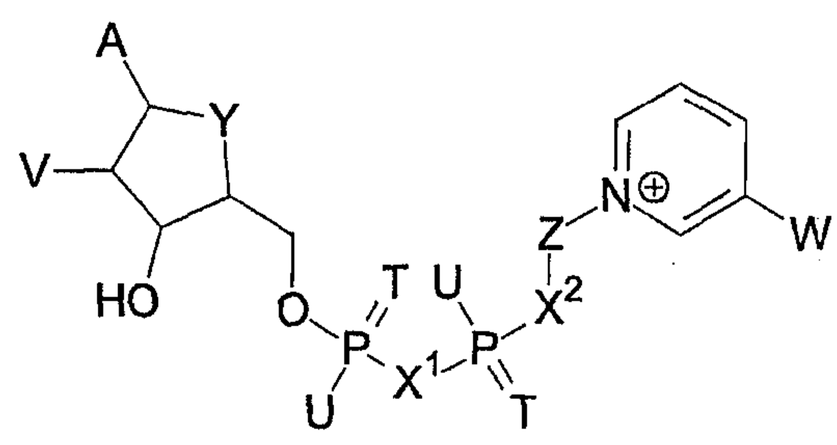
provided that Z and the pyridine residue are not linked by a glycosidic bond, or a salt or optionally a reduced form thereof; and

(b) determining at least one of the presence and the amount of the analyte.

It is further provided a diagnostic element for determining an analyte, comprising a dry reagent layer which contains

(a) a mutated glucose dehydrogenase (EC 1.1.1.47) which is specific for the analyte and having an increased in at least one of thermal and hydrolytic stability compared to the corresponding wild-type glucose dehydrogenase, the mutated glucose dehydrogenase comprising a mutation at position 170, position 252, or at positions 170 and 252 of the amino acid sequence of the corresponding wild type glucose dehydrogenase, and

(b) an artificial coenzyme, the artificial coenzyme being a compound of formula (II):



(II)

in which

A = adenine or an analogue thereof,

T = in each case independently denotes O, S,

U = in each case independently denotes OH, SH, BH_3^-, $BCNH_2^-$,

V = in each case independently denotes OH or a phosphate group, or two groups which form a cyclic phosphate group;

W = COOR, $CON(R)_2$, COR, $CSN(R)_2$ where R in each case independently denotes H or C_1-C_2 alkyl,

X^1,X^2 = in each case independently denote O, CH_2, $CHCH_3$, $C(CH_3)_2$, NH, NCH_3,

Y = NH, S, O, CH_2,

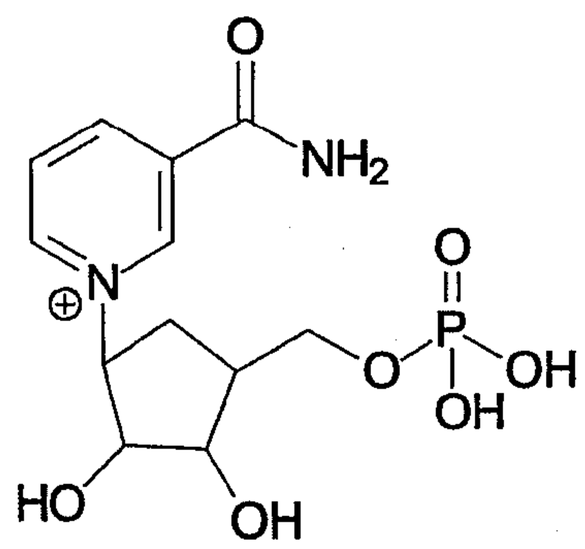
Z = is a linear or cyclic organic residue,

provided that Z and the pyridine residue are not linked by a glycosidic bond, or a salt or optionally a reduced form thereof.

According to the invention, the diagnostic elements described herein furthermore comprise an artificial coenzyme in addition to a mutated dehydrogenase which is specific for the analyte. An artificial coenzyme within the sense of the present invention is a coenzyme which is chemically modified compared to the native coenzyme and has a higher stability towards moisture, temperatures especially in the range of 0°C to 50°C, acids and bases especially in the range pH 4 to pH 10 or/and nucleophiles such as alcohols or amines at atmospheric pressure compared to the native coenzyme, and is thus able to exhibit its activity over a longer period than the native coenzyme under identical environmental conditions.

The artificial coenzyme preferably has a higher hydrolytic stability compared to the native coenzyme, with a complete resistance to hydrolysis under the test conditions being particularly preferred. The artificial coenzyme can have a reduced binding constant for the dehydrogenase compared to the native coenzyme, e.g. a binding constant that is reduced by a factor 2 or more.

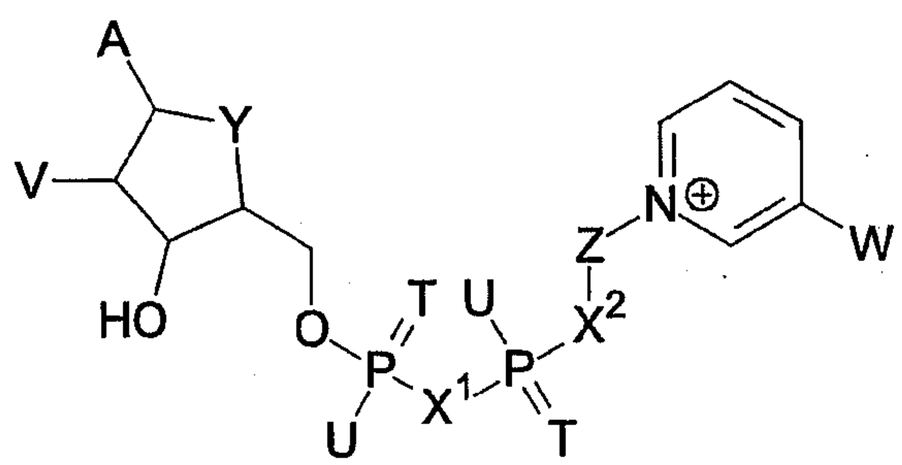
Preferred examples of artificial coenzymes which can be used within the scope of the method according to the invention are artificial NAD(P)/NAD(P)H compounds, i.e. chemical derivatives of native nicotinamide adenine dinucleotide (NAD/NADH) or native nicotinamide adenine dinucleotide phosphate (NADP/NADPH) or the compound of formula (I)



(I)

If the artificial coenzyme is an artificial NAD(P)/NAD(P)H compound, the artificial NAD(P)/NAD(P)H compound preferably comprises a 3-pyridine carbonyl or a 3-pyridine thiocarbonyl residue which is linked without a glycosidic bond to a phosphorus-containing residue such as for example a phosphate residue via a linear or cyclic organic residue, in particular via a cyclic organic residue.

The artificial coenzyme is particularly preferably selected from a compound of the general formula (II):



(II)

in which

A = adenine or an analogue thereof,

T = in each case independently denotes O, S,

U = in each case independently denotes OH, SH, BH_3^-, $BCNH_2^-$,

V = in each case independently denotes OH or a phosphate group, or two groups which form a cyclic phosphate group;

W = COOR, $CON(R)_2$, COR, $CSN(R)_2$ where R in each case independently denotes H or C_1-C_2 alkyl,

X^1,X^2 = in each case independently denote O, CH_2, $CHCH_3$, $C(CH_3)_2$, NH, NCH_3,

Y = NH, S, O, CH_2,

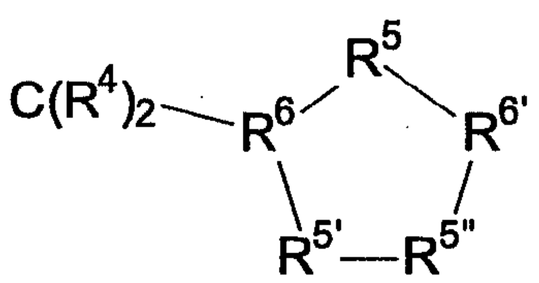
Z = is a linear or cyclic organic residue,

provided that Z and the pyridine residue are not linked by a glycosidic bond,

or a salt or optionally a reduced form thereof.

In the compounds of formula (II), Z is preferably a linear residue having 4-6 C atoms, preferably having 4 C atoms in which 1 or 2 C atoms are optionally replaced by one or more heteroatoms selected from O, S and N, or a residue comprising a cyclic group having 5 or 6 C atoms which optionally contains a heteroatom selected from O, S and N and optionally one or more substituents, and a residue CR^4_2, where CR^4_2 is bound to the cyclic group and to X^2, with R^4 being in each case independently H, F, Cl, CH_3.

Z is particularly preferably a saturated or unsaturated carbocyclic or heterocyclic five-membered ring, in particular a compound of the general formula (III),



(III)

wherein a single or double bond may be present between $R^{5'}$ and $R^{5''}$, in which

R^4 = in each case independently denotes H, F, Cl, CH_3,

R^5 = CR^4_2,

$R^{5'}$ = O, S, NH, NC_1-C_2 alkyl, $CR4_2$, CHOH, $CHOCH_3$, and $R^{5''}$ = CR^4_2, CHOH, $CHOCH_3$ if a single bond is present between $R^{5'}$ and $R^{5''}$,

$R^{5'} = R^{5''} = CR^4$, if a double bond is present between $R^{5'}$ and $R^{5''}$,

and

R^6, $R^{6'}$ = in each case independently denote CH or CCH_3.

In a preferred embodiment, the compounds according to the invention contain adenine or adenine analogues such as e.g. C_8-substituted and N_6-substituted

adenine, deaza variants such as 7-deaza, aza variants such as 8-aza or combinations such as 8-deaza or 7-aza or carbocyclic analogues such as formycin, wherein the 7-deaza variants can be substituted in the 7 position by halogen, C_{1-6} alkinyl, C_{1-6} alkenyl or C_{1-6} alkyl.

In a further preferred embodiment the compounds contain adenosine analogues which contain for example 2-methoxydeoxyribose, 2'-fluorodeoxyribose, hexitol, altritol or polycyclic analogues such as bicycle sugars, LNA sugars and tricyclo sugars instead of ribose.

In particular, (di)-phosphate oxygens can also be isotronically replaced in the compounds of formula (II) such as e.g. O^- by S^- or BH_3^-, O by NH, NCH_3, or CH_2 and =O by =S. In the compounds of formula (II) according to the invention, W is preferably $CONH_2$ or $COCH_3$.

R^5 is preferably CH_2 in the groups of formula (III). Furthermore, it is preferred that $R^{5'}$ is selected from CH_2, CHOH and NH. In a particularly preferred embodiment, $R^{5'}$ and $R^{5''}$ are in each case CHOH. In yet a further preferred embodiment, $R^{5'}$ is NH and $R^{5''}$ is CH_2. A compound of formula (III) in which R^4 = H, $R^5 = CH_2$, $R^{5'} = R^{5''}$ = CHOH and $R^6 = R^{6'}$ = CH is most strongly preferred.

In the most strongly preferred embodiment, the artificial coenzyme is the compound carbaNAD known from the literature (J.T. Slama, Biochemistry (1988), 27, 183 and Biochemistry (1989), 28, 7688). Other stable coenzymes which can be used according to the invention are described in WO 98/33936, WO 01/49247, WO 2007/012494, US 5,801,006, US11/460,366 and the publication by Blackburn et al. (Chem. Comm. (1996), 2765),

The diagnostic element used in the method according to the invention can be any diagnostic element which comprises a dry reagent layer containing the mutated dehydrogenase and the artificial coenzyme and can be wetted by the sample containing the analyte. In addition to the mutated dehydrogenase and the artificial coenzyme, the reagent layer can optionally contain further reagents which are used for the qualitative detection or quantitative determination of the analyte, such as e.g. a suitable mediator as well as suitable auxiliary substances or/and additives.

Diagnostic elements on which the analyte can be applied in the form of an aqueous or non-aqueous solution are preferably used within the scope of the present invention. In a particularly preferred embodiment of the invention, the diagnostic element is a test tape, a test disk, a test pad, a test strip, a test strip drum, or the diagnostic elements mentioned in WO 2005/084530 A2 to which reference is herewith explicitly made. The diagnostic elements described in the present application comprise in each case at least one test area which can be brought into contact with a sample containing the analyte and enables a qualitative or/and quantitative determination of the analyte using suitable means.

The term "test tape" as used herein refers to a tape-like diagnostic element which usually comprises more than one individual test area, preferably at least 10 individual test areas, more preferably at least 25 individual test areas and most preferably at least 50 individual test areas. The individual test areas are preferably each arranged at a distance of a few millimetres to a few centimetres, for example at a distance of < 2.5 cm from one another, and the test tape can optionally comprise marker areas between consecutive test areas to record the distance travelled during tape transport or/and for calibration. Such test tapes are for example described in EP 1739 432 A1.

The term "test disk" as used herein refers to a disk-shaped diagnostic element which can comprise one or more individual test areas, for example at least 10 individual

test areas. In one embodiment, the test disk is coated with a thin layer of the test chemistry, e.g. with a layer having a thickness of about 20 µm, on which a sample of the analyte can be applied whereby an area of the test disk of greater or lesser size is wetted by the sample depending on the volume of the sample and can be used to determine the analyte. The non-wetted area of the test disk which can be partly or completely wetted due to passage of moisture through the test chemistry layer is subsequently available for further determinations of the analyte.

The method according to the invention can be used to determine any biological or chemical substance which can be detected photochemically or electrochemically. The analyte is preferably selected from the group consisting of malic acid, alcohol, ammonium, ascorbic acid, cholesterol, cysteine, glucose, glucose-6-phosphate, glutathione, glycerol, urea, 3-hydroxybutyrate, lactic acid, 5'-nucleotidase, peptides, pyruvate, salicylate and triglycerides, with glucose being particularly preferred. In this connection, the analyte can originate from any source but is preferably contained in a body fluid comprising, but not limited to, whole blood, plasma, serum, lymph fluid, bile, cerebrospinal fluid, extracellular tissue fluid, urine, as well as glandular secretions such as saliva or sweat. The presence and/or the amount of an analyte in a sample from whole blood, plasma, serum or extracellular tissue fluid is preferably determined by means of the diagnostic elements described herein.

The qualitative or/and quantitative determination of the analyte can take place in any manner. For this purpose, all methods for detecting enzymatic reactions which are known from the prior art and which generate a measurable signal that can be analysed or read-out manually or by using suitable means can basically be used. Within the scope of the present invention, optical detection methods which for example comprise the measurement of absorption, fluorescence, circular dichroism (CD), optical rotation dispersion (ORD), refractometry etc. as well as electrochemical techniques are preferably used. The presence or/and the amount of the analyte is particularly preferably determined photometrically or fluorometrically,

e.g. indirectly by means of a fluorometrically detectable change of the artificial coenzyme.

In a further aspect, the invention concerns a diagnostic element for determining an analyte, comprising a dry reagent layer which contains

(a) a mutated dehydrogenase which is specific for the analyte, and

(b) an artificial coenzyme.

With regard to preferred embodiments of the diagnostic element as well as of the mutated dehydrogenase contained therein or the artificial coenzyme contained therein, reference is made to the embodiments in conjunction with the description of the inventive method.

The invention is further elucidated by the following figures and examples:

Description of the figures

Figure 1: Kinetics of wild type glucose dehydrogenase from Bacillus subtilis in the presence of NAD/NADH as the coenzyme at glucose concentrations of 0.0 mg/dl, 35.2 mg/dl, 54.2 mg/dl, 146.6 mg/dl, 249.0 mg/dl, 338.6 mg/dl and 553.6 mg/dl (shown from top to bottom).

Figure 1A: enzyme activity 1556.2 kU/100 g mass

Figure 1B: enzyme activity 1004.0 kU/100 g mass

Figure 1C: enzyme activity 502.0 kU/100 g mass

Figure 1D: enzyme activity 251.0 kU/100 g mass

Figure 1E: enzyme activity 25.10 kU/100 g mass.

Figure 2: Kinetics of a glucose dehydrogenase double mutant GlucDH_E170K_K252L obtained by mutation of wild type glucose dehydrogenase from Bacillus

subtilis in the presence of carbaNAD/carbaNADH as the coenzyme at glucose concentrations of 0.0 mg/dl, 34.4 mg/dl, 141.2 mg/dl, 236.6 mg/dl, 333.8 mg/dl and 525.8 mg/dl (shown from top to bottom). Enzyme activity: 4.60 kU/100 g mass.

Figure 3: Fluorescence spectrum of the complex glucose dehydrogenase (GlucDH)/NADH before and after titration with gluconolactone.

Figure 4: Fluorescence spectrum of the complex glucose dehydrogenase (GlucDH)/NADH before and after titration with glucose.

Figure 5: Kinetics of the conversion of glucose in the presence of wild type glucose dehydrogenase and NADH at various glucose concentrations.

Figure 5A: Kinetics without additionally added gluconolactone at glucose concentrations of 77.0 mg/dl, 207.0 mg/dl, 300.0 mg/dl and 505.0 mg/dl (shown from top to bottom).

Figure 5B: Kinetics with additionally added gluconolactone at glucose concentrations of 96.2 mg/dl, 274.0 mg/dl, 399.0 mg/dl and 600.0 mg/dl (shown from top to bottom).

Figure 6: Representation of the amino acid sequence of the glucose dehydrogenase double mutant GlucDH_E170K_K252L.

Examples

Example 1: Preparation of a double mutant of glucose dehydrogenase from Bacillus subtilis with amino acid substitutions E170K and K252L (GlucDH_E170K_K252L)

In order to generate an enzyme which is stabilized compared to native dehydrogenase, the nucleic acid sequence of glucose dehydrogenase from Bacillus subtilis was introduced in the plasmid pKK177 (cloned via EcoRI and HindIII). The mutations E170K and K252L were introduced by site-specific mutagenesis firstly at position 170 and subsequently by site-specific mutagenesis at position 252 of the amino acid sequence of the wild type glucose dehydrogenase. The respective mutagenesis steps were carried out with the aid of specifically designed primers as part of a PCR reaction.

The PCR product obtained was transformed into Escherichia coli XL1blue MRF'. The cells were plated out, clones containing plasmid were cultured overnight and the enzyme activity was determined before and after temperature stress (stress test: 30 min at 50°C). The results are shown in table 1.

Table 1: Residual activity of wild type glucose dehydrogenase from Bacillus subtilis and the mutants GlucDH_E170K and GlucDH_E170K_K252L after stress (tested in Escherichia coli XL1blue MRF')

	residual activity after stress (%)
wild type glucose dehydrogenase	23
mutant GlucDH_E170K	80
mutant GlucDH_E170K_K252L	130

Positive clones were tested before sequencing a further two times. The double mutant GlucDH_E170K_K252L obtained in this manner was transformed into the production strain Escherichia coli NM522 using pUBS-520 as a helper plasmid.

Example 2: Purification of the double mutant GlucDH_E170K_K252L

10 g biomass of each was taken up in 50 ml of a 30 mM potassium phosphate buffer pH 6.5 and disrupted at about 800 bar. After separation of the cell debris, a chromatography was carried out on DEAE sepharose (GE Healthcare Company) at a loading of < 40 mg protein/ml column volume and using a linear gradient of buffer A (30 mM potassium phosphate buffer pH 6.5) to buffer B (buffer A + 500 mM NaCl). The fractions which exhibited glucose dehydrogenase activity were combined and adjusted with ammonium sulfate (Aldrich Company) to a conductivity of 230 mS/cm.

After centrifugation, the clean supernatant was chromatographically separated on phenyl sepharose FF (GE Healthcare Company) at a maximum loading of 10 mg protein/ml column volume. The elution was carried out using a linear gradient of buffer A which was adjusted with ammonium sulfate to a conductivity of 230 mS/cm to pure buffer A. The fractions were tested for their enzyme activity, combined, rebuffered to a concentration of about 50 mg/ml in 60 mM potassium phosphate buffer pH 6.5, concentrated and lyophilized.

Example 3: Determination of the activity of wild type glucose dehydrogenase from Bacillus subtilis and of the double mutant GlucDH_E170K_K252L in the cuvette test

In order to examine the specific activity or lyophilisate activity of wild type glucose dehydrogenase from Bacillus subtilis as well as of the double mutant

GlucDH_E170K_K252L generated in example 1 in the presence of NAD/NADH or carbaNAD/carbaNADH, a glucose dehydrogenase activity test was carried out for both enzymes.

Preparation of reagent solutions

Tris buffer (0.1 M, pH 8.5; 0.2 M NaCl)

11.68 g NaCl (Sigma-Aldrich Company) and 12.11 g Tris (Sigma-Aldrich Company) were dissolved in about 900 ml double distilled water, adjusted with 1 N HCl to a pH of 8.5 and filled up to 1000 ml with double distilled water.

Dilution buffer (3.8 mM NAD; 0.1 M Tris, pH 8.5; 0.2 M NaCl)

250 mg NAD (Roche Company) was dissolved in 100 ml Tris buffer (0.1 M, pH 8.5; 0.2 M NaCl).

Glucose solution

2 g D(+) glucose monohydrate (Sigma-Aldrich Company) was dissolved in 10 ml double distilled water. The solution was ready-to-use after a standing time of 2 hours at room temperature and adjustment of the mutarotation equilibrium.

NAD solution (15 mM)

10 mg NAD (Roche Company) was dissolved in 1 ml double distilled water.

carbaNAD solution (15 mM)

10 mg carbaNAD (Roche Company) was dissolved in 1 ml double distilled water.

Sample preparation

In order to prepare for the measurement, 10 mg of the enzyme to be examined was dissolved in 1 ml dilution buffer and kept for 60 min at room temperature, in order

to allow a reconstitution. Subsequently it was diluted with dilution buffer to 0.12 to 0.23 U/ml.

Measurement procedure

In order to carry out the measurement 1.35 ml Tris buffer, 0.1 ml glucose solution and 0.05 ml NAD solution or 0.05 ml carbaNAD solution (each incubated to 25°C) were pipetted into a plastic cuvette, mixed together and incubated to 25°C in a cuvette carriage.

After the absorbance of the solution no longer changed (blank value), the reaction was started by introducing 0.025 ml sample into the cuvette, and the absorbance of the sample was monitored for a period of 5 min.

Measurement wavelength: 340 nm
test volume: 1.525 ml
path length: 1 cm
temperature: 25°C
evaluation range: 1 - 5 min

Evaluation

The activity of the respective enzyme in aqueous solution was evaluated using the following equation:

$$\text{activity} = (1.525 \times \Delta A/\text{min} \times \text{dilution factor}) / (\varepsilon_{340} \times 0.025 \times 1) \text{ U/ml}$$

in which:

$\Delta A = A_t - A_0$ = slope of the change in absorbance over time

$\varepsilon_{340} = 6.3$ [l x $mmol^{-1}$ x cm^{-1}]

The results of the determination are shown in table 2.

Table 2: Activity of wild type glucose dehydrogenase from Bacillus subtilis (WT-GlucDH) and of the double mutant GlucDH_E170K_K252L

		WT-GlucDH	GlucDH_E170K_K252L
NAD	U/mg lyophilisate	203	167
	U/mg enzyme	484	270
	Km mM	0.08	0.07
	V_{max} (U/mg lyophilsate)	122	144
carbaNAD	U/mg lyophilisate	3.4	2.3
	U/mg enzyme	8.2	3.7
	% U/mg lyophilisate relative to NAD	1.7 %	1.4 %
	Km mM	0.3	1.4
	V_{max} (U/mg lyophilisate)	3	13

As shown in table 2, the activity of the system WT-glucDH/carbaNAD (8.2 U/mg enzyme) under standardized conditions in a cuvette is two orders of magnitude lower than the activity of the system WT-GlucDH/NAD (484 U/mg enzyme). Likewise it is found that the activity of the double mutant GlucDH_E170K_K252L in the presence of the artificial coenzyme carbaNAD is about two orders of magnitude lower (3.7 U/mg enzyme) than in the presence of the native coenzyme NAD (270 U/mg enzyme).

Example 4: Determination of the kinetics of wild type glucose dehydrogenase from Bacillus subtilis (WT-GlucDH) and of the double mutant GlucDH_E170K_K252L in a dry reagent layer

Various test strips were prepared which either contained the native glucose dehydrogenase from Bacillus subtilis (WT-GlucDH) or the double mutant GlucDH_E170K_K252L prepared in example 1 in combination with NAD/NADH or carbaNAD/carbaNADH as the coenzyme, in order to determine the kinetics of enzymes.

Specifically, firstly a partial solution 1 consisting of 18.4 g 1 M phosphate buffer pH 7.0, 1.4 g Gantrez S97 (International Specialty Products Company), 2.94 g 16 % NaOH solution, 0.34 g Mega 8 (Sigma-Aldrich Company), 0.039 g Geropon T77 (Rhone-Poulenc Company) and 1.90 g polyvinyl pyrrolidone 25000 (Fluka Company) was prepared for this purpose.

This partial solution was subsequently admixed with a partial solution 2 consisting of 0.50 g sodium chloride, 21.3 g double distilled water, 4.43 g Transpafill (Evonik Company) and 2.95 g Propiofan (BASF Company) as well as with a partial solution 3 stored overnight in a refrigerator, the latter consisting of 17.4 g 1 M phosphate buffer pH 7.0, 0.5 g sodium chloride, 14.35 g 2 M dipotassium hydrogen phosphate and the respective amounts of dehydrogenase, coenzyme and optionally bovine serum albumin (BSA; Roche Company) stated in each case in the following table 3. The enzymatically inactive bovine serum albumin was added to the formulation when reduced amounts of native dehydrogenase were used in order to keep the matrix properties of the test strip as constant as possible.

Table 3: Content of dehydrogenase and coenzyme in the test strips used

enzyme	mass enzyme (g)	lyophilisate activity (U/mg)	coenzyme	mass coenzyme (g)	mass BSA (g)	kU/100 g mass
WT-GlucDH	6.2	251	NAD	7.36	0	1556.2
WT-GlucDH	4.0	251	NAD	7.36	2.2	1004.0
WT-GlucDH	2.0	251	NAD	7.36	4.2	502.0
WT-GlucDH	1.0	251	NAD	7.36	5.2	251.0
WT-GlucDH	0.1	251	NAD	7.36	6.1	25.10
GlucDH double mutant	2.0	2.3	carbaNAD	2.1	0	4.60

The test strips obtained in this manner were measured on laboratory measuring instruments (self-made Roche Company) which comprised an excitation LED (375 nm) and conventional detectors (BPW34 blue-enhanced). The applied sample material was blood containing adjusted glucose values. The results of the determination are shown in figures 1 and 2.

As shown in figures 1A-1E, the kinetics of the conversion of glucose by wild type glucose dehydrogenase in the presence of NAD worsened with a decreasing enzyme content in the test strips and thus a decreasing enzyme activity. Thus, one would have expected that the kinetics of the conversion of glucose by means of the double mutant GlucDH_E170K_K252L in the presence of carbaNAD would yield even worse results due to the considerably lower enzyme activity of only 4.60 kU/100 g mass (see table 3).

Figure 2 shows the kinetics of the mutated glucose dehydrogenase obtained according to example 1 in the presence of carbaNAD as the coenzyme. As shown in figure 2, the impairment of the kinetics expected with a reduced enzyme activity does not occur. Rather, the double mutant exhibits better kinetics in the presence of carbaNAD than all formulations listed in table 3 which contain the corresponding wild type glucose dehydrogenase and the native coenzyme NAD.

If one takes into consideration the results described in examples 3 and 4 it appears that it is not the activity of the enzyme but rather the state of the complex formation between enzyme, coenzyme, reduced coenzyme, glucose and gluconolactone that is decisive for the turnover rate of glucose in dry reagent layers, which state of complex formation is apparently better in the case of the mutant prepared in example 1 with carbaNAD/carbaNADH than in the case of the wild type enzyme with native NAD/NADH.

Example 5: Detection of ternary complexes consisting of glucose dehydrogenase, NADH and glucose or gluconolactone

In order to check the existence of ternary complexes consisting of enzyme, reduced coenzyme and glucose or gluconolactone, experiments on the binding of the analyte based on the fluorescence properties of NADH were carried out in the cuvette test.

For this purpose, 1 mg NADH (Roche Company) was dissolved in 1 ml phosphate buffer and the associated fluorescence spectrum was recorded. Subsequently, 10 mg wild type glucose dehydrogenase (GlucDH) from Bacillus subtilis was added whereupon the complex GlucDH-NADH known from the literature was formed, which complex, due to a considerably longer life time of NADH (3 ns compared to 0.4 ns in the free state), yielded a shifted emission maximum at 450 nm (see figure 3). Titration of this complex with gluconolactone lowered the fluorescence while, at the same time, shifting the emission maximum to 427 nm, the latter indicating the presence of a new complex that is the ternary complex GlucDH-NADH-gluconolactone (see figure 3).

The lowering of the fluorescence which also corresponds to a shortening of the life time is presumably due to the rapid energy depletion by the redox pair NADH-gluconolactone. If the binary complex GlucDH-NADH is tritated with glucose, then an ineffective ternary GlucDH-NADH-glucose complex is formed which, due to the

lack of a reducible species, cannot degrade energy in a special manner and therefore tends to exhibit an even longer life time and higher intensity at the same emission maximum (see figure 4).

If the cleavage of the ternary complex GlucDH-NADH-gluconolactone and thus the re-availability of the enzyme is decisive for the rate of conversion of glucose into gluconolactone in a dry reagent layer, addition of gluconolactone should slow down the conversion of glucose because additional gluconolactone (in addition to the gluconolactone formed in the reaction) should inhibit further enzyme complexes.

This assumption was confirmed in a kinetic measurement in which blood was applied to a dry reagent layer according to example 4 of the present application in the absence (see figure 5A) or presence (see figure 5B) of gluconolactone. Figure 5B shows a considerable slowing down of the conversion compared to the sample measured in figure 5A.

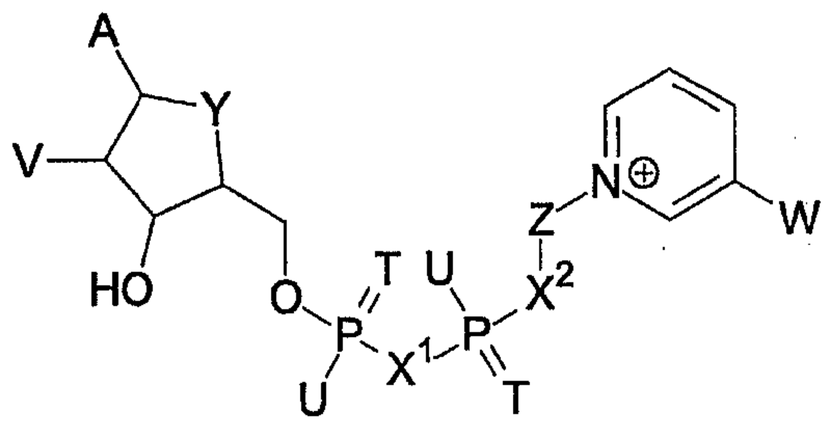
Claims

1. Method for determining at least one of the presence and the amount of an analyte comprising, the steps of:

(a) contacting a sample containing the analyte with a diagnostic element comprising a dry reagent layer which contains;

(i) a mutated glucose dehydrogenase (EC 1.1.1.47) which is specific for the analyte and having an increased in at least one of thermal and hydrolytic stability compared to the corresponding wild-type glucose dehydrogenase, said mutated glucose dehydrogenase comprising a mutation at position 170 consisting of an amino acid substitution of glutamic acid by arginine or lysine, and/or at position 252 consisting of an amino acid substitution of lysine by leucine, of the corresponding wild type glucose dehydrogenase, and

(ii) an artificial coenzyme, said artificial coenzyme being a compound of formula (II):



(II)

in which

A = adenine or an analogue thereof,

T = in each case independently denotes O, S,

U = in each case independently denotes OH, SH, BH_3^-, $BCNH_2^-$,

V = in each case independently denotes OH or a phosphate group, or two groups which form a cyclic phosphate group;

W = COOR, $CON(R)_2$, COR, $CSN(R)_2$ where R in each case independently denotes H or C_1-C_2 alkyl,

X^1,X^2 = in each case independently denote O, CH_2, $CHCH_3$, $C(CH_3)_2$, NH, NCH_3,

Y = NH, S, O, CH_2,

Z = is a linear or cyclic organic residue,

provided that Z and the pyridine residue are not linked by a glycosidic bond, or a salt or optionally a reduced form thereof; and

(b) determining at least one of the presence and the amount of the analyte.

2. The method of claim 1, wherein the turnover rate of the analyte in the diagnostic element is equal or higher than the turnover rate of the analyte in a corresponding diagnostic element which comprises the corresponding wild type coenzyme instead of the artificial coenzyme.

3. The method of claim 2, wherein the turnover rate is at least 20% higher than the turnover rate of the analyte in a corresponding diagnostic element.

4. The method of any one of claims 1 to 3, wherein the mutated dehydrogenase used has a reduced specific enzyme activity compared to the corresponding wild type dehydrogenase.

5. The method of any one of claims 1 to 4, wherein the mutation at position 170 consists of an amino acid substitution of glutamic acid by lysine.

6. The method of any one of claims 1 to 5, wherein the mutated glucose dehydrogenase is a glucose dehydrogenase obtained by mutation of a wild type glucose dehydrogenase from Bacillus megaterium, Bacillus subtilis or Bacillus thuringiensis.

7. The method of any one of claims 1 to 6, wherein the mutated glucose dehydrogenase is a glucose dehydrogenase obtained by mutation of a wild type glucose dehydrogenase from Bacillus subtilis.

8. The method of any one of claims 1 to 7, wherein the mutated glucose dehydrogenase has the amino acid sequence shown in SEQ ID NO:1.

9. The method of any one of claims 1 to 8, wherein the artificial coenzyme is carbaNAD.

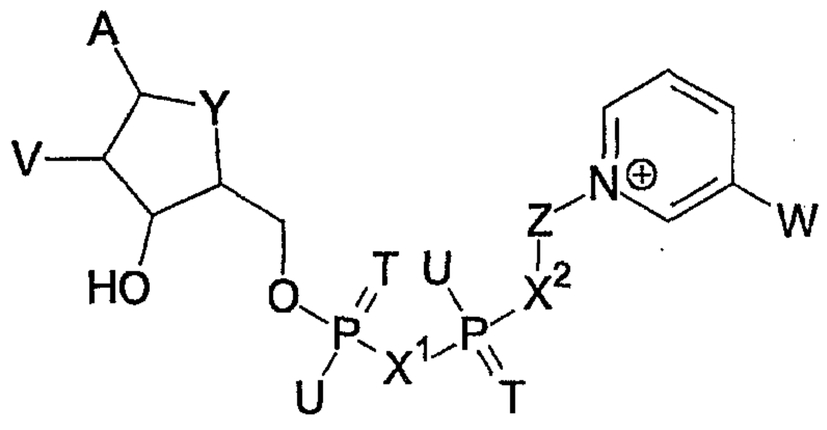
10. The method of any one of claims 1 to 9, wherein a test tape, a test disk, a test pad, a test strip or a test strip drum is used as the diagnostic element.

11. The method of any one of claims 1 to 10, wherein the presence or the amount of the analyte is determined photometrically or fluorometrically.

12. A diagnostic element for determining at least one of the presence and the amount of an analyte, comprising a dry reagent layer which contains

(a) a mutated glucose dehydrogenase (EC 1.1.1.47) which is specific for the analyte and having an increased in at least one of thermal and hydrolytic stability compared to the corresponding wild-type glucose dehydrogenase, said mutated glucose dehydrogenase comprising a mutation at position 170 consisting of an amino acid substitution of glutamic acid by arginine or lysine, and/or at position 252 consisting of an amino acid substitution of lysine by leucine, of the amino acid sequence of the corresponding wild type glucose dehydrogenase, and

(b) an artificial coenzyme, said artificial coenzyme being a compound of formula (II):



(II)

in which

A = adenine or an analogue thereof,

T = in each case independently denotes O, S,

U = in each case independently denotes OH, SH, BH_3^-, $BCNH_2^-$,

V = in each case independently denotes OH or a phosphate group, or two groups which form a cyclic phosphate group;

W = COOR, $CON(R)_2$, COR, $CSN(R)_2$ where R in each case independently denotes H or C_1-C_2 alkyl,

X^1,X^2 = in each case independently denote O, CH_2, $CHCH_3$, $C(CH_3)_2$, NH, NCH_3,

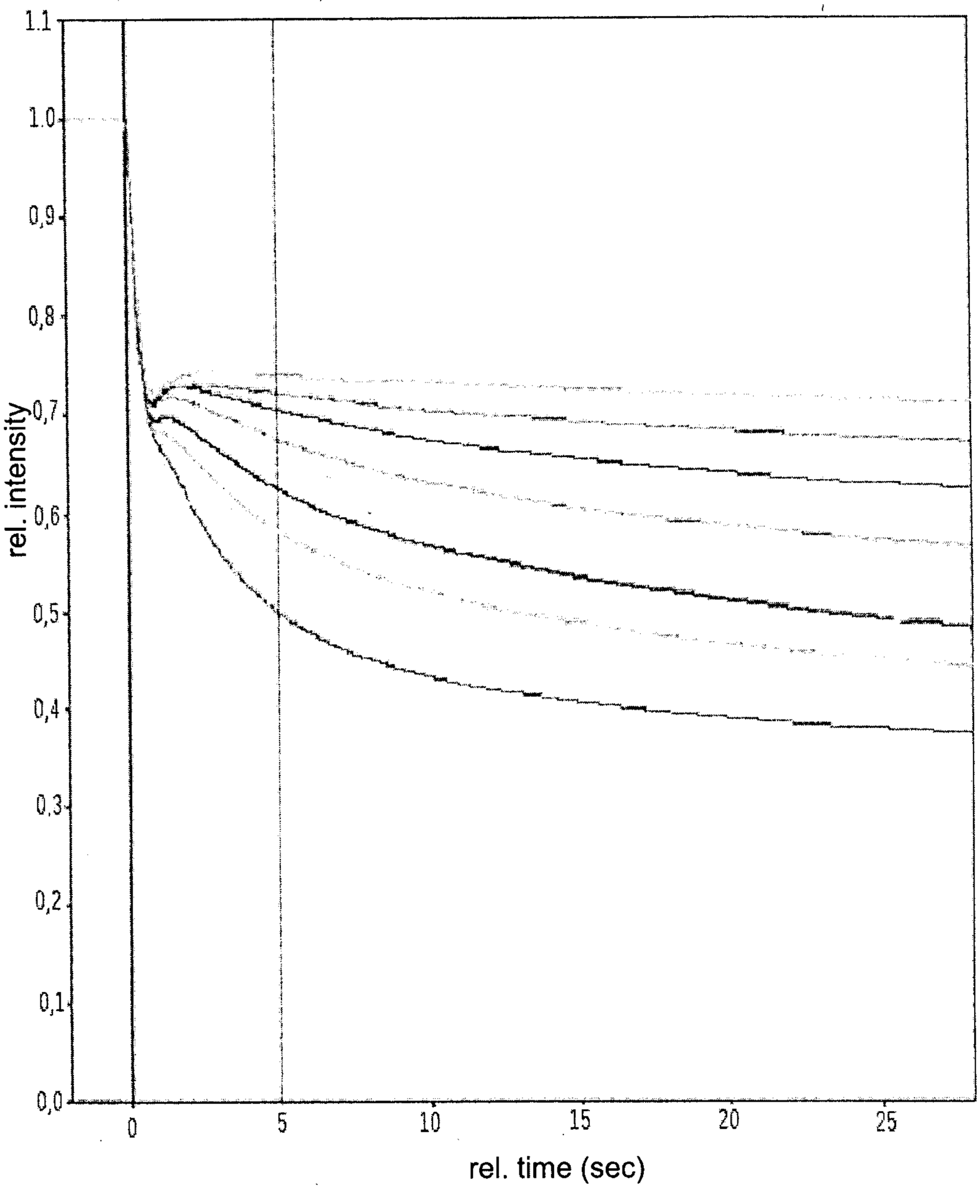
Y = NH, S, O, CH_2,

Z = is a linear or cyclic organic residue,

provided that Z and the pyridine residue are not linked by a glycosidic bond, or a salt or optionally a reduced form thereof.

13. The diagnostic element of claim 12, wherein the mutated glucose dehydrogenase has the amino acid sequence shown in SEQ ID NO:1.

Figure 1A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0,6
0,5
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rel. intensity
0
5
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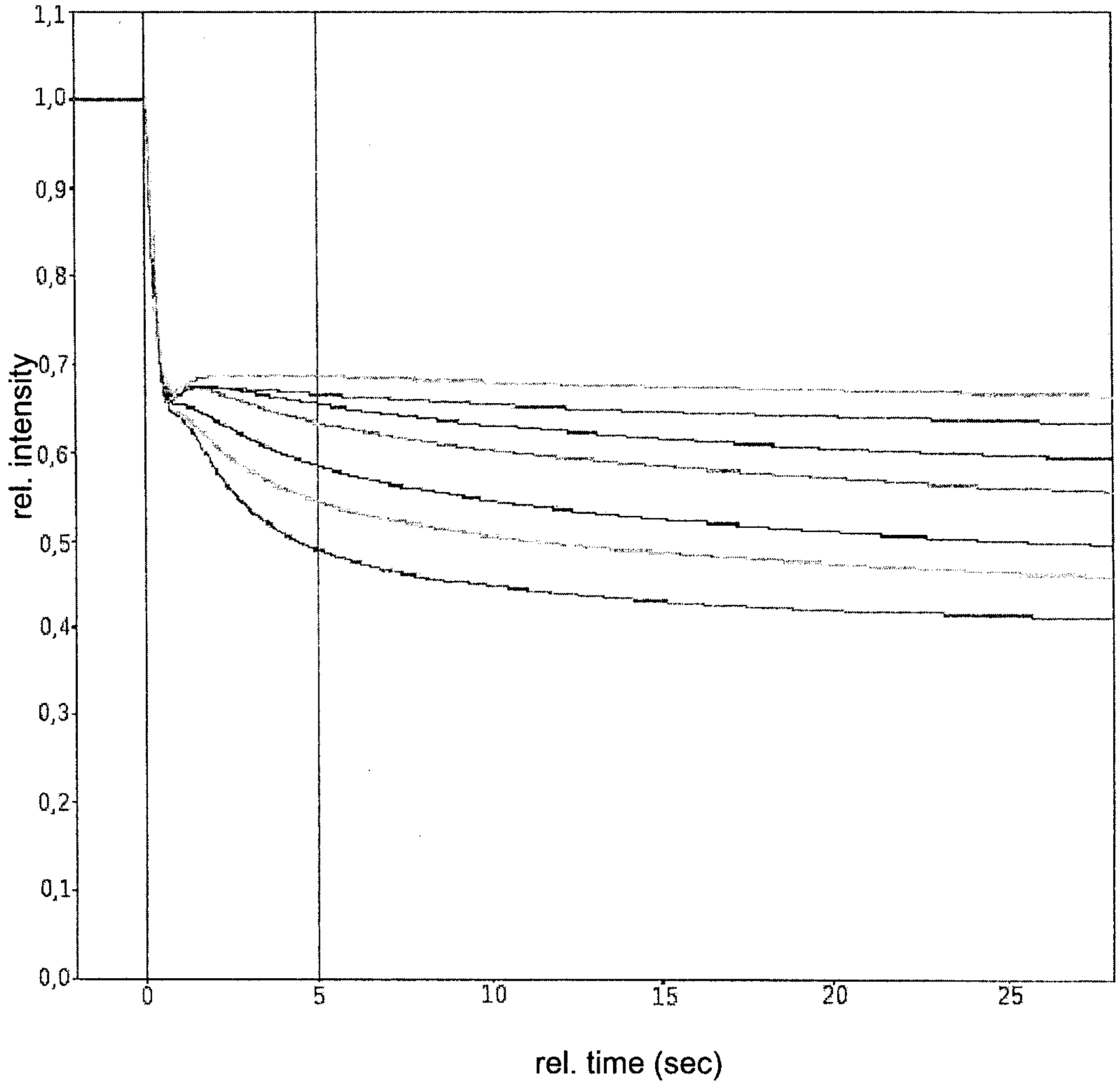
15
20
25
rel. time (sec)

Figure 1B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rel. intensity
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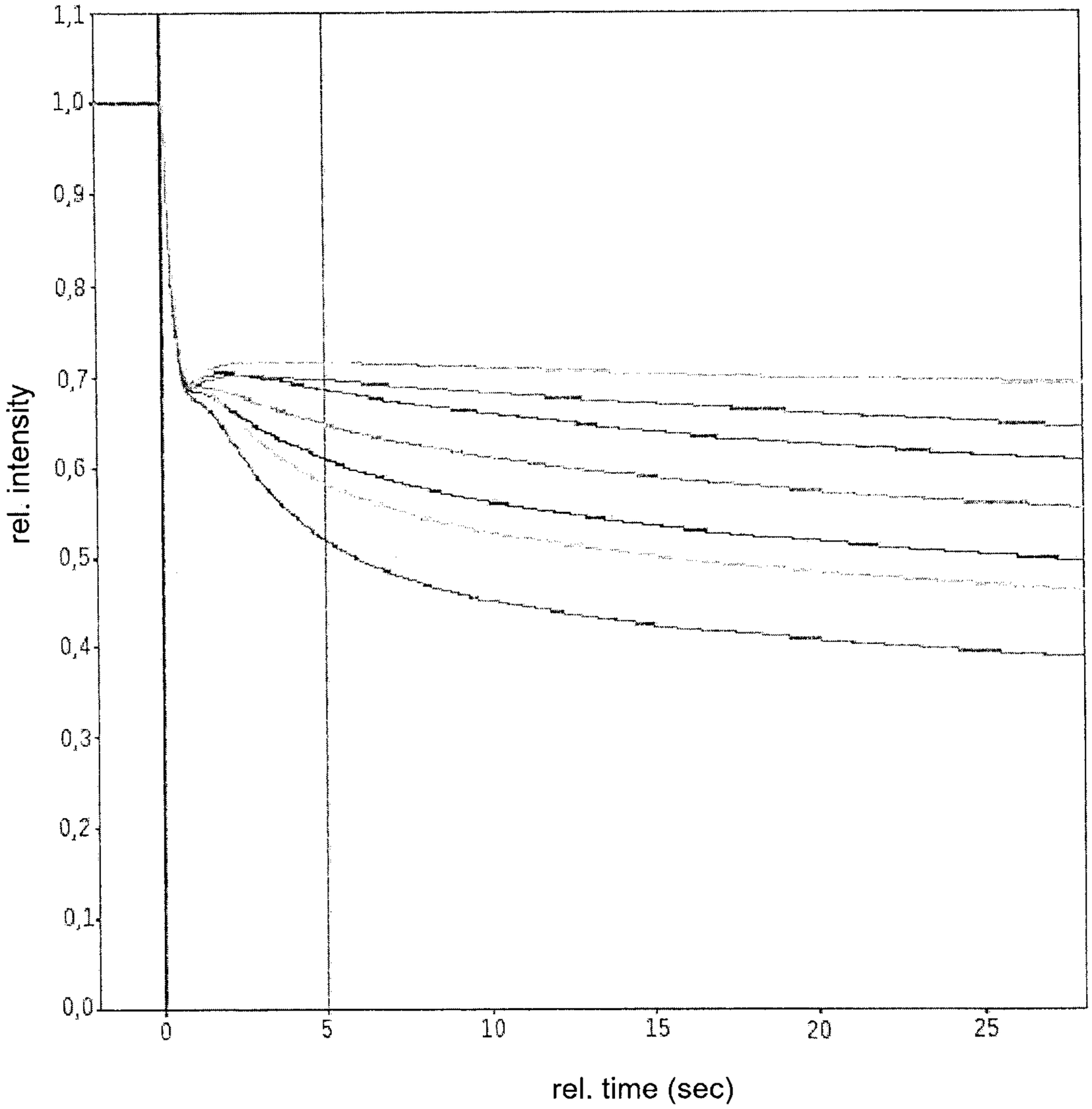
rel. time (sec)

Figure 1C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rel. intensity
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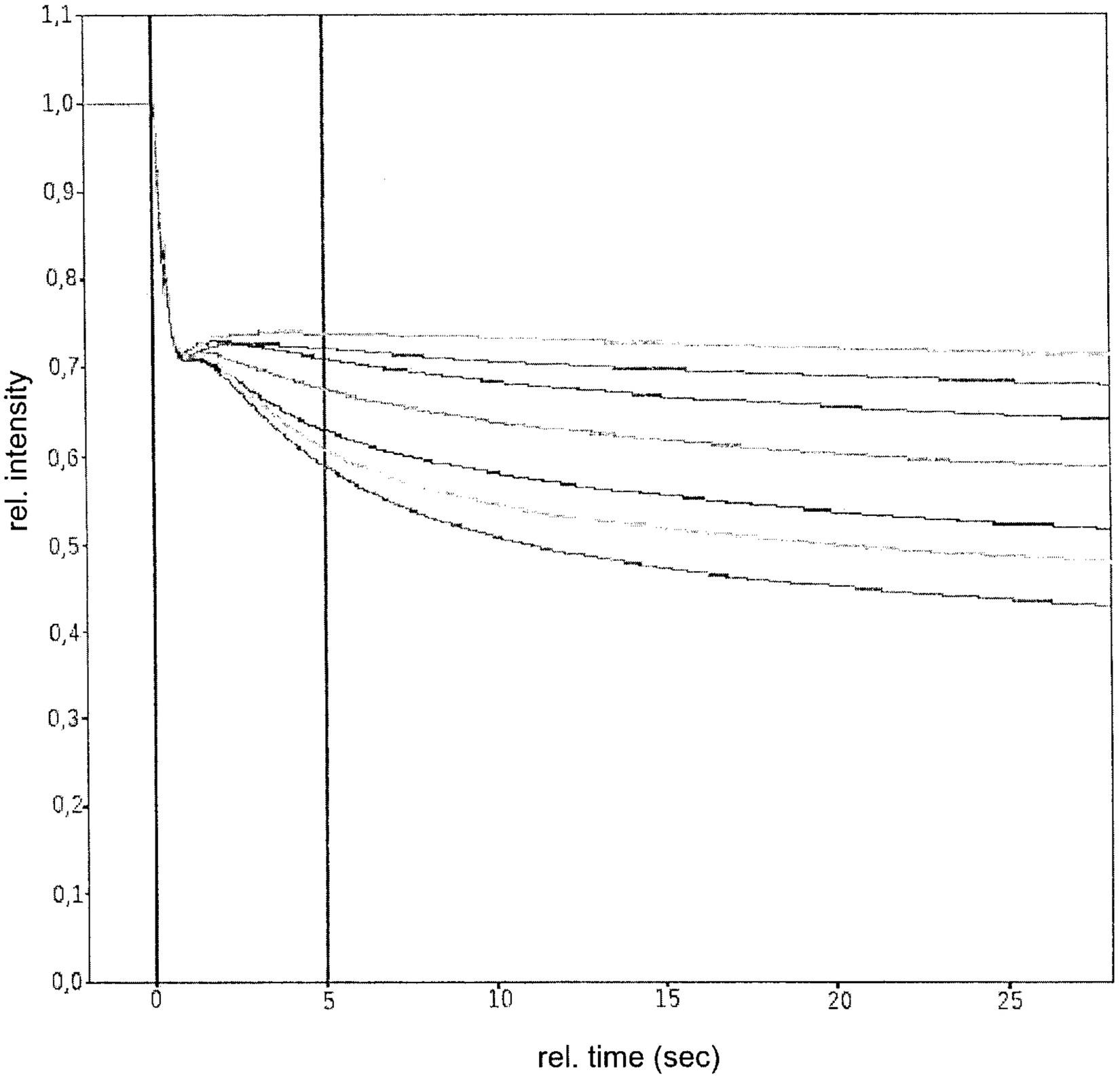
rel. time (sec)

Figure 1D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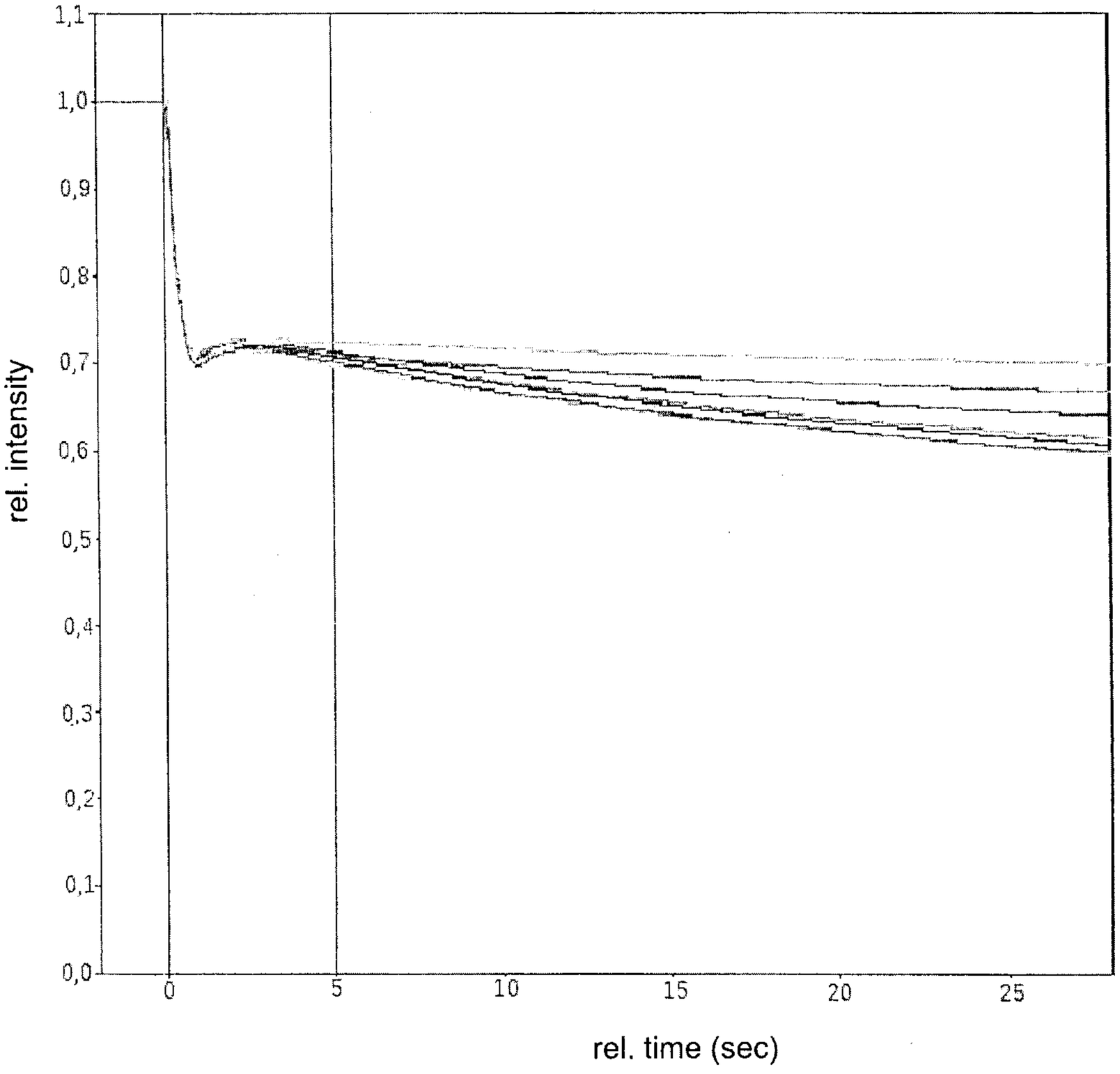
rel. time (sec)

Figure 1E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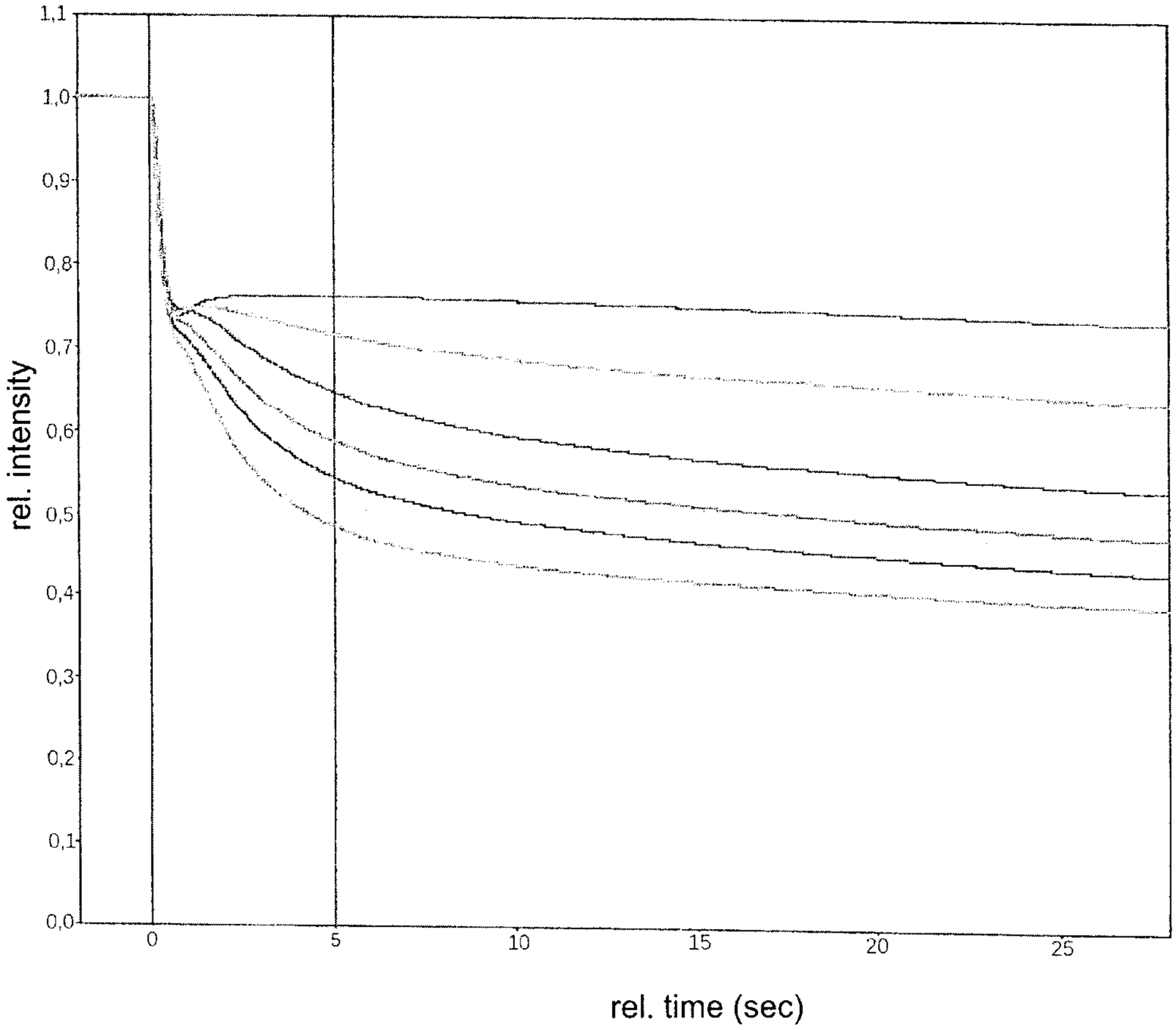
rel. time (sec)

Figure 2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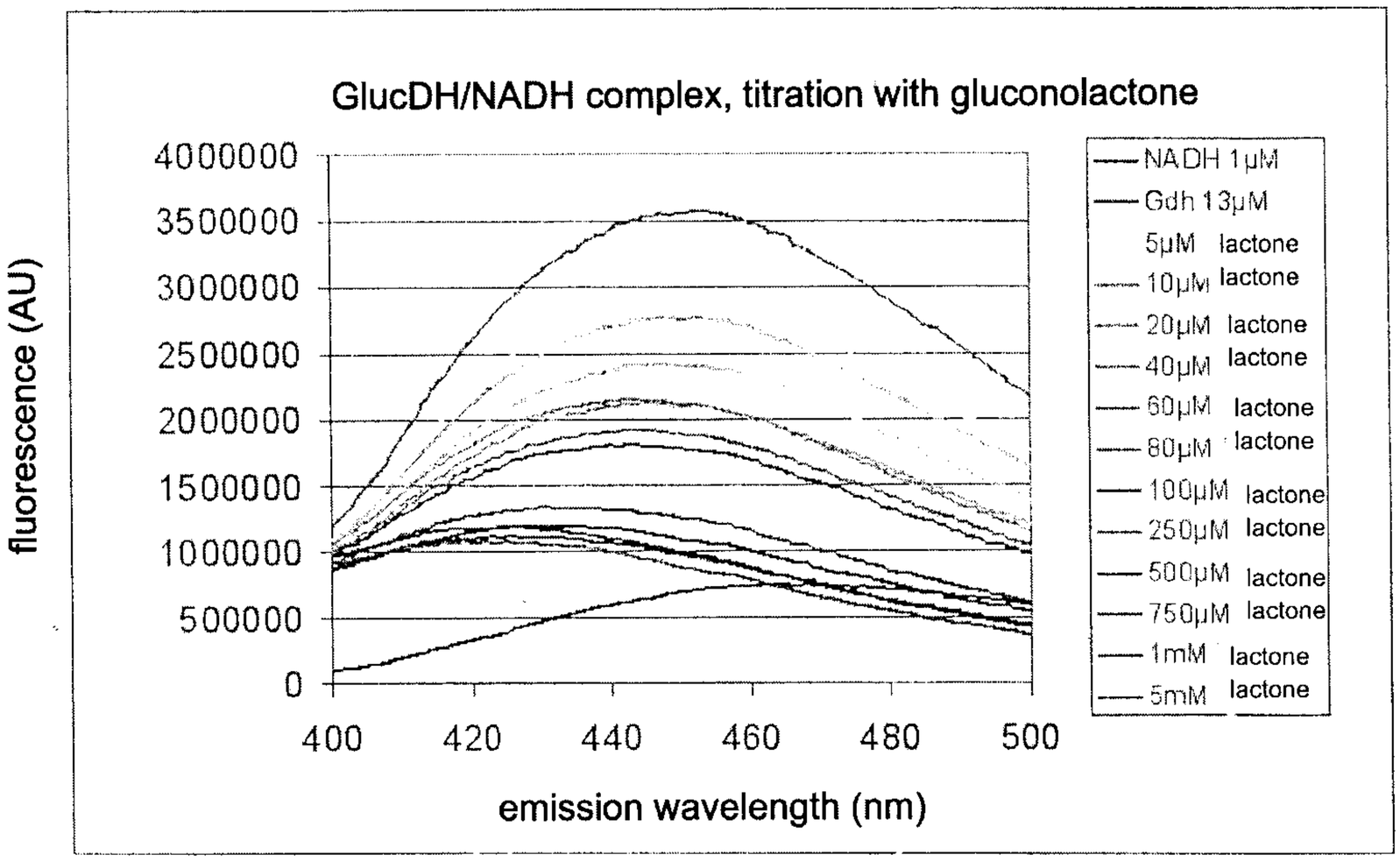
rel. time (sec)

Figure 3


GlucDH/NADH complex, titration with gluconolactone
fluorescence (AU)
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emission wavelength (nm)
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Gdh 13µM
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20µM lactone
40µM lactone
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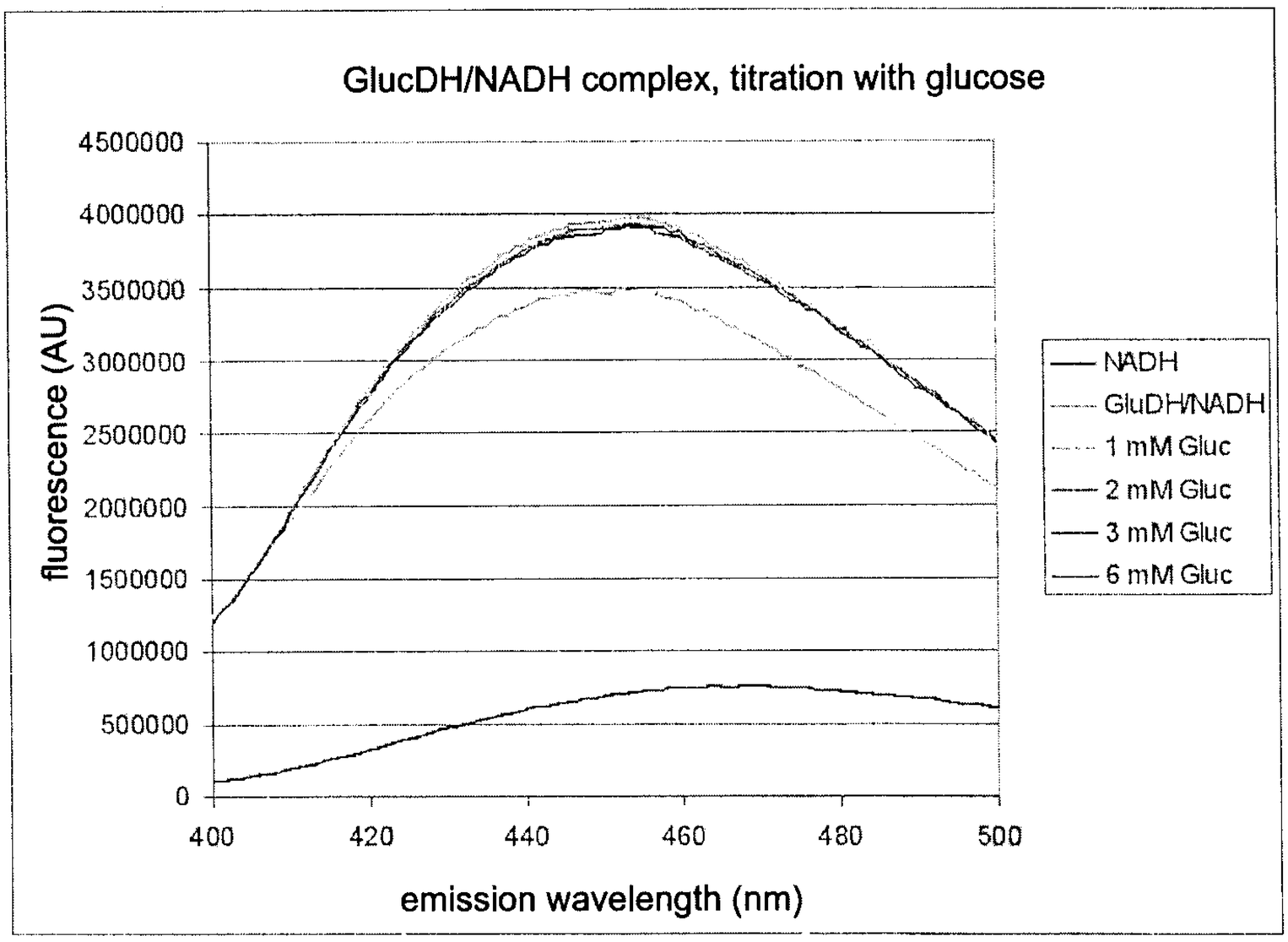
Figure 4


GlucDH/NADH complex, titration with glucose
fluorescence (AU)
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3000000
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2000000
1500000
1000000
500000
0
400
420
440
460
480
500
emission wavelength (nm)
NADH
GluDH/NADH
1 mM Gluc
2 mM Gluc
3 mM Gluc
6 mM Gluc

Figure 5A

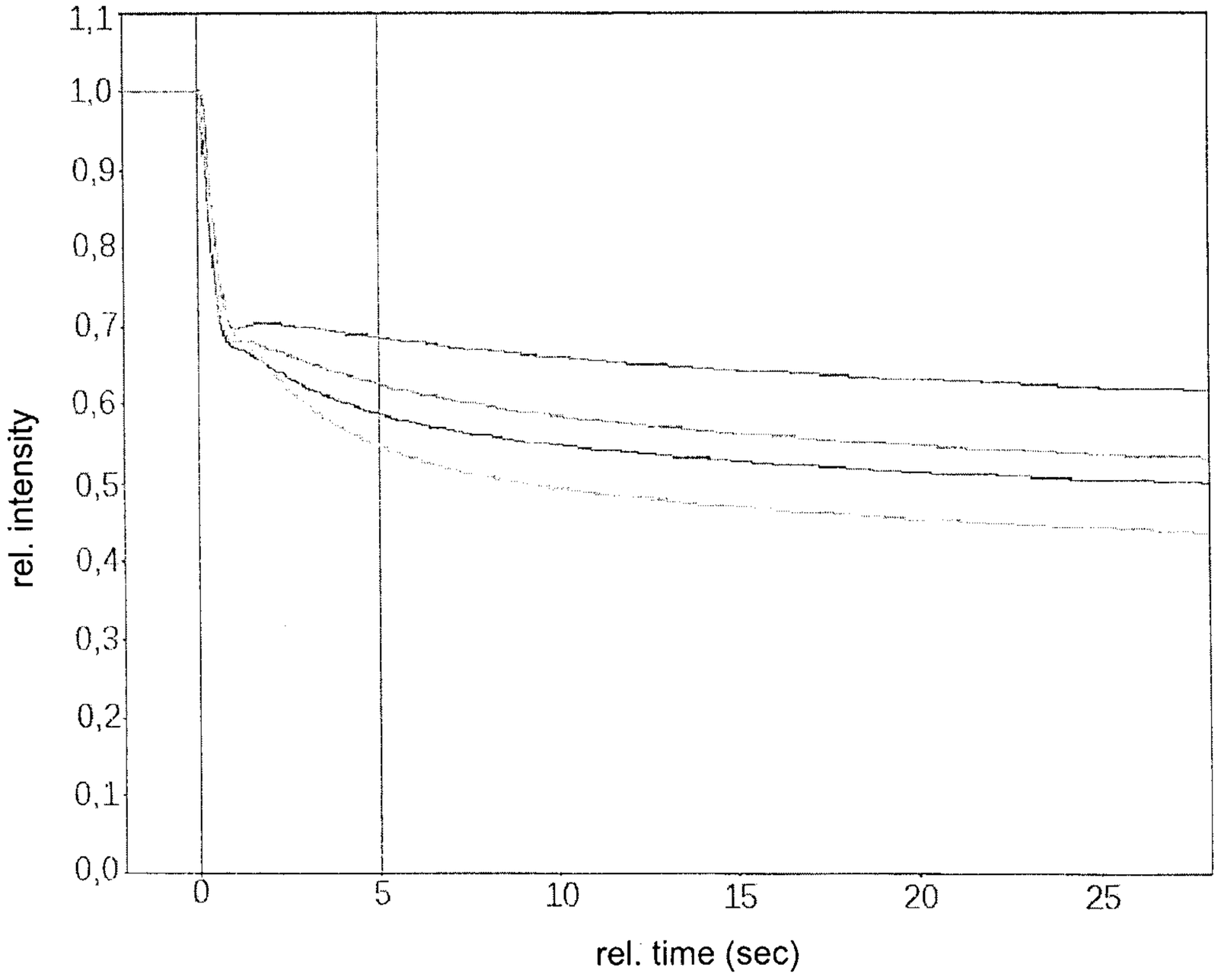
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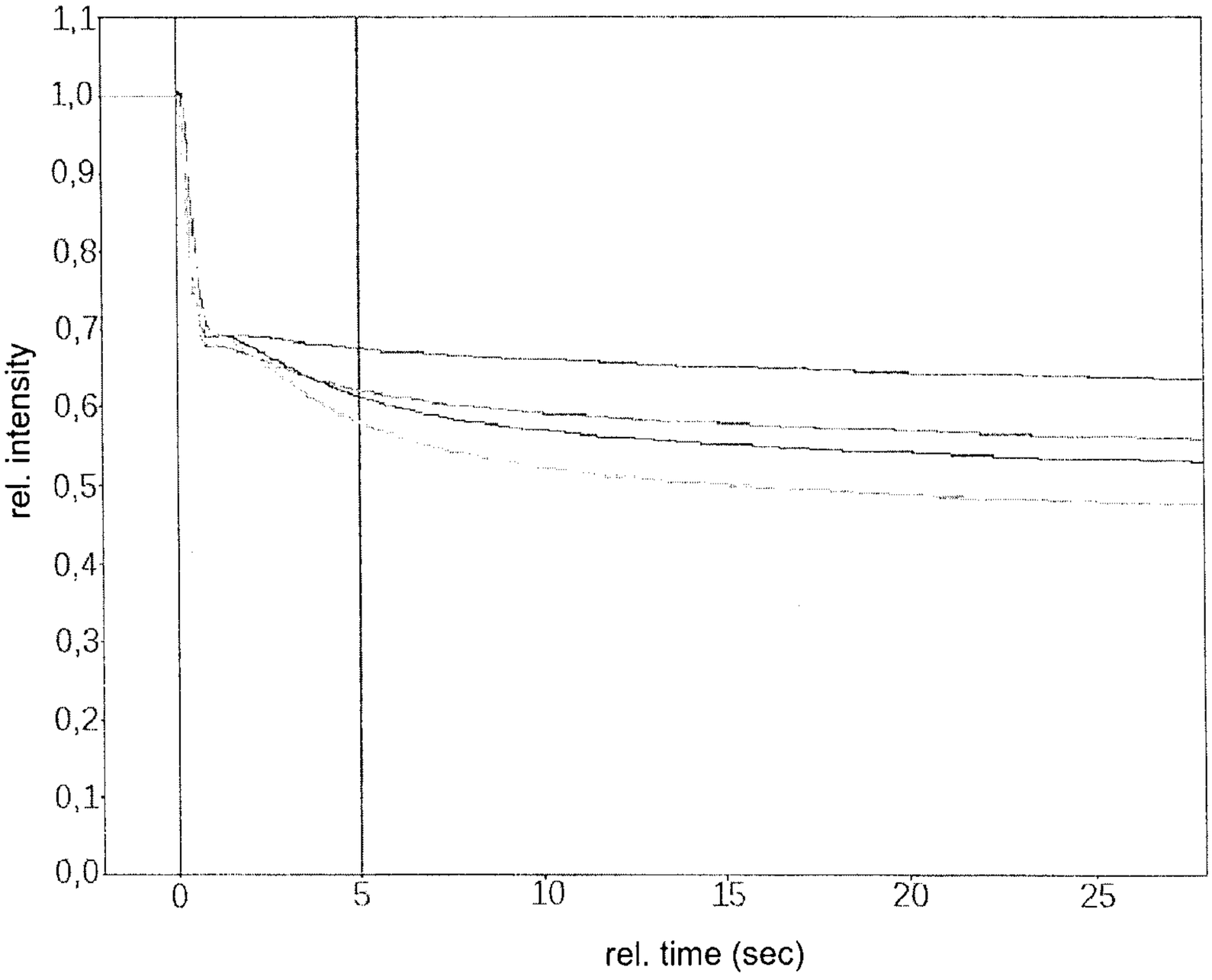
Figure 5B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rel. intensity
0
5
10
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20
25
rel. time (sec)

Figure 6

GlucDH E170K K252L

M Y P D L K G K V V A I T G A A S G L G K A M A I R F G K E
Q A K V V I N Y Y S N K Q D P N E V K E E V I K A G G E A V
V V Q G D V T K E E D V K N I V Q T A I K E F G T L D I M I
N N A G L E N P V P S H E M P L K D W D K V I G T N L T G A
F L G S R E A I K Y F V E N D I K G N V I N M S S V H E V I
P W P L F V H Y A A S K G G I K L M T K T L A L E Y A P K G
I R V N N I G P G A I N T P I N A E K F A D P K Q K A D V E
S M I P M G Y I G E P E E I A V A V W L A S K E S S Y V T
G I T L F A D G G M T L Y P S F Q A G R G