Velocity Factor

The velocity factor is the ratio of the antigen-antibody complex association rate constants determined at 37 °C and 13 °C with which it is possible to classify antibodies e.g. to be an entropic or enthalpic binder without the need of detailed thermodynamic determinations and calculations.

Background of the Invention

The generation of high-affinity antibodies with distinguished antigen specificity and extraordinary antigen complex stability is a major goal in diagnostic and therapeutic antibody development.

Regarding thermodynamic analyses of protein-protein interactions the prevailing technology is the calorimetric assay (Chaires, J.B., Ann. Rev. Biophys. 37 (2008) 135-51; Perozzo, R., et al., J. Recept. Signal Transduct. Res. 24 (1-2) (2004) 1-52; Li, L., et al., Chem. Biol. Drug Des. 71(6) (2008) 529-32; Liang, Y., Acta Biochim. Biophys. Sin. (Shanghai) 40(7) (2008) 565-76; Thielges, M.C., et al., Biochemistry 47(27) (2008) 7237-47). The required sample amount for a reaction calorimeter determination is high, such as an antibody concentration of at least 125 µg/ml and sample volumes of at least 150 µl. Furthermore reaction calorimetry requests high sample purity and does not tolerate any sample impurities or sample heterogeneity. Additionally the sample buffers directly influence the results of the determined thermodynamic parameters. Further, reaction calorimetry is solely able to resolve equilibrium thermodynamics.

Surface Plasmon Resonance (SPR) instrumentation (Roos, H., et al., J. Mol. Recognit. 11(1-6) (1998) 204-210; Van Regenmortel, M.H., et al., J. Mol. Recognit. 11(1-6) (1998) 163-167; Gunnarsson, K., Curr. Prot. Immunol. Chapter 18 (2001) Unit 18.6; Drake, A.W., et al., Anal. Biochem. 328(1) (2004) 35-43; Kikuchi, Y., et al., J. Biosci. Bioeng. 100(3) (2005) 311-7) allows the rapid determination of temperature-dependent kinetic profiles in a high throughput manner (see e.g. Canziani, G.A., et al., Anal. Biochem. 325 (2004) 301-307; Saefsten, P., et al., Anal. Biochem. 353 (2006) 181-190; Leonard, P., et al., J. Immunol. Methods 323 (2007) 172-9).

Wassaf, D., et al. (Anal. Biochem. 351 (2006) 241-253) report high-throughput affinity ranking of antibodies using surface plasmon resonance microarrays. A

thermodynamic analysis of protein interactions with biosensor technology is reported in Roos, H., et al., J. Mol. Recognit. 11 (1998) 204-210.

Wark, K.L., et al. (Adv. Drug. Deliv. Rev. 58 (2006) 657-670) report technologies for the enhancement of antibody affinity. High-resolution characterization of antibody fragment/antigen interactions using BIAcore T100 is reported by Papalia, G.A., et al. in Anal. Biochem. 359 (2006) 112-119. Sagawa, T., et al. (Mol. Immunol. 39 (2003) 801-808) report thermodynamic and kinetic aspects of antibody evolution during the immune response to hapten. An overview of BIAcore systems and application is reported by Jason-Moller, L., et al. in Curr. Prot. Prot. Sci. (2006) page unit 19.13. Gelb, W., reports microcalorimetric methods for peptides studies (Proc. 4th Int. Peptide Sympos. (2007) page 1).

Summary

It has been found that for the characterization of antibodies no throughout kinetic characterization is required but furthermore the determination of the association rate constant at 37 °C and the association rate constant at 13 °C is required. The ratio thereof can be used to classify antibodies according to their binding properties.

In a first aspect, there is provided a method for obtaining an antibody comprising the following steps a) providing a multitude of antibodies, b) determining the association rate constant of each antibody to its antigen at 37 °C and the association rate constant of each antibody to its antigen at 13 °C, c) calculating the ratio of the association rate constant at 37 °C to the association rate constant at 13 °C, d) selecting an antibody with a ratio of 10 or less and thereby obtaining an antibody.

In a second aspect, there is provided a method for obtaining an antibody with cross-reactivity for at least two antigens comprising the following steps a) providing a multitude of antibodies, b) determining the association rate constant of each antibody to an antigen at 37 °C and the association rate constant of each antibody to the same antigen at 13 °C, c) calculating the ratio of the association rate constant at 37 °C to the association rate constant at 13 °C, d) selecting an antibody with a ratio of 50 or more and thereby obtaining an antibody with cross-reactivity for at least two antigens.

In a third aspect, there is provided a method for producing an antibody comprising the following steps: a) providing a multitude of antibody producing cells, b) determining the association rate constant for each antibody produced by each antibody producing cell to an antigen at 37 °C and 13 °C, c) calculating the ratio of the association rate constant at 37 °C to the association rate constant at 13 °C, d) selecting an antibody producing cell producing an antibody with a ratio of 10 or less, e) cultivating the selected cell, f) recovering the antibody from the cultivated selected cell or the cultivation medium thereof and thereby producing an antibody.

In a fourth aspect, there is provided a method for obtaining a humanized antibody comprising a) providing a parent antibody, b) providing a set of humanized forms of the parent antibody, c) determining the association rate constant for each humanized antibody form to its antigen at 37 °C and at 13 °C, d) calculating the ratio of the association rate constant at 37 °C to the association rate constant at 13 °C, e) comparing the ratio of the humanized forms with the ratio of the parent antibody, f) selecting a humanized form as humanized antibody with a ratio that is less than twice the ratio of the parent antibody and thereby obtaining a humanized antibody.

The present disclosure relates to a method for selecting an antibody comprising the step of selecting an antibody with a velocity factor of 10 or less.

In one embodiment the method for selecting an antibody comprises the following steps

a) providing a set of or a population or a multitude of antibodies,
b) determining temperature-dependent kinetic data,
c) calculating the velocity factor for all provided antibodies,
d) selecting an antibody with a velocity factor of 10 or less.

In one embodiment the velocity factor is of from 0 to 10. In another embodiment the velocity factor is the ratio of the association rate constant at 37 °C to the association rate constant at 13 °C. In still another embodiment the temperature-dependent kinetic data is determined at 13 °C and 37 °C. In another embodiment the determining temperature-dependent kinetic data is with surface plasmon resonance. In a further embodiment the method is a high-throughput method. In still another embodiment the selecting is by the velocity factor and by the $\Delta S°‡ass$

value. In one embodiment in the surface plasmon resonance the antigen is immobilized on the surface plasmon resonance chip. In a further embodiment the temperature-dependent kinetic data is thermodynamic equilibrium data and transition state thermodynamic data. In still another embodiment the temperature-dependent kinetic data is calculated with the linear forms of the van't Hoff and Eyring and Arrhenius equations. In one embodiment an antibody is selected with a $\Delta S°‡ass$ of less than 200 J/mol*K ($\Delta S°‡ass < 200$ J/mol*K). In one embodiment an antibody is selected with a $\Delta S°‡ass$ in the range of from -200 J/mol*K to 200 J/mol*K. In also an embodiment an antibody is selected with a $\Delta S°‡ass$ in the range of from -150 J/mol*K to +100 J/mol*K. In another embodiment the surface plasmon resonance is with the HBS-EP buffer supplemented with 1 mg/ml carboxymethyldextrane. In still another embodiment each of the antibodies is produced by a single hybridoma or B-cell.

The present disclosure also relates to a method for obtaining an antibody with cross-reactivity for at least two antigens comprising selecting an antibody with a velocity factor of 50 or more. In one embodiment the velocity factor is of from 50 to 300.

The present disclosure also relates to a method for producing an antibody comprising the following steps:

a) providing a set/population/multitude of antibody producing cells,
b) selecting an antibody producing cell producing an antibody with a velocity factor of 10 or less,
c) cultivating the selected cell,
d) recovering the antibody from the cultivated selected cell or the cultivation medium thereof and thereby producing an antibody.

In one embodiment the antibody producing cells are deposited as single cells. In a further embodiment the deposited single cells are cultivated prior to the selecting. In another embodiment the antibody is purified prior to the selecting. In a further embodiment the antibody is purified after the recovering. In a specific embodiment the purifying is by Protein A chromatography.

The present disclosure also relates to a method for selecting a humanized form of a parent antibody comprising

a) providing a parent antibody,

b) providing a set/population/multitude of humanized forms of the parent antibody,

c) determining temperature-dependent kinetic data of all provided antibodies,

d) calculating the velocity factor for all provided antibodies,

e) comparing the velocity factors of the humanized forms with the velocity factor of the parent antibody,

f) selecting a humanized form of a parent antibody by selecting an antibody with a velocity factor that is less than twice of the velocity factor of the parent antibody.

In one embodiment the selecting is of a humanized from with a velocity factor that is less than 125% of the velocity factor of the parent antibody, in another embodiment less than 110% of the velocity factor of the parent antibody.

The present disclosure also relates to the use of an antibody selected with a method as reported herein as therapeutic or diagnostic agent. The present disclosure also relates to a pharmaceutical composition comprising an antibody obtained with a method as reported herein.

Detailed Description of the Invention

The methods reported herein are based on the determination of the velocity factor. Based on the velocity factor antibodies can be classified, i.e. antibodies can be characterized according to their binding properties as e.g. entropic or enthalpic antigen binder. It has been found that a velocity factor based classification does not require detailed thermodynamic to be determined and/or calculated, thus, reducing the amount of parameters to be determined and the number of calculations to be performed. The velocity factor is the ratio of the antigen-antibody complex association rate constants (ka) independently determined at 37 °C and at 13 °C. As only two experimental determinations are required to calculate the velocity factor this is a fast and high-throughput suited method.

The velocity factor can be used e.g. to compare a humanized antibody with the parent antibody from which it is derived. It can also be used to evaluate antibodies directly from hybridoma and/or B-cell supernatants. This does not require a preliminary purification as the determination can be made directly with the crude supernatant.

Surface plasmon resonance based kinetic methods have several advantages over conventional calorimetric assays:

- high throughput processing is possible,
- low sample consumption,
- measurement of affinity instead of avidity, and
- use of crude cell supernatants or complex cultivation mixtures.

The surface plasmon biosensor surface is an affinity matrix, which is used for example for antibody capturing from crude cell cultivation supernatants. Thus, crude and complex mixtures can be used as samples. As one of the interacting partners is immobilized on the sensor's surface and the second compound is injected into the flow system, the measurement of equilibrium and transition state thermodynamics is possible, as the FIA (Flow Injection Analysis) system allows for and can separately monitor the complex association and dissociation phase.

With this technology the calculation of the thermodynamic parameters

- free standard binding enthalpy $\Delta G°$,
- standard binding enthalpy $\Delta H°$,
- standard binding entropy $\Delta S°$,

and of the transition state parameters

- free standard association enthalpy $\Delta G°‡ass$,
- standard association enthalpy $\Delta H°‡ass$,
- standard association entropy $\Delta S°‡ass$,
- activation energy Eaass,
- free standard dissociation energy $\Delta G°‡diss$,
- standard dissociation enthalpy $\Delta H°‡diss$,
- standard dissociation entropy $\Delta S°‡diss$, and
- dissociation energy Eadiss

is possible. In case of using the non-linear van't Hoff equation also a ΔC_p value can be determined.

Generally an SPR-based kinetic antibody screening (see e.g. Steukers, M., et al., J. Immunol. Methods 310 (2006) 126-135; Rich, R.L., et al., Anal. Biochem. 361 (2007) 1-6) is succeeded by a second step of higher resolution thermodynamic SPR analyses.

Antibody producing cell are cultured and the produced cell culture supernatants are subjected to, in one embodiment high throughput, analyses, in which the temperature-dependent kinetic data is generated in order to calculate the velocity factor and transition state (TS) thermodynamic properties. The selection of the antibody according to the method as reported herein is done based on its thermodynamic behavior.

A high affinity antibody is characterized by a temperature-dependent acceleration of the antigen complex association rate constant ka [1/Ms] and a remaining or decelerated antigen complex dissociation rate constant kd [1/s]. Surprisingly, such an antibody is characterized by an antigen interaction mechanism, which shows a large entropy-change in the binding equilibrium. Therefore, a risk assessment must take place by other means than calorimetry to determine from which effect the entropic contribution originates. From the complex association phase, which is risky due to promiscuitive antigen binding, or from the complex dissociation phase, which just indicates a complex antigen interaction, comprising a potentially induced conformational change of the antigen. In one embodiment the entropic contribution comes from the antibody-antigen complex dissociation step, wherein a large positive or a large negative change of the dissociation entropy $\Delta S°‡diss$ takes place.

A high affinity antibody may also have a thermodynamic anomaly originating from the antigen dissociation phase, where the dissociation rate constant kd [1/s] surprisingly and unexpectedly decreases with increasing temperature. Such an antibody is characterized by thermodynamic parameters such as i) a dissociation phase showing a negative or nearby zero dissociation activation energy Eadiss [kJ/mol], ii) a negative dissociation enthalpy $\Delta H°‡diss$ [kJ/mol], and iii) a large negative dissociation entropy $\Delta S°‡diss$ [kJ/mol]. It has to be pointed out that this is a completely theoretical treatment of this effect and shall not be construed as limitation. Thus, in one embodiment the dissociation activation energy Eadiss, the dissociation enthalpy $\Delta H°‡diss$, and the dissociation entropy $\Delta S°‡diss$ are determined and an antibody is selected that has i) a negative or nearby zero dissociation activation energy Eadiss [kJ/mol], ii) a negative dissociation enthalpy $\Delta H°‡diss$ [kJ/mol], and iii) a large negative dissociation entropy $\Delta S°‡diss$ [kJ/mol].

Thus, a method as reported herein allows for the selection of an antibody from a multitude of high affinity antibodies, based on the velocity factor and the $\Delta S°‡ass$ value.

Single cell deposited clones can be cultivated prior to screening as in one embodiment in 100 ml spinner culture flasks using RPMI 1640 medium. In another embodiment the antibodies are purified from the supernatant by Protein A Sepharose(TM) column chromatography prior to the determination of the temperature-dependent kinetic data, i.e. the thermodynamic screening. In one embodiment the system buffer is HBS-EP for the thermodynamic screening. In another embodiment the sample buffer is supplemented with 1 mg/ml carboxymethyldextrane to reduce unspecific sensor matrix effects.

Most publications using SPR-based measurements don't use antibody capture systems as sensor surface presentation technology. Usually the antibody or fragments thereof are covalently immobilized on the sensor. This technology can't be used in a high throughput format, since the surface is not suitable for multi purpose antibody presentation, but it is technically limited by the number of sensors being immobilized with ligands.

In order to perform a thermodynamic screening a species specific capture system with appropriate temperature-dependent secondary antibody complex stability can be established. The biosensor has to be calibrated by an optimization procedure to determine the binding characteristics of antibodies with varying epitope specificities in a high throughput format. The thermodynamic screening provides a temperature-dependent set of data (see Figure 3). At lower temperatures less response is observed, since the capture system's association rate is reduced (see Example 5). At higher temperatures the association rate accelerates.

Further has it been found that at temperatures below 13 °C the capture system's association kinetics are too slow for a sufficient antibody response. Below 13 °C and above 37 °C, the antibody's antigen binding kinetics determination provide for non-linearizable data according to the van't Hoff, Eyring and Arrhenius equations.

In one embodiment the thermodynamic screening is performed at temperatures of 13 °C or 17 °C and 37 °C. It has been found that in this temperature range a simple calculation of the thermodynamic equilibrium data according to the linear form of the van't Hoff equation and a simple calculation of transition state thermodynamics according to the linear Eyring and linear Arrhenius equations is possible (Wear, M.A., et al., Anal. Biochem. 359 (2006) 285-287; see also Figure 5). In one embodiment all measurements are performed under the same conditions in order to make high-throughput-screening (HTS) amenable.

For the calculation the following formulas can be used:

a) Arrhenius equation:

(I) $k = A*e^{(-Ea/R*T)}$

b) van't Hoff calculations:

(II) $\Delta G° = \Delta H° - T*\Delta S°$

(III) $\Delta G° = - R*T*\ln K_D$

(IV) $\ln K_D = -1/T * (\Delta H°/R)/\text{slope} - (\Delta S°/R)/\text{intercept}$

(V) $R*T*\ln K_D = \Delta H°_{T0} - T*\Delta S°_{T0}+\Delta C°_p(T-T_0)-T*\Delta C_p° \ln(T/T_0)$

c) Eyring association phase:

(VI) $ka = (k_b*T/h)*e^{(-\Delta G°‡/R*T)}$

(VII) $\ln ka/T = -1/T * (\Delta H°‡/R)/\text{slope} + (\Delta S°‡*R + \ln k_b/h)/\text{intercept}$

(VIII) $ka = A*e^{-Ea/R*T}$

(IX) $\ln ka = \ln A/\text{intercept} - (1/T*Ea/R)/\text{slope}$

d) Eyring dissociation phase:

(X) $kd = (k_b*T/h)*e^{(-\Delta G°‡/R*T)}$

(XI) $\ln kd/T = -1/T * (\Delta H°‡/R)/\text{slope} + (\Delta S°‡/R + \ln k_b/h)/\text{intercept}$

(XII) $k_d = A*e^{-Ea/R*T}$

(XIII) $\ln kd = \ln A/\text{intercept} - (1/T*Ea/R)/\text{slope}$

with

$\Delta H°$ - standard binding enthalpy,

$\Delta S°$ - standard binding entropy,

$\Delta G°$ - free standard binding enthalpy,

$T*\Delta S°$ - entropic term,

$\Delta H°‡ass$ - standard association binding enthalpy,

$\Delta S°‡ass$ - standard association binding entropy,

$\Delta G°‡ass$ - standard association free binding enthalpy,

Eaass - Arrhenius Parameter for the association,

$\Delta H°‡diss$ - standard dissociation binding enthalpy,

$\Delta S°‡diss$ - standard dissociation binding entropy,

$\Delta G°‡diss$ - standard dissociation free binding enthalpy,

Eadiss - Arrhenius Parameter for the dissociation,
K_D - affinity constant,
ka - association rate constant,
k_b - Boltzmann Constant = ($1.3806503 \times 10^{-23}$ m^2 kg s^{-2} K^{-1}),
kd - dissociation rate constant,
h – Planck constant,
C_p- molar heat capacity.

The temperature dependency of the free binding enthalpy ΔG° can be calculated for each temperature in the range from 13 °C to 37 °C with the formula ΔG° = -R*T*ln K_D. If the value is constant, the linear form of the van't Hoff equation can be used. If ΔG° changes the non-linear form is preferred.

The data obtained in the thermodynamic screening can be visualized in a double logarithmic plot as depicted in Figure 6 a) wherein the kinetic rate constants (k_{on}) ka [1/Ms] and (k_{off}) kd [1/s] are denoted on the X- and Y-axis, respectively. Isometric lines (solid lines) indicate areas of the same affinities, which are plotted in bold at the right side of the diagram. Since the ratio of kd/ka provides for the equilibrium constant K_D [M], each data point is equivalent to an affinity at a respective temperature. The arrow above symbolizes the temperature gradient in steps of + 4 °C starting at 13 °C or 17 °C, respectively, and ending at 37 °C. Temperature-dependent affinity trends of each antibody are connected by a line.

Three exemplary affinity trends are shown in Figure 6 b). In this rate map the K_Ds in steps of + 4 °C of three example antibodies are shown, whereof one is an antibody with increasing affinity with increasing temperature, one is an antibody with constant affinity with increasing temperature, and one is an antibody with decreasing affinity with increasing temperature. Most antibodies show an affinity loss due to lacking antigen complex stability (like those represented by the square in Figure 6 b)). The affinity remains constant when k_{on} and k_{off} increase (circles). A therapeutic or diagnostic antibody preferably shows an increase in k_{on} and k_{off} with increasing temperature or shows a k_{on} acceleration and a k_{off} deceleration. Likewise the affinity increases and the complex gains stability at higher temperatures (filled circles).

The monitoring of the temperature-dependent kinetics as shown in Figure 6 is the basis for the selection of antibodies with temperature-independent or temperature-increasing antigen complex stability.

Figure 7 exemplarily shows two different temperature-dependent kinetics of two antibody forms 1 and 2, which can be obtained in a humanization process. In such a process different combinations of human germline heavy and light chain based sequences can be used to replace murine parental antibody framework sequences. The aim is to select humanized monoclonal antibodies (mAbs) with, preferably, unchanged thermodynamics, specificities and stabilities, when compared to the parental antibody. It can be seen, that antibody 2, in contrast to antibody 1, strongly accelerates its association rate constant ka in the given temperature gradient.

Both antibodies bind the antigen at 37 °C with similar affinity, so that affinity is no suitable selection parameter to discriminate the antibodies.

Since both antibodies strongly differ in their entropic contributions, a much better additional selection parameter is the $\Delta S°‡ass$. For example, the humanized antibody form 1 shows $\Delta S°‡ass$ = 70 +/- 15 J/mol*K (Eyring equation, R^2 = 0.9601) and humanized antibody form 2 shows a binding entropy of $\Delta S°‡ass$ = 350 +/- 70 J/mol*K (Eyring equation, R^2 = 0.9530). The higher binding entropy of humanized antibody form 2 is reflected by a strong acceleration of the association rate constant ka in the temperature gradient of from 13 °C to 37 °C.

It has been found that the acceleration of the antibody-antigen complex formation by a temperature increase, respectively the increase of the association rate constant ka at elevated temperature, correlates with the antibody-antigen association phase entropy $\Delta S°‡ass$.

The antigen-binding interactions of 28 antibodies were thermodynamically quantified. The association phase entropy $\Delta S°‡ass$ and the velocity factor VF (ka(37 °C)/ka(13 °C)) were calculated (see Table 1).

Table 1: $\Delta S°‡ass$ of 28 antibodies calculated according to the Eyring equation; all values show R^2 > 95%. SE: error of the calculation. VF: Velocity Factor.

antibody	antigen	VF	ΔS°‡ass [J/mol*K]	SE [J/mol*K]
antibody 3	4	2	-85	7
antibody 4	5	2	-53	5.1
antibody 5	3	2	-77	5.4
antibody 6	4	2	-73	6.9
antibody 7	4	2	-83	1.2
antibody 8	2	3	-18	3.5
antibody 9	2	3	-10	5.8
antibody 10	5	3	-66	14
antibody 11	1	2	-35	4.1
antibody 12	5	2	-38	13
antibody 13	1	5	-14	1.3
antibody 14	2	5	9	6.6
antibody 15	1	5	-5.3	5.6
antibody 16	1	6	71	23
antibody 17	2	8	77	15
antibody 18	2	9	80	16
antibody 19	2	12	110	29
antibody 20	2	21	160	41
antibody 21	2	74	350	70
antibody 22	2	137	370	78
antibody 23	2	4	6	5.5
antibody 24	2	4	3.6	4.2
antibody 25	2	5	26	9.2
antibody 26	6	5	38	13
antibody 27	2	5	24	6.3
antibody 28	2	6	34	12
antibody 29	2	6	28	12
antibody 30	6	7	62	14
antibody 31	2	8	77	15

Table 1 shows data of 28 different murine, human chimeric and humanized murine antibodies, Fab and Fab'2 fragments from murine or human origin, binding to six different antigens. The antigens are all proteinogenous, conformational antigens,

differing in their molecular weight. The Velocity Factor VF correlates with $\Delta S^{\circ}\ddagger ass$, whereby small values of VF correlate with negative or small values for $\Delta S^{\circ}\ddagger ass$. It has been found that a negative $\Delta S^{\circ}\ddagger ass$ indicates an enthalpy-driven antibody-antigen interaction, a positive $\Delta S^{\circ}\ddagger ass$ values indicate entropic/enthalpic interactions and three digit $\Delta S^{\circ}\ddagger ass$ values stand for fully entropy-driven interactions. Thus, herein is reported a method for selecting a specifically binding antibody comprising the following steps

a) providing a multitude of antibodies,
b) determining the association rate constant of each antibody to its antigen at 37 °C and the association rate constant of each antibody to its antigen at 13 °C,
c) calculating the ratio of the association rate constant at 37 °C to the association rate constant at 13 °C ,
d) selecting a specifically binding antibody by selecting an antibody with a ratio of 10 or less and a $\Delta S^{\circ}\ddagger ass$ value of 100 J/mol*K or less.

In one embodiment the selecting is of an antibody with a $\Delta S^{\circ}\ddagger ass$ value of 50 J/mol*K or less.

The higher $\Delta S^{\circ}\ddagger ass$ the higher is the probability of promiscuitive antibody binding.

Small or negative $\Delta S^{\circ}\ddagger ass$ correlates to specificity and monospecific binding.

It has been found that it is not necessary to determine association phase thermodynamics by linearizing the data according to the Eyring and Arrhenius equations, where it is the task to produce linearizable high quality data in order to calculate the parameters with acceptable errors. Using the methods as reported herein it is sufficient to determine kinetic data at only two temperatures, i.e. at 13° C and at 37 °C.

Based on the experimental data the values ka(37 °C) and ka(13 °C) are determined by using an appropriate kinetic model. Thereafter the Velocity Factor (VF) = ka(37 °C)/ka(13 °C) is calculated and $\Delta S^{\circ}\ddagger ass$ is assessed according to the characteristic curve presented in Figure 8.

Double digit VF-values correlate with three digit $\Delta S^{\circ}\ddagger ass$ values and indicate a higher risk of promiscuitive binding. The data in Figure 8 were modeled by an exponential association function $y = y0 + A1 * (1-\exp(-x/t1)) + A2 * (1-\exp(-x/t2))$.

According to the graph of the exponential equation, the risk to select an unwanted entropy-driven binder can be minimized. $\Delta S°‡ass$ is estimated by the surrogate parameter VF. In contrast to $\Delta S°‡ass$, VF is an instrumentally easy to access parameter.

Figure 8 shows that $\Delta S°‡ass$ reaches a plateau value at the equation parameter A1 = 399.74088 +/- 36.60886 J/mol*K.

The more relevant data is characterized by small VF values of less than 10 and show smaller standard deviations of $\Delta S°‡ass$.

The fitting parameter A2 (A2 = 198.50729 +/- 29.933 J/mol*K) is $\Delta S°‡ass$ at the inflexion of the curve.

Antibodies with $\Delta S°‡ass$ of more than A2 ($\Delta S°‡ass > A2$) are critical, i.e. not well suited, for further processing efforts. Figure 8 indicates, that most of the antibodies analyzed show VF values of less than 10 (< 10). These antibodies can be diagnostically or pharmaceutically used.

Antibodies with $\Delta S°‡ass$ of more than A2, which correlates to a VF of more than 50 show e.g. cross reactivity to further antigens.

Thus, herein is reported a method for selecting a cross-reactive antibody comprising the following steps

a) providing a multitude of antibodies,
b) determining the association rate constant of each antibody to its antigen at 37 °C and the association rate constant of each antibody to its antigen at 13 °C,
c) calculating the ratio of the association rate constant at 37 °C to the association rate constant at 13 °C ,
d) selecting a cross-reactive antibody by selecting an antibody with a ratio of 50 or more and a $\Delta S°‡ass$ value of 200 J/mol*K or more.

In one embodiment the selecting is of an antibody with a $\Delta S°‡ass$ value of 300 J/mol*K or more.

Figure 8 can be segmented into four corridors (see Figure 9). Factors $0 < VF < 20$ indicate a selected screening result. Antibodies in segment 3 show VF factors of more than 20 (VF > 20) and are not selected due to their increased $\Delta S°‡ass$ values.

These antibodies are suspicious to promiscuitive antigen binding and require more detailed analyses to clarify the target binding mechanism.

The classification according to Figure 9 can be used to realize high throughput $\Delta S°‡ass$ based screenings of large antibody populations. For example in the humanization process of antibodies, where the optimal human heavy and light chain compositions are systematically tested for their optimal performance, similar to the parental antibody, the method as reported herein can be used.

The antibodies 8, 9, 17, 18, 20, 21, 22, and 31 as comprised in Table 1 correspond to different stages of a humanization process. From the VF values the process of humanization can be followed. The parental murine antibody 8 shows a low VF value and a negative $\Delta S°‡ass$. The chimeric antibody 9 shows still unchanged VF as well as $\Delta S°‡ass$. The fully humanized antibodies, which are of different VL/VH chain sequence combinations, show discrepancies of VF and therefore of $\Delta S°‡ass$. Finally selected was antibody 31, which shows the lowest VF as well as $\Delta S°‡ass$, whereas antibody 21 and antibody 20 were not selected due to thermodynamic instability, lower functionality in cell culture assays, and/or promiscuitive antigen binding.

Table 1 indicates that the diagram shown in Figure 9 can also be used for antibody fragments.

The method as reported herein can also be used for obtaining antibodies or antibody producing cells e.g. directly originating from cell mixtures, in one embodiment hybridoma or B-cells, or in another embodiment from a single deposited hybridoma or B-cell, wherein antibodies of potentially inhomogeneous maturation status are excluded, because it is of importance to identify fully mature antibodies with enthalpic binding behavior.

The method as reported herein can be used in high throughput format. Generally, $\Delta S°‡ass$ values are calculated from time-consuming measurements by using a wide-spanning temperature-range and as many data spots as possible in order to get a good linear correlation to calculate transition state parameters according to the Eyring equation with $R^2 > 95\%$.

In the methods as reported herein by using only two temperatures, 13 °C and 37 °C, and the reduced kinetic "2 over 2" model precise velocity factors for the association rate can be obtained. This is essential for high throughput $\Delta S°‡ass$

measurements. When combining the high throughput kinetic screening for the identification of highly stable antigen complex forming antibodies with the high throughput determination of the VF factors, best in class antibodies can be selected.

For example, when the VF values in Table 2 are referenced to the ΔS°‡ass/VF diagram it can be seen, that the monoclonal antibody denoted as 1F8 does not show elevated association phase entropy. This has been confirmed also in a different experimental format. The mean VF value of all 16 buffer conditions is 4. The VF value in the reference Table 1 is 4 under similar buffer conditions. This example gives evidence for the applicability of high throughput ΔS°‡ass analyses by using the VF factors as a ΔS°‡ass surrogate.

Table 2: Matrix showing the Velocity Factors (VF = ka(37 °C)/ka(13 °C)) of the antibody 1F8/antigen interactions for 16 different buffer conditions. All interactions were measured in PBS with varying KCl concentrations and different pH values. Antibody 1F8 would therefore populate segment 1 of Figure 9.

1F8	**pH ↓**				
KCl [mM] →		2.7	54	162	324
	6.8	2	4	6	4
	7.0	4	3	4	3
	7.4	4	5	4	4
	7.8	4	4	4	5

The antibody selected with a method as reported herein can be produced recombinantly. Methods for recombinant production of antibodies are known in the state of the art and comprise protein expression in prokaryotic and eukaryotic cells with subsequent isolation of the antibody and usually purification to a pharmaceutically acceptable purity. For the expression of the antibodies as aforementioned in a host cell, nucleic acids encoding the respective light and heavy chains can be inserted into expression vectors by standard methods. Expression can be performed in appropriate prokaryotic or eukaryotic host cells like CHO cells, NS0 cells, SP2/0 cells, HEK293 cells, COS cells, PER.C6(R) cells, yeast, or E.coli cells, and the antibody can be recovered from the cells (supernatant or cells after lysis). General methods for recombinant production of antibodies are well-known in the state of the art and described, for example, in the review articles of Makrides,

S.C., Protein Expr. Purif. 17 (1999) 183-202; Geisse, S., et al., Protein Expr. Purif. 8 (1996) 271-282; Kaufman, R.J., Mol. Biotechnol. 16 (2000) 151-160; Werner, R.G., Drug Res. 48 (1998) 870-880.

The term "host cell" as used in the current application denotes any kind of cellular system which can be engineered to generate the antibodies according to the current invention. In one embodiment HEK293 cells and CHO cells are used as host cells. As used herein, the expressions "cell," "cell line," and "cell culture" are used interchangeably and all such designations include progeny. Thus, the words "transformants" and "transformed cells" include the primary subject cell and cultures derived there from without regard for the number of transfers. It is also understood that all progeny may not be precisely identical in DNA content, due to deliberate or inadvertent mutations. Variant progeny that have the same function or biological activity as screened for in the originally transformed cell are included.

Expression in NS0 cells is described by, e.g., Barnes, L.M., et al., Cytotechnology 32 (2000) 109-123; Barnes, L.M., et al., Biotech. Bioeng. 73 (2001) 261-270. Transient expression is described by, e.g., Durocher, Y., et al., Nucl. Acids Res. 30 (2002) E9. Cloning of variable domains is described by Orlandi, R., et al., Proc. Natl. Acad. Sci. USA 86 (1989) 3833-3837; Carter, P., et al., Proc. Natl. Acad. Sci. USA 89 (1992) 4285-4289; and Norderhaug, L., et al., J. Immunol. Methods 204 (1997) 77-87. A transient expression system (HEK 293) is reported by Schlaeger, E.-J., and Christensen, K., in Cytotechnology 30 (1999) 71-83 and by Schlaeger, E.-J., in J. Immunol. Methods 194 (1996) 191-199.

The term "specifically binding" denotes the binding of an antibody or Fab fragment with a dissociation constant (=K_{Diss}) of at least 10^{-8} mol/l or less, i.e. 10^{-9} mol/l, or in the range of 10^{-8} to 10^{-13} mol/l.

The following examples and figures are provided to aid the understanding of the present invention, the true scope of which is set forth in the appended claims. It is understood that modifications can be made in the procedures set forth without departing from the spirit of the invention.

Description of the Figures

Figure 1 Illustration of the Binding Late (BL) and Stability Late (SL) data of exemplary antibodies.

Figure 2 Binding Late/Complex Stability plot of 549 hybridoma primary cultures: the encircled data spot shows sufficient antigen response signal and 100% complex stability, whereas the enframed data spot shows no sufficient antigen response.

Figure 3 Secondary antibody response of the <IgGFCγM>R antibody capture system versus the analyte monoclonal antibody at 25 nM, 50 nM, 75 nM and 100 nM and at increasing temperatures.

Figure 4 a) Exemplary concentration–dependent sensogram of the temperature-dependent antibody-antigen interaction of antibody M D1.1. The kinetics were measured in HBS-EP pH 7.4 at 25 °C, 3 min. association time, 5 min. dissociation time, fitting according to Langmuir model;
b) Exemplary concentration–dependent sensogram of the temperature-dependent antibody-antigen interaction of antibody M 9.3.1. The kinetics were measured in HBS-EP pH 7.4 at 25 °C, 3 min. association time, 15 min. dissociation time, fitting according to a Langmuir 1.1. model.

Figure 5 Calculation of thermodynamic parameters according to the linear equations of van't Hoff, Eyring and Arrhenius. Exemplary plots shown for antibody M D1.1.

Figure 6 a) Double logarithmic plot of the temperature-dependent characteristics of 34 exemplary antibodies;
b) Double logarithmic plot of the temperature-dependent characteristics of three exemplary antibodies: filled circles – antibody with increasing affinity with increasing temperature, open circles - antibody with constant affinity with increasing temperature, squares - antibody with decreasing affinity with increasing temperature.

Figure 7 Rate map with double logarithmical plotting of the antigen dissociation constant kd [1/s] versus the antigen association rate constant ka [1/Ms]; isometric lines indicate areas of constant affinity; the temperature-dependent kinetics of two humanized antibodies, antibody 1 (filled triangles) and antibody 2 (hollow stars) are plotted in the temperature gradient 13 °C to 37 °C in steps of +4 °C, beginning at slower association rates at the left of the graphic.

Figure 8 Data of Table 1. ΔS°‡ass is plotted versus VF. The data was fitted with an exponential association model according to the equation y = y0 + A1 * (1-exp(-x/t1)) + A2 * (1-exp(-x/t2)); Y0 = -185.05789 +/- 36.83135; A1 = 399.74088 +/- 36.60886; t1 = 41.93317 +/- 9.834; A2 =

198.50729 +/- 29.933; t2 = 3.13298 +/- 1.32572; R^2 = 0.97575. The dashed line at VF = 10 indicates, that most of the suitable interactions show VF < 10.

Figure 9 Segmentation of the plot of Figure 8 in four corridors by short dotted lines. The segments are numbered 1, 2, 3 and 4.

Example 1

Immunization of mice

Balb/c mice 8-12 weeks old were subjected to intraperitoneal immunization with 100 µg of the antigen formulated as a KLH (keyhole limpet haemocyanine) fusion in complete Freud's adjuvant.

The immunization was performed 4 times: initial boost, 6 weeks, 10 weeks and 14 weeks after the initial boost. The second and third immunization was done using incomplete Freud's adjuvant. The final boost was done i.V. using 100 µg antigen three days before the hybridoma fusion took place. The production of hybridoma primary cultures was done according to Köhler and Milstein (Kohler, G., et al., Nature 256 (1975) 495-497). The hybridomas were isolated in 96-well micro titer plates (MTPs) by limited dilution and were screened for antigen binding by ELISA methods according to the manufacturer's manual. Primary hybridoma cell cultures, which showed a positive color formation upon antigen binding in ELISA, were transferred into the kinetic screening process.

Example 2

Preparation of the CM5 sensor chip

The BIAcore A100 system under the control of the Software V.1.1 was prepared like follows: A BIAcore CM5 sensor (series S) was mounted into the system and was hydrodynamically addressed according to the manufacturer's recommendations.

In case of analyzing a murine antibody, the polyclonal rabbit anti-IgG antibody <IgGFCγM>R (Jackson ImmunoResearch Laboratories Inc.) was immobilized on the flow cells via EDC/NHS chemistry according to the manufacturer's instructions.

In case of using human chimeric or fully humanized antibodies, the polyclonal goat antibody pAb<h-lgG,Fcg-Frag>G-IgG(IS) (Jackson ImmunoResearch Laboratories

Inc.) was immobilized on all flow cells via EDC/NHS chemistry according to the manufacturer's instructions.

In case of using murine IgG Fab or Fab'2 fragments, the polyclonal goat antibody <MFab> G-IgG(IS) (Bethyl L. Cat. No. A90-100A-5 v. 9.8.2000) was immobilized on all flow cells via EDC/NHS chemistry according to the manufacturer's instructions.

In case of using human or humanized IgG Fab or Fab'2 fragments, the polyclonal goat antibody <huFab'2>G-IgG (Jackson Immuno Research Laboratories Inc.) was immobilized on all flow cells via EDC/NHS chemistry according to the manufacturer's instructions.

Example 3

Kinetic screening of primary hybridoma culture supernatants

Hybridoma culture supernatants from different immunization campaigns conducted according to Example 1 were processed as outlined below.

The spots 2 and 4 of a sensor chip obtained according to Example 2 were used as a reference (1-2, 5-4). In order to capture antibody on the sensor surface hybridoma culture supernatants were diluted 1:5 with running buffer HBS-EP (10 mM HEPES pH 7.4, 150 mM NaCl, 1 mM EDTA, 0.05% P20, BIAcore) and were injected at 30 µl/min. for 1 min. Subsequently, the respective antigen was injected at 30 µl/min. for 2 to 3 min. association time. The dissociation phase was monitored for 5 to 15 min. Finally the surface was regenerated with a 2 min. injection of 100 mM phosphoric acid.

The sensor was preconditioned by repeated cycles of antibody capturing and regeneration.

For the selection of primary hybridomas the following procedure was used: A Binding Late (BL) reference point was set shortly before the antigen's injection ended. A Stability Late (SL) reference point was set shortly before the end of the complex dissociation phase. The BL and SL data were graphically visualized (Figure 1). The data was used to calculate the antigen complex stability using formula (XIV):

$$(1-[BL\ (RU) - SL\ (RU) / BL\ (RU)]) \quad \text{(XIV)}$$

(see Figure 2). E.g. the encircled data spots show sufficient antigen response signal and 100 % complex stability, whereas the enframed data spot shows no sufficient antigen response.

Thus, the top 10% hybridomas according to antigen response signal and complex stability have been selected.

Example 4

Hybridoma cloning and antibody production

Antibody producing hybridoma primary cultures selected according to Example 3 were subcloned using the cell sorter FACSAria (Becton Dickinson) under the control software V4.1.2. The deposited single clones were incubated under suitable conditions for further proliferation in 24 well plates and were subsequently transferred to the thermodynamic screening process according to Example 5 after having determined the antibody concentration in solution using ELISA methods according to the instruction of the manufacturer.

Example 5

Thermodynamic screening

Secreted antibodies were characterized by a thermodynamic screening employing the determination of the temperature-dependent kinetics in order to determine the antigen-antibody complex thermostability and in order to calculate thermodynamic properties.

A CM5 sensor series S was mounted into the BIAcore T100 System driven under the control software V1.1.1 and preconditioned by 1 min. injection at 100 µl/min. of a mixture comprising 0.1 % SDS, 50 mM NaOH, 10 mM HCl and 100 mM H_3PO_4.

In case of analyzing a murine antibody, the polyclonal rabbit anti-IgG antibody <IgGFCgammaM>R (Jackson ImmunoResearch Laboratories Inc.) was immobilized on the flow cells via EDC/NHS chemistry according to the manufacturer's instructions.

In case of using human chimeric or fully humanized antibodies, the polyclonal goat antibody pAb<h-lgG,Fcg-Frag>G-IgG(IS) (Jackson ImmunoResearch Laboratories

Inc.) was immobilized on all flow cells via EDC/NHS chemistry according to the manufacturer's instructions.

In case of using murine IgG Fab or Fab'2 fragments, the polyclonal goat antibody <MFab> G-IgG(IS) (Bethyl L. Cat. No. A90-100A-5 v. 9.8.2000) was immobilized on all flow cells via EDC/NHS chemistry according to the manufacturer's instructions.

In case of using human or humanized IgG Fab or Fab'2 fragments, the polyclonal goat antibody <huFab'2>G-IgG (Jackson Immuno Research Laboratories Inc.) was immobilized on all flow cells via EDC/NHS chemistry according to the manufacturer's instructions.

The concentration values of the reference antibody were adjusted in order to achieve similar secondary antibody response levels at different temperatures.

Kinetic measurements at different temperatures were performed at 20 µl/min., the flow rate was 30 µl/min., 50 µl/min., 100 µl/min., respectively. The sample injection of the antigen was done for 30 sec., 90 sec., 180 sec., respectively, or other suitable injection times in order to achieve ligand saturation or entry into the binding equilibrium during the complex association phase (see Figure 4 a)). The dissociation rate was monitored first for up to 300 sec. and further for 15 min. (see Figure 4 b)). The antigen injections were repeated in different concentration steps of at least five concentrations. As control one concentration step was analyzed twice to control the reproducibility of the assay. Flow cell 1 served as a reference. A buffer injection was used instead of an antigen injection to double reference the data by buffer signal subtraction. The capture system was regenerated using 100 mM H_3PO_4 by a 2 min. injection at 100 µl/min. The regeneration procedure was optimized to guarantee quantitative surface regeneration also at 13 °C, 17 °C and 21 °C. At these temperatures the regeneration solution was injected three times whereas at 25 °C, 29 °C, 33 °C and 37 °C the regeneration solution was injected one time.

The data obtained was evaluated according to a 1:1 binary Langmuir interaction model in order to calculate the association rate constant ka [1/Ms], the dissociation rate constant kd [1/s] and the resulting affinity constant K_D [M] at different temperatures. Thermodynamic equilibrium data was calculated according to the linear form of the Van't Hoff equation. Transition State thermodynamics were

calculated according to the Eyring and Arrhenius equations using e.g. the BIAcore T100 evaluation software V.1.1.1 or the program Origin 7SRI v. 7.0300.

Example 6

Example for the effect of the adjustment to homogeneous RU_{MAX} values

A BIAcore T100 device was mounted with a CM5 series-S BIAcore sensor, and was immobilized with 6000 RU <IgGFCyM>R (Jackson ImmunoResearch Laboratories Inc., USA) on each flow cell according to the manufacturer's instructions. The non-optimized experiment used 40 nM capture antibody at 20 µl/min., in HBS-EP buffer (0.05 % P20). The sample buffer was the system buffer, supplemented with 1 mg/ml CMD (carboxymethyldextrane).

The antigen was injected after the capturing of the secondary antibody in six concentration steps of 1.2 nM, 4 nM, 11 nM, 33 nM, 100 nM and 300 nM, whereby 11 nM were used as a double control and 0 nM were used as reference. The antigen was injected at 100 µl/min. for 2 min. association and 5 min. dissociation, followed by a HBS-EP wash of 15 min. at 30 µl/min. and a regeneration with 10 mM glycine pH 1.7 at 3µl/min. for 3 min. Concentration-dependent measurements were done at 4 °C, 11 °C, 18 °C, 25 °C, 32 °C, and 40 °C.

The optimized system was used like described above, but with the exceptions that the antibody to be captured was injected for 3 min. association time at different concentration steps of 100 nM at 15 °C, 80 nM at 20 °C, 60 nM at 25 °C, 50 nM at 30 °C, 40 nM at 35 °C and 40 nM at 40 °C.

Finally kinetics and thermodynamics were determined using the BIAcore evaluation software.

Example 7

Determination and calculation of the velocity factor

A CM5 sensor series S was mounted into the BIAcore T100 System.

In case of using murine antibodies, the polyclonal rabbit IgG antibody <IgGFCyM>R (Jackson ImmunoResearch Laboratories Inc.) was immobilized on the flow cells via EDC/NHS chemistry according to the manufacturer's instructions.

In case of using human chimeric or fully humanized antibodies, the polyclonal goat antibody pAb<h-IgG,Fcg-Frag>G-IgG(IS) (Jackson ImmunoResearch Laboratories Inc.) was immobilized on all flow cells via EDC/NHS chemistry according to the manufacturer's instructions.

In case of using murine IgG Fab or Fab'2 fragments, the polyclonal goat antibody <MFab> G-IgG(IS) (Bethyl L. Cat. No. A90-100A-5 v. 9.8.2000) was immobilized on all flow cells via EDC/NHS chemistry according to the manufacturer's instructions.

In case of using human or humanized IgG Fab or Fab'2 fragments, the polyclonal goat antibody <huFab'2>G-IgG (Jackson Immuno Research Laboratories Inc.) was immobilized on all flow cells via EDC/NHS chemistry according to the manufacturer's instructions.

The sample buffer was the system buffer supplemented with 1 mg/ml carboxymethyldextrane to reduce unspecific sensor matrix effects. Kinetic measurements in the temperature gradient 13 °C to 37 °C were performed at 100 µl/min.

The analyte injections of recombinant synthetic human full length antigen 1, recombinant human antigen 2 (10 kDa), recombinant human antigen 3 human FC-chimera (R&D Systems, 160 kDa), recombinant human antigen 4 (29 kDa), or recombinant human antigen 5 (72 kDa) were done for 180 sec. The dissociation rate was monitored for up to 900 sec. The antigen injections were repeated in different concentration steps of at least five concentrations. As a control one concentration step was analyzed twice to control the reproducibility of the assay. Flow cell 1 was used as reference. A blank buffer injection was used instead of an antigen injection to double reference the data by buffer signal subtraction.

Prior to each assay, homogenous RU_{MAX} values in the temperature range 13 °C – 37 °C were adjusted by titration experiments with the respective antibodies to be presented on the sensor surface (see section in which the temperature-dependent titration experiments are described in detail). The kinetics were measured in the temperature gradient 13 °C, 17 °C, 21 °C, 25 °C, 29 °C, 33 °C and 37 °C. The capture systems were regenerated using a buffer wash with HBS-ET buffer at 30 µl/min. for 15 sec., prior to regeneration with 10 mM glycine pH 1.5 at 30 µl/min.

for 15 sec., followed by a 1 min. injection and a 30 sec. injection of 10 mM glycine at pH 1.7.

The data obtained was evaluated according to a 1:1 binary Langmuir interaction model in order to calculate the association rate constants ka [1/Ms], the dissociation rate constants kd [1/s] and the resulting affinity constants K_D [M] at the respective temperatures. Thermodynamic equilibrium data was calculated according to the linear and non linear form of the Van't Hoff equation. Transition State thermodynamics were calculated according to the Eyring and Arrhenius equations using e.g. the BIAcore T100 evaluation software V.1.1.1. Graphic evaluation was done using Origin 7SRI v. 7.0300.

The Velocity Factor (VF) was calculated as the quotient of the antigen complex association rates ka (1/Ms) at 37 °C and 13 °C. The exponential association fitting curve y = y0 + A1 *(1-exp(-x/t1)) + A2 * (1-exp(-x/t2)) was used.

Example 8

High throughput Velocity Factor analysis

A CM5 sensor series S was mounted into the BIAcore A100 System and the detection spots were hydrodynamically addressed according to the manufacturer's instructions.

The polyclonal rabbit IgG antibody <IgGFCyM>R (Jackson ImmunoResearch Laboratories Inc.) was immobilized at 4 kRU on the detection spots 1 and 5 in each flow cell. 800 RU <IgGFCyM>R were immobilized on spots 2 and 4 in each flow cell. Coupling was done via EDC/NHS chemistry according to the manufacturer's instructions. The sample buffer was the system buffer supplemented with 1 mg/ml carboxymethyldextrane to reduce unspecific sensor matrix effects.

The basic buffer system was PBS (phosphate buffered saline). The buffer was adjusted to four different pH conditions: pH 6.8, pH 7.0, pH 7.4 and pH 7.8, and four different KCl concentrations: 2.7 mM, 54 mM, 162 mM and 324 mM. Sixteen different sample buffer conditions were tested.

The mAbs to be captured were injected at 10 µl/min. for 1 min. at different concentration steps between 60 nM at 37 °C and 240 nM at 13 °C to ensure homogenous RU_{MAX} values in the subsequent antigen interaction measurements.

Kinetic measurements in the temperature gradient 13 °C to 37 °C were performed at 30 µl/min. The analyte injections of recombinant human antigen 1-84 (9.4 kDa), were done for 180 sec. The dissociation rate was monitored for 600 sec. The antigen injections were repeated in two concentration steps at 60 nM and 240 nM. A blank buffer injection was used instead of an antigen injection to double reference the data by buffer signal subtraction.

The capture system was regenerated using a 15 sec. buffer wash with HBS-ET buffer at 30 µl/min., prior to regeneration with 10 mM glycine pH 1.5 at 30 µl/min. for 90 sec., followed by 2 x 30 sec. injection of the same buffer.

The kinetic data obtained was evaluated according to a "2 over 2" kinetic model, which uses two different ligand densities of the sensor matrix to calculate kinetics by just using two antigen concentrations. The association rate constants ka [1/Ms], the dissociation rate constants kd [1/s] and the resulting affinity constants KD [M] at the respective temperatures were calculated using the BIAcore A100 evaluation software 1.1.

Thermodynamic equilibrium data was calculated from the kinetic data according to the linear form of the Van't Hoff equation. Transition State thermodynamics were calculated according to the Eyring equations using Excel. Graphic evaluation was done using Origin 7SRI v. 7.0300. The Velocity Factor (VF) for the association phase was calculated as the quotient of the antigen complex association rates ka (1/Ms) at 37 °C and 13 °C. The Velocity Factor for the dissociation rates were calculated as the quotient of kd (1/s) at 13 °C and 37 °C.

Patent Claims

1. Method for obtaining an antibody comprising the following steps

   a) providing a multitude of antibodies,
   b) determining the association rate constant of each antibody to its antigen at 37 °C and the association rate constant of each antibody to its antigen at 13 °C,
   c) calculating the ratio of the association rate constant at 37 °C to the association rate constant at 13 °C ,
   d) selecting an antibody with a ratio of 10 or less and thereby obtaining an antibody.

2. Method according to claim 1, characterized in that the determining is with surface plasmon resonance.

3. Method according to any one of the preceding claims, characterized in that the selection is by the calculating the ratio and by determining $\Delta S^{\circ\ddagger}ass$.

4. Method according to any one of claims 2 to 3, characterized in that in the surface plasmon resonance the antigen is immobilized on the surface plasmon resonance chip.

5. Method according to any one of claim 3 or 4, characterized in that an antibody is selected with a $\Delta S^{\circ\ddagger}ass$ of less than 200 J/mol*K.

6. Method according to any one of the preceding claims, characterized in that each of the antibodies is produced by a single hybridoma or B-cell.

7. Method for obtaining an antibody with cross-reactivity for at least two antigens comprising the following steps

   a) providing a multitude of antibodies,
   b) determining the association rate constant of each antibody to an antigen at 37 °C and the association rate constant of each antibody to the same antigen at 13 °C,
   c) calculating the ratio of the association rate constant at 37 °C to the association rate constant at 13 °C ,
   d) selecting an antibody with a ratio of 50 or more and thereby obtaining an antibody with cross-reactivity for at least two antigens.

8. Method for producing an antibody comprising the following steps:

a) providing a multitude of antibody producing cells,
b) determining the association rate constant for each antibody produced by each antibody producing cell to an antigen at 37 °C and at 13 °C,
c) calculating the ratio of the association rate constant at 37 °C to the association rate constant at 13 °C,
d) selecting an antibody producing cell producing an antibody with a ratio of 10 or less,
e) cultivating the selected cell,
f) recovering the antibody from the cultivated selected cell or the cultivation medium thereof and thereby producing an antibody.

9. Method according to claim 8, characterized in that the antibody producing cells in step a) are deposited as single cells.

10. Method according to any one of claim 8 or 9, characterized in comprising the additional step of cultivating the deposited single cells prior to the selecting.

11. Method for obtaining a humanized antibody comprising

a) providing a parent antibody,
b) providing a set of humanized forms of the parent antibody,
c) determining the association rate constant for each humanized antibody form to its antigen at 37 °C and at 13 °C,
d) calculating the ratio of the association rate constant at 37 °C to the association rate constant at 13 °C ,
e) comparing the ratio of the humanized forms with the ratio of the parent antibody,
f) selecting a humanized form as humanized antibody with a ratio that is less than twice the ratio of the parent antibody and thereby obtaining a humanized antibody.