Quote Proposal

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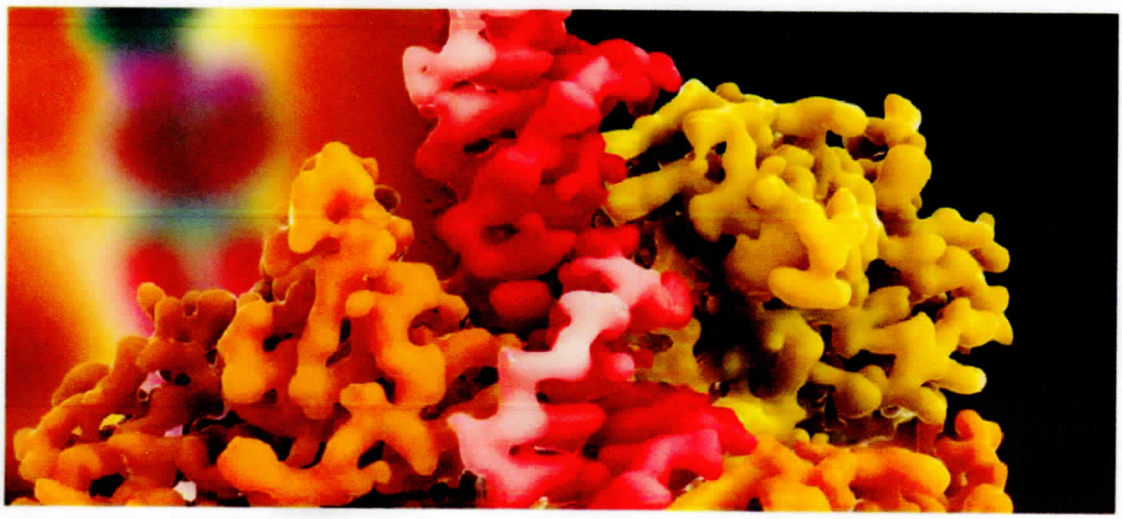
Quote Proposal

For

Antibody Maturation

Using Phage Display Selection

Quote Number: CBLJ08211501



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Creative Biolabs

Proposal CBLJ08211501

for

Antibody Maturation

Using Phage Display Selection

From:

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Date: August , 2015

Contents

Thank you very much for your interest in our antibody maturation services. We would like to present this antibody affinity maturation service proposal for you to consider our quality service, which should be much more cost-effective than in-house operation.

PROJECT OUTLINE

You currently have one antibody directed against a phosphorylated peptide and would like to increase the affinity. Your antibody currently has an affinity in nM range and you want to bring it to low nM (possibly) sub nM. This affinity range is the best fit for our phage display mutant library based affinity-maturation technology.

Depending on the final applications of the affinity-matured antibodies, we may introduce mutations to the entire variable regions [VL and VH] if affinity is the only concern, e.g., for antibodies intended for in vitro diagnostic use. If the final antibody is a human antibody for therapeutic use, we may introduce mutations mainly to CDR regions [with selection RF positions] since the humanness of the antibody human RF regions cannot be altered. Also, to produce humanized antibodies with lower immunogenic potential, we may integrate our humanized antibody germinalization service to have human antibodies with excellent germinality index (GI).

Our major antibody affinity maturation approach and our major antibody humanization approach share the same key step: antibody mutant library creation and screening, our antibody humanization service essentially includes affinity maturation, except for the minor issue that we mainly introduce mutations to CDR regions in humanization service [after CDR grafting]; whereas for affinity maturation, we may introduce mutations to RF regions too.

Supposedly, both affinity-maturation and humanization services should not change the epitope specificity of the parental antibody. In fact, when affinity-maturation or humanization is conducted, the introduction of mutations on each antibody molecule is very minor: only 1-5 some mutations on each antibody molecule. Epitope-shifting should be avoided in all these services.

Below is how we use peptide immunogen to raise phospho-specific monoclonal antibodies. We recommend to use the same screen strategy for antibody affinity maturation.

Peptide immunogens to raise phospho-specific monoclonal antibodies:

To raise phospho-specific monoclonal antibodies, we need synthesize two kinds of polypeptides for each phosphorylation site: one is phosphorylated and the other is

non-phosphorylated. Note that we need each kind of peptides in multiple forms: naked peptides, KLH-conjugated and BSA-conjugated as well as [optional] biotinylated. Frequently, we add a PEG spacer between the biotin and the peptide in case the peptide target is a very short one.

- The "hot" phosphorylated peptide should be in three forms: naked, KLH-conjugated and [BSA-conjugated or biotinylated] form.
- The "cold" non-phosphorylated peptide should be in two forms: naked and [BSA-conjugated or biotinylated] form.

We need KLH- [or BSA]-conjugated peptides for animal immunization. We need BSA- [or KLH-] conjugated peptides to test the immune response [before we proceed to antibody library construction]. We also need free peptides to set up competitive ELISA. Biotinylated peptides are good to have but not required for anti-serum titration.

We are well aware that KLH-coupling peptide is a very challenging task for most players in the field. Note that the challenge stays in the step of KLH-conjugation! Peptide synthesis is not challenging. This KLH conjugation is so critical for such an expensive project. Note that we cannot use the same conjugated peptides used in immunization to test the immune response. We can use naked peptides, biotinylated peptides or another carrier-conjugated peptides to do so.

Conjugation to a big protein, such as KLH, BSA, and conjugation to biotin are necessary for bio-panning. Peptide targets cannot be immobilized on a solid support efficiently; also due to the short length of a peptide, steric hindrance that interferes with the interaction between the target and the binder can be an issue. For these reasons, we usually biotinylate the target peptide [the phosphorylated one] and capture it via avidin-coated beads. Also, we may perform in solution screening instead of on surface screening. WE will then elute the captured binders. We also need the peptides in naked form since we may run competitive elution in bio-panning and we may use high concentrations of the naked [non-biotinylated] phosphorylated peptide to competitively wash away weak binders. Therefore, we need a pair of phosphorylated peptides, one is biotinylated, and the other is not. As controls, we need a similar pair of non-phosphorylated peptides to perform negative selection [depletion] and set up negative controls in binder validation assays. This negative validation using non-phosphorylated peptides is required [although widely forgotten] since peptide phosphorylation is never 100% complete. IN other words, some final binders may be captured by biotinylated non-phosphorylated peptide molecules and eluted by non-biotinylated non-phosphorylated peptide molecules. Therefore, we must use biotinylated non-phosphorylated peptide to invalidate them! For each peptide, we need about 4mg at >90% purity. Even if the naked peptide is not used in bio-panning, it can be used in validation of the binders in a competitive ELISA to confirm that the binders do bind to the peptide sequence.

The current KD and antigen status of the antibody:

1. If the antigen is an integral cell surface protein, the affinity maturation story can be much more complicated.
2. What is the current Ka and Kd? If the starting KD is better than one digital low nM, it is not that easy to increase the affinity 10 fold. However, it is easy to increase the affinity of a 10nM-100nM antibody 10-100 fold.
3. It is difficult to further increase the affinity of a very strong antibody binder. If the current KD is lower than 0.5nM, we may have to employ alanine scanning to find a key binding position in CDR regions and then mutate this key position to alanine to create a weaker binder. We then perform affinity maturation on this weaken antibody. In the end, back-mutate the alanine to the original residue. This is the method we have to increase the affinity of very strong antibody binders.
4. It is also tough to increase the affinity of very weak antibody binders. If the starting affinity is lower than 200nM, with which the antibody can barely bind, employing a monovalent system showed here can be problematic. We may have to use a multiple-valent system.
5. If the target antibody is a human or humanized antibody, we will not change the humanness of its framework regions. This rule applies to antibodies of other species.

If the target antigen is a soluble protein, and the current KD is around 6-20nM, we can present the following proposal. Of note, affinity maturation is very expensive and only used on antibodies intended for therapeutic purposes. It is not worth to increase the affinity of an antibody that is not well documented. We charge around $58,000 [or $88,800 using the backup method] to perform affinity maturation. Although we have good track record, the increase in affinity for 10 fold is not guaranteed.

In fact, it is very cost-effective to raise new mouse [using hybridoma technology at $5,800] or human [using phage display platform at $16,000-$36,000] antibodies against your target. We guarantee good affinity [e.g., low nM]. In vitro methods in affinity maturation are usually worse than in vivo methods; a poor mouse can do a much better job!

In our hands, using one of both of the methods described below in combination, we were able to increase antibody affinity ranging from 1.0nM-20nM to 10pM-100pM in three cases.

The First Strategy [quoted]: to create mutations at all positions in 6 CDR regions mutated.

A. This antibody affinity maturation strategy is based on our unparalleled expertise in mutagenesis and high-throughput phage display antibody library screening. We propose

to generate a combinatorial phage-display mutant antibody library that targets diversity to positions in all 6 CDR regions. We will use an oligonucleotide-directed mutagenesis approach to introduce mutations simultaneously in these positions. The randomization strategy will favor the original residues (wild type) at each targeted position. Degenerate oligonucleotides will be synthesized with the wild type nucleotide [at each nt position] in excess. This conservative, mild randomization approach, in which many variants maintain binding activity, results in the wild type residue at each targeted position with a frequency of approximately over 90%. We propose a mild-mutation approach thus on each target molecule only a very small number of residues are mutated, such as 1-3 [are mutated]. As a result, the affinity-maturated variants will have a very small number of mutations in comparison with the parental antibody molecule/sequence. [We may do this work using NNK method, or Trimer codons.]

B. Here, we propose no change on the length of the target antibody molecule.

C. We then screen the [phage display mutant] library using the wild-type antibody [and antigen] to wash away equal or weaker binder variants.

This proposal below is based on our unparalleled phage display antibody library expertise. WE must use oligonucleotide-directed mutagenesis approach since we must keep the humanness of your antibody. We usually charge **$84,000** to do one cycle of affinity maturation to increase the affinity 16-160 or more folds. An additional fee of $2,000 is required if cell line pairs are involved in antibody validation using FACS. An additional fee of $12,000 is required if cell line pairs are involved in bio-panning and antibody FACS validation. A second cycle may be necessary to further increase the affinity.

We understand that you do not have the structure of the antibody/antigen complex. WE need the sequence of the antibody to analyze. We need know the binding affinity constant [KD] of your current antibody. It is important to increase both the KD and koff [with koff in particular] of your antibody. We are fully aware that the dissociation (off) rate constant [koff] is as important as KD to define a good antibody for both antibody-based diagnostics and therapy. As elaborated below, we have good experience in creating antibody mutants and subsequently selecting antibodies with slow off rates.

We usually take scFv as the antibody format in affinity maturation. Also, a mono-valent phage display antibody library system is used to reduce the avidity effects during antigen-binding screening. Two methods, untargeted mutagenesis and oligonucleotide-directed mutagenesis, are employed to construct random or sequence-tailored sub-libraries to introduce a large number of mutants of the original antibody. Antibody binders of higher affinity are then selected by increasing the screening stringency. By constructing a series of sub-libraries of a scFv antibody, our proprietary protocol allows increase of the affinity of the scFv antibodies from 10 [-8] to 10 [-10]M. We have successfully obtained a scFv antibody that has an extremely high affinity of 10 [-12]M, whose binding to the antigen is essentially irreversible.

Since your target antibody is a humanized one intended for therapeutic use, introduction of mutations into the framework regions of the human antibody is unwanted as it may generate immunogenicity to the final affinity-improved antibodies. For this reason, oligonucleotide-directed mutagenesis is the best method to choose and CDR regions are the primary target regions to introduce mutations. Sometimes, we study the AA sequence of the antibody to find out conserved frame work positions [in comparison with germ-line and antibody subfamily sequences]. We may then introduce mutations to the positions in the frame work regions that are not conserved. Supposedly, these regions will be antigen-specific and change in these regions may increase affinity but not immunogenicity.

In fact, our experience also confirmed the notion that targeting mutations to the CDRs is effective in increasing antibody affinity. In particular, we found that mutagenesis in the CDR3 regions, especially HCDR3, is the most efficient means to improve affinity by phage display library screening.

Since the limit for a phage display library is smaller than 10[11], decisions must be made as to which amino acids to diversify and to what extent so that there are fewer nonsense antibody mutants that waste the capacity of the library. For LCDR3 and HCDR3, if the structure of the antibody/antigen complex is available or modeling the structure of the antibody/antigen is possible, we will distinguish between residues with solvent accessible side chains from those with buried side chains. We confirmed that randomization of residues with buried side chains is a waste of library sequence space. Also, we do not mutate glycines and tryptophans since changes in these residues usually abolish binding. Frequently, the randomized positions are biased for wild-type residues so that fewer non-binding mutants are introduced in the library.

Mutations can be introduced in CDRs using either error-prone PCR integrated DNA-shuffling approach, nucleotides NNK or NNS. Of note, Trinucleotide Phosphoramidites [trimer codon technology] are the best method to introduce defined randomness at defined positions with least bias and greatest diversity.

Trimer codon technology allows building up the library with each AA position randomized with a defined AA composition. Also, this is the best method to introduce region length variation. In order to achieve the defined AA distribution at each position to be randomized, it is required to take the incorporation efficiency of each trimer codon into consideration when prepare trimer condon mixture for each position to be randomized. Moreover, if the sites to be randomized are located within a region of 120bp [the limit for DNA synthesis using trimer codons], we can make one single strand DNA with Trimer Codons. If the sites to be mutated are located within a region longer than 120bp, 2 ssDNA are required and the cost for cDNA insert preparation will be doubled. Also, one independent ssDNA is required to incorporate one region length variation. Also, we are able to incorporate the length variations into the library at defined percentages.

Once the mutant library is made at the biggest possible size, high-affinity mutants are selected by bio-panning using "solution-sorting" strategies, in which a labeled antigen in

solution is used. We have two approaches: selection based on the equilibrium constant (Kd) and selection based on binding kinetics. In the first approach, sub-library phage is incubated with biotinylated antigen at controlled concentrations and bound phages are captured by immobilized NeutrAvidin. Selection based on binding kinetics is also termed off-rate [koff] selection, in which phage population is allowed to saturate the labeled antigen before a large molar excess of unlabeled antigen is added to the mix for controlled periods of time. This allows the selection of mutant antibodies that have slower off-rates. Since a reduction in koff usually results in a higher affinity, this selection approach singles out antibody variants with improved Kd.

We [and other companies in the field] usually look for antibody variants with slower koff. We may also try to look for antibody variants with fast kon. Note that shorter antibody-antigen incubation time and low concentration of antigen will favor the selection of antibodies with fast kon in our "solution-sorting" biopanning procedure, in which library phage is incubated with biotinylated antigen and bound phages are captured by immobilized NeutrAvidin.

After stringent selections, a fraction of ELISA positive mutant binders will be ranked using the Surface Plasmon Resonance instrument Biacore. Top mutants with increased KD and the best off-rate, koff, will be selected.

A second round of affinity maturation, including mutant library construction and screening, is necessary if the increase in affinity achieved in the first round of affinity maturation is inadequate for the desired application. Sequence analysis of the binding clones derived from the first round of affinity maturation can identify both conserved structural and functional residues and the residues that modulate affinity. These results are used to guide subsequent mutagenesis efforts in the second round of affinity maturation.

We need the AA sequences of the antibody and the antigen in mgs to start the project. We use the antigen to screen the sub-libraries. If you don't have the antibody sequence information yet, please refer to related service below.

Decode the preexisting monoclonal antibodies *[OPTIONAL]*:

http://www.creative-biolabs.com/antibody-sequencing-service.html

We also offer a *de novo* antibody sequencing service to decode the AA sequences of any monoclonal antibodies at $28,000. Using this approach, only 50-100 μg of antibody is required to sequence a purified monoclonal antibody. Sequencing can be performed in 3-10 working days. In some cases, there is BSA in the sample, which we can remove. We can also sequence mouse IgG in hybridoma supernatant that contains bovine serum antibody. We recently successfully sequenced an antibody made of ascites.

http://www.creative-biolabs.com/next-generation-antibody-sequencing.html

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Hybridoma-based antibody mRNA cloning and sequencing:

If you have the hybridoma cell line of an antibody, we charge $2,400 to sequence/clone the antibody gene and charge another $2,600 to express the gene into a recombinant IgG plus ELISA validation.

http://www.creative-biolabs.com/antibody-sequencing-service.html

The Backup Strategy [at $108,800]: to combine better mutations at each key position onto final antibody molecules

A. We do alanine scanning to find out which residues [in the CDR regions] are critical for antigen binding. [This step is optional since the critical residues identified by structure information can be informative.] Both the critical positions and their flanking positions are target for mutation.

B. After that, we mutate one critical position [or one flanking position] on one molecule to see which new mutations [in total 18 if cysteine is excluded] can increase the affinity. We do this on all critical positions and positions flanking the critical positions. [If there are 15 critical residues and flanking residues, we need do this work 15 times.] Of note, we mutate each position separately. On each mutant molecule, there will be only one mutation residue in comparison with the original molecule.

C. In the end, we combine the "better" mutant residues [at each critical and flanking position] onto individual molecules to see which molecules [with combination] will show the best affinity. For example, on position 10, we have 2 mutation residues that can increase affinity, and on position 20, there are 3. In the end, we have 6 final variants that bear the combinations of the "better" residues. The principle of this strategy is that "better" residues plus "better" residues will produce the "best" molecules. However, this assumption is not always true, but frequently works. Sometimes, "better" mutations in critical positions plus "useless" mutations in non-critical positions may increase the affinity better. Sometimes, mutations in non-critical positions increase the affinity. There is still no single gold-standard in affinity maturation.

Sample Questions and Answers

Q1: The form of recombinant antigen-X as an Fc-fusion protein is somewhat aggregated and may not be totally physiologic. Though antigen-specific antibodies bind to it in ELISA and Biacore formats, the more relevant form may be cell-surface expressed. We would like to know more about the possible forms of the antigens? a. Is it possible to screen using cells

instead of purified protein? b. What are the challenges of using cells vs. protein? c. Are there differences in cost and time?

A1: It can be very challenging to perform affinity maturation work against a whole cell antigen. We do affinity maturation using phage display antibody library construction and screening method. Therefore, we are able to isolate antibody mutants that have better affinity than the parental antibody against whole cells. However, it is hard to measure the actual affinity of the final antibodies against whole cells. We succeeded in a similar project for Dr. Philipp Wolf, in which the antigen is a conformational epitope on cell surface [and the mouse antibody does not recognize the recombinant form of the protein antigen]. We were contracted to humanize the mouse antibody and deliver humanized antibodies that had better affinity than the parental mouse antibody. Since we could not use the antigen protein in a recombinant protein format, we had to use antigen-expressing whole cells as the antigen to perform affinity maturation on the humanized antibody variants. We did capture humanized antibody variants that have better binding affinity against the whole cells [in comparison with the parental mouse antibody in FACS]; however, we were not able to measure the exact affinity of the humanized antibodies; we had to use FACS to compare the affinity of the humanized antibodies and the parental antibody. In other words, we obtained a comparative affinity for the humanized antibodies in the end.

The full contact of Dr. Philipp Wolf is shown as follows.

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We just completed another similar affinity-maturation project for a customer in Japan, in which the parental human antibody and the affinity-matured human antibodies can recognize the antigen protein in both a recombinant soluble protein format and on cancer cell surface. We used the soluble protein to capture affinity-matured antibody variants and measure their exact affinity. WE finally used the cancer cell lines expressing the antigen to confirm the binding [via FACS]. We increased the affinity from ~5nM to 0.25nM for the best antibody variants. The customer is from Toray Industries, Inc., 6-10-1, Tebiro, Kamakura, Kanagawa, 248-0036, JAPAN.
Please see the section entitled "[Phage Display Peptide/Antibody Library Screening against Whole Cells]" below to know our expertise in screening antibody libraries against whole cells and the challenges of using cells versus purified recombinant proteins.

If you provide the receptor target in a recombinant protein format and the receptor protein is functional [and is able to bind to the ligand protein], we do not have to use whole cells in bio-panning. In that case, we use whole cells for binder validation alone. An additional fee of

$2,000 is required if cell line pairs are involved in antibody validation using FACS. An additional fee of $12,000 is required if cell line pairs are involved in bio-panning and antibody FACS validation. Also, we will surely use more time when whole cells are involved in bio-panning and FACS based antibody validation.

Q2: Can a 10-fold increase in affinity be achieved?

A2: Yes. The key deliverable is the sequence of at least one affinity-matured antibody that has the antigen-binding affinity at least ten (10) times higher than that of the parental antibody. Otherwise, we will lose 20% of the service fee for the second half of the project as quoted.

Q3: How long will the process take?

A3: We quoted for 3 months. If whole cells are involved in bio-panning, we may need more time.

Q4: What are the deliverables to customer?

A4: Please see the quote table: 5 candidate affinity-matured antibodies with at least one affinity-matured antibody that has the antigen-binding affinity at least 10 times higher than that of the parental antibody.

Q5: What methods, if any, are employed to mitigate against introduction of antigenic motifs?

A5: We usually do not touch regions outside of CDR regions, so no introduction of antigenic motifs. Also, on each particular affinity-matured antibody variants, there will be an extremely small number of residues changed [usually 1-3], thus minimal alteration to the parental antibody.

Q6: What reagents and information will be required from customer?

A6: We need the sequences of the two parental antibodies. We also need the antigen protein in both purified soluble ECD protein format and in stable cell line format [preferably with a negative control cell line with identical genetic background for negative selection]. We also need the parental antibodies in a purified form in the amount of 5mg for each. You have shown us the current affinity of the parental antibodies.

Q7: What is your pricing structure? E.g., what are the milestones of the process? What are the costs of each milestone, and what happens should a milestone fail?

A7: As quoted, we decided to divide the project into two parts. There is a non-refundable fee of 80% to start each part of the project. If a particular part is successful, we will invoice customer for the 20% remaining fee of that part. Otherwise, we take the risk of the remaining 20% fee.

Q8: What happens if 10-fold affinity increase cannot be achieved?

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A8: The key deliverable is the sequence of at least one affinity-matured antibody that has the antigen-binding affinity at least ten times higher than that of the parental antibody. Otherwise, we will lose 20% of the service fee for the second half of the project as quoted above. This is negotiable.

Q9: An agreement on a definition of the "affinity" parameter that will be used to gauge the success of the project.

A9: There are two ways to define the affinity of an antibody: KD and Kd, both can be called Equilibrium dissociation constant. [In both cases, K is in upper case.]

KD is a kinetic term; it is the ratio of the off-rate (kback) and on-rate (kforward) constants. KD= k_{back}/ $k_{forward}$ =k_{off}/$_{kon}$=kd/ka. k_{back}= k_{off} = kd= dissociation rate constant, while $k_{forward}$ = k_{on} =ka=association rate constant in our field; k is in lower case in k_{on}, k_{off}, kd and ka here. [It is very confusing in the field since people use these symbols interchangeably.]

Kd [K in upper case] is calculated with the concentrations of antigen, antibody and antibody/antigen complex at equilibrium. Kd=[Ab][Ag]/[AbAg]; it is equal to KD in value.

In this proposal, we use KD to define affinity. Two antibodies can have the same affinity [ie, KD or Kd], but one may have both a high on- and off-rate constant, while the other may have both a low on- and off-rate constant.

Q10: IgG is bivalent, how can you determine the affinity KD in mono-valent IgG format? It seems the KD will be from monovalent IgG if you coat IgG direct on the chip in Biacore, am I right?

A10: Yes. We consider the KD is monovalent if IgG is immobilized on chip and the antigen is a monomer. Note that even if the antibody is bivalent [like IgG, scFv-Fc or a Fc-tagged nanobody], using the method in which the bivalent antibody is immobilized onto the sensor chip of a biacore system, the final affinity KD is a monovalent antibody KD since each arm of the bivalent antibody can bind only one antigen molecule, a 1:1 stoichiometric ratio, just like when you immobilize scFv/Fab onto the sensor chip. In this way the monovalent affinity of the bivalent antibody is measured. Also, if an IgG is immobilized, its monovalent affinity should be similar to that of its Fab/scFv format. Therefore, avidity effect is not an issue in measuring the affinity of the bivalent antibody this way [when the antigen is monovalent too].

Q11: What can be the difference of the affinity measured in scFv/Fab format in comparison with the affinity measured in IgG format?

A11: Besides valency, scFv and IgG may still have some difference in KD due to the fact that scFv is an artificial structure. Nevertheless, the affinity rank order is usually preserved between scFv, Fab and IgG.

Q12: IF the Koff of the Parental Antibody is already very slow, how can you increase the affinity by changing the Kon?

A12: We will use equilibrium affinity selection, using biotinylated soluble antigen with reducing concentration. So the phage selection itself will select for lower KD, instead of just koff. ScFv with better KD will be enriched over wild-type in the output phage population, which can be revealed by sequencing.

Selected References:

We have run antibody affinity maturation projects for following customers.

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Using essentially the same antibody library screening approaches, we recently completed humanization, caninization and human antibody discovery projects for the following customers.

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Please also be advised that we are the only company that can do antibody murinization, antibody caninization [with the reference shown below], simianization, camelization and so on. Also, we are the only company that offers rabbit and chicken antibody humanization! For Dr. Morsey, we caninized his mouse antibody into dog antibodies. Importantly, the final dog antibody variants has affinities that are better than the parental mouse antibody, an admirable achievement in the field.

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Service Terms:

Any work conducted will be performed on a fee-for-service basis and under a confidentiality agreement. In addition customer will be the sole owner of all commercial rights, IP and materials generated during the project. We will not seek any royalty for any of the services performed. Please see our Service Terms in the last page, we provide the services on a strict fee-for-service basis.

If you need any further information please do not hesitate to contact the undersigned.

Look forward to working with you!

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FEATURED PRODUCTS/SERVICE

The issues of the membrane protein targets

We are probably the only company in the field that has the expertise in raising functional high-affinity antibodies against membrane proteins on cell surface using phage display antibody library technology.

It is very challenging to have good peptide-binders/antibodies [especially functional binders] against membrane proteins. For GPCRs, agonist-like functional antibodies are almost impossible to have, while antagonist-like antibodies can be possible. To have simple binders will be easier; even so, to have antibodies that can recognize the native conformation of the target membrane protein can be difficult too, such as antibodies to be used in IP, IF, FACS, ELISA and cell based functional assays. It is important to know the exact target membrane protein to design the right binder-generation strategy. If the target protein has no long extracellular domains and has many, such as 7, 11 or 12, transmembrane domains, to have antibodies against the native conformation could be extremely challenging.

Note that we usually have three phage display approaches to obtain peptide-binders/antibodies that can recognize transmembrane antigens in their native confirmation [such as those used in FACS, IP, IF, ELISA and therapeutic applications].

If there are big extracellular domains available from the target protein, we can simply design a peptide or a recombinant protein immunogen based on one of the extracellular domains and use the recombinant protein as immunogen---the resultant binders frequently recognize the native antigen. This is the most inexpensive method. The recombinant protein will surely share epitopes with the native target. Therefore, the best strategy to get specific binders is to screen our libraries rotationally against the target in the recombinant protein form and native form [on whole cells]. This way, only specific binders that recognize the epitopes shared by the two forms of the target will be isolated. To discover high-affinity peptide binders against the integral targets in their native conformation by screening peptide/antibody libraries using a recombinant target ECD protein, and a cell line expressing the target protein, we do not have to use a negative cell line with identical genetic background.

1. We may have to rely on one Partner Company to produce recombinant membrane protein in a purified protein form [at around $80,000] to be used as immunogen for binder production. We may rely on another partner company to make the membrane protein in lipoparticle impure form [at around $40,000] to be used as immunogen.

2. The third method is using phage display peptide/antibody libraries. Using this method, we usually use whole cells to present the target protein. Sometimes, we use liposomes, lipoparticles or VLP to present the target membrane protein. In particular, we have extensive experience in screening peptide/antibody libraries against whole cells. Please see

the following section entitled: [Phage Display Peptide/Antibody Library Screening against Whole Cells].

Phage Display Peptide/Ab Library Screening against Whole Cells

Frequently, high-affinity binders that can recognize the target in its native/physiological forms are desired. In order to have binders of this property, targets [antigens] that retain the properties of their native form are necessary. In many cases, whole cells are the only resource to have membrane-associated or membrane-bound antigens in its physiological conformation.

We have extensive experience in screening phage display libraries using live or fixed cells instead of purified protein targets, especially in the cases in which the target antigens are transmembrane receptors. The chance will be better if the receptor-expression cells are used in screening, since transmembrane-domain-containing receptors can keep a native conformation only on the surface of the cells. Note that extraction and purification of thansmembrane proteins may change their structure. The cell membrane protein extraction and purification process may affect the folding of the target receptors, leading to the selection of binders targeting conformation-altered receptors that may have lower or no affinity to the receptors under physiological conditions. It is also very tough to get transmembrane protein in a pure form and at a good yield. For these reasons, we recommend using receptor-expressing whole cells instead of purified transmembrane proteins to do the work. We normally screen a phage display library against the live cells that express the target receptor, while using the control cells [empty vector transfected] to subtract the background. There are many examples that employed receptor-expressing cells as the targets in phage display library screening.

Note that transmembrane proteins can only be expressed in mammalian cells to get the 3D structure and modification in its native form. Even so, it is not easy to express receptors in a functional form in mammalian cells by transient or stable transfection. Our experience reveals that the best choice is to find a cancer cell line that highly expresses the target receptor; while employ RNAi technology to create a control cell line that knock-down the receptor. This is the best approach that can create the desired cell line pair, whose major difference is the expression of the target receptor, otherwise sharing identical genetic background.

We had an example. We did a project using a pair of CHO cell lines: one was transfected by insulin receptor; the other was regular CHO control cell line. We screened a 6-mer peptide library [provided by Dr. George P. Smith's lab at University of Missouri] against the CHO-expressed insulin receptor. We used the control CHO cell line to deplete non-specific binders for three rounds; after that we applied the subtracted library to the insulin-receptor-expressing cell line and eluted the binders using an elution butter of low pH. After that, we conducted another 3 rounds of screening against the

insulin-receptor-expressing cell line while conducted competitive elution [using free insulin, the ligand of the insulin receptor]. Our results indicated that the peptide binders had affinities of nM range to the insulin receptor. This is a good piece of evidence that using intact cells to do phage display library screening is feasible.

In the end of the screening process [using the paired cell lines], either ligand or soluble form of the target receptor is used to competitively elute the specific binders. If you only have a pair of cell populations and do not have a specified target, the final binders will target a diverse range of antigens on [or in] the cells. Due to the complexity of this cell-based screening projects [in cell culture, screening condition optimization—adherent or suspension cell culture, live or fixed cells---and binder validation], it is important to have a receptor-specific third molecule [e.g. a soluble receptor or a ligand] to narrow down the binders.

Sometimes, isolation of functional [antagonist or agonist type of binders, e.g. neutralizing or inhibitory binders] binders [that have more activities than simple binding] against membrane proteins is desired. In these cases, a ligand to the membrane protein [e.g. a receptor] or a soluble portion of the membrane protein may be required. For sure, the ligand is required to validate the "functional binders" in the end. Also, in our "Stanford Biopanning Strategy", a ligand is required to selectively isolate inhibitory binders that have higher affinity than the ligand [to the receptor].

Using whole cells plus protein ligands, we have successfully isolated high affinity antibody binders against quite a few membrane-bound receptors, including EGFR, CD20, NOTCH1, insulin receptor and HER2 and CD33.

We may also use one of the following two animal-involved methods to raise murine antibodies for transmembrane proteins: [MPAT Platform for Multi-spanner Antibodies] and [Genetic Immunization].

http://www.creative-diagnostics.com/MPATTM-platform-for-multi-spanner-Antibodies.html

http://www.creative-diagnostics.com/Antibody-Production-through-DNA-Immunization-Genetic-Immunization.html

Peptide immunogens derived from loops of the membrane proteins:

The last possible method is to design, synthesize and conjugate peptides as immunogens, and then use these peptide immunogens to screen the human antibody libraries to get human therapeutic-potential binders. In fact, most of research use antibodies against integral targets were raised using peptide immunogens derived from the extracellular [mainly] and intracellular loops of the target proteins! Below is an example.

http://www.creativebiomart.net/symbolsearch_SLC2A1.htm

Peptide immunogens:

Note that we need the peptides in multiple forms: naked peptides, KLH-conjugated and BSA-conjugated as well as [optional] biotinylated. It is much safer for you to order KLH-coupled peptide [in the amount of 4-5mg]. We are well aware that this is a very challenging task for most players in the field. Note that the challenge stays in the step of KLH-conjugation! Peptide synthesis is not challenging. This KLH conjugation is so critical for such an expensive project. Note that we cannot use the same KLH-conjugate peptides to test the immune response before we proceed to antibody library construction. We can use naked peptides, biotinylated peptides or BSA-conjugated peptides to do so.

Conjugation to a big protein, such as KLH, BSA, and conjugation to biotin are necessary for bio-panning. We also need the peptides in naked form since we may run competitive elution in bio-panning and we may do competitive ELISA in binder validation. Peptide targets cannot be immobilized on a solid support efficiently. Also, due to the short length of a peptide, steric hindrance that interferes with the interaction between the target peptide and a binder can be an issue too. For these reasons, we usually biotinylate the target peptide and capture it via avidin-coated beads in biopanning. Also, we would like to perform in solution screening instead of on surface screening. WE will then elute the captured binders. Alternatively, we use high concentrations of the naked [non-biotinylated] peptide to competitively elute the specific binders. Therefore, we may need a pair of peptides, one is biotinylated, and the other is naked. As controls, we may need a similar pair of control peptides to perform negative selection [depletion] and set up negative controls in binder validation assays. For each peptide, we need about 1mg at >90% purity. Even if the naked peptide is not used in bio-panning, it can be used in validation of the binders in a competitive ELISA to confirm that the binders do bind to the peptide sequence. Frequently, we add a PEG spacer between the biotin and the peptide in case the peptide target is a very short one.

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TIMELINE AND PRICE

[Quote Number: CBLJ08211501]

Antibody affinity maturation **[Phage Display Mutant Library Technology]**			
Featured Service	**Description or Quantity**	**Timeline [weeks]**	**Price**
Validation of the parent antibody	1. Antibody expression 2. ELISA or other immune assays 3. Affinity KD measurement	4 weeks	$8,000 [OPTIONAL]
Parental antibody affinity measurement in scFv format in two orientations	We run affinity maturation in scFv format. The first step is to express the parent antibody in scFv format using our system and then measure its affinity. This step is an exit point, which is quoted at $4,000+$4,000.	4 weeks	$8,000
Design and construction of a mutant phage display scFv library	Design a diversified scFv library (phage display) by degenerating/randomizing residues in CDR and/or framework regions 1. Create phage display mutant library using our optimized Kunkel-like mutagenesis approach. Library size will be over 10^{10}. 2. The randomization strategy would favor the original resides (wild type). Approximately 1-2 base changes per 100 base pairs were introduced.	4 weeks	$28,000
Biopanning against the scFv library by solution-sorting	1. 3-4 rounds of screening are performed 2. The mutants with higher binding affinity will be selected.	2 weeks	$16,000
Phage ELISA to compare the binding affinity of phage-displayed humanized scFvs with that of phage-displayed parent scFv	Phage amplification [$40 /clone]	1 week	$20,000
	Phage ELISA against the target [$20 /clone]		
	Phage DNA extraction [$20 /clone]		
	Phage DNA sequencing [$20 /clone]		
Antibody ELISA validation	Individual antibodies in the phage virion format will be expressed into a	1 week	

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	soluble protein format [phage-free] and the binding of the antibodies to the antigen will be examined with ELISA.		
Sequence analysis of top binders	In silico antibody modeling, sequence analysis to get rid of the mutants that include unacceptable mutations in the framework regions.	0.5 weeks	
Affinity Ranking and Measurement in KD in scFv format	In the end, we compare and measure the affinity of affinity-matured mutants with the parental scFv, for top 3-5 clones.	1 week	
Conversion of scFv into full IgG	4 affinity matured IgG clones, 1 mg each. Select the proper isotype of constant antibody sequences to the client's request for 4 clones, 1mg for each clone	3 weeks	$12,000
Affinity Ranking and Measurement in monovalent IgG format	for 4 clones	2 weeks	$6,000 **[OPTIONAL]**
Affinity-matured antibody delivery [Free]	Affinity-matured antibody sequences are released free of charge.	0	$0
	Subtotal:	**3 months**	**$84,000 + [OPTIONAL]**

Notes:

1. More than one affinity-matured antibody sequences will be generated.
2. These variants may have slightly different binding affinity against the target.
3. We offer a fee-for-service scheme for this service and will not claim intellectual property rights for results generated.
4. A non-refundable fee of 80% will be due at project [or each experimental step] initiation.

Deliverables:

1. Three candidate affinity-matured antibodies with at least one affinity-matured antibody that has the antigen-binding affinity at least 10 times higher than that of the parental antibody.
2. A written report containing a summary of the key findings in the project.

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BILLING AND MAILING ADDRESS

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The Bank Account Information:

BANK Name: CITIBANK
The bank routing No.: 021000089
The bank swift code: CITI US 33
Account No: 4992896329
Company Name: Creative Biolabs Inc.

APPENDIX

[TERMS AND CONDITIONS FOR PROVISION OF SERVICES]

1. **Governing Provisions.** Creative Biolabs, Inc ("CREATIVE BIOLABS") that is processing this order for purchase of services (the "Services") agrees to provide to Client certain services identified and agreed upon by the parties expressly conditional on Client's assent to the terms and conditions contained herein. Client agrees that the terms of Client's purchase order or any other document supplied by Client to CREATIVE BIOLABS shall not be of any force or effect, except to the extent CREATIVE BIOLABS agrees in writing thereto. These Terms and Conditions, including all writings incorporated herein by reference, any quotation issued to Client by CREATIVE BIOLABS, and those specific terms of a purchase order or other document (e.g., a work order) that are either consistent with these conditions or expressly agreed upon by CREATIVE BIOLABS in writing, constitute the entire contract between the Client and CREATIVE BIOLABS (the "Services Agreement"), and supersede all prior agreements and understandings between the parties, whether written or oral, relating to the subject matter hereof. In the event of a conflict, a quotation takes precedence over the Terms and Conditions, and a written contract signed by both parties takes precedence over either. If one or more of these Terms and Conditions are held to be invalid, illegal, or unenforceable by a court of competent jurisdiction, the remaining Terms and Conditions shall be unimpaired. For purposes of the Services Agreement, "Deliverables" means (a) Data (defined below) generated by CREATIVE BIOLABS as a direct result of the performance of the Service; and/or (b) materials made by CREATIVE BIOLABS pursuant to the Services Agreement derived and/or made from Client Materials (defined below), and in each case as may be delivered to the Client.
2. **Standard of Performance.** All Services will be performed using due care in accordance with (a) the Services Agreement and (b) generally prevailing industry standards applicable to such Services. A good faith effort to start and complete all Services on time will be made, and Client will be notified if CREATIVE BIOLABS determines that there are likely to be substantial delays. CREATIVE BIOLABS expressively guarantees to perform all its procedures with professional diligence, and strives to perform quality work acceptable to all its Clients. Should quote proposal and study scope not align with Client's expectations, it is the Client's responsibility to notify CREATIVE BIOLABS in writing of any changes to this proposal prior to initiation of the project. CREATIVE BIOLABS is only responsible for performing services as outlined in this quote, unless other agreed upon in writing by CREATIVE BIOLABS and Client.
3. **Changes.** Changes to the Services Agreement must be in writing and signed by authorized representatives of CREATIVE BIOLABS and Client. If such changes result in an increase in the cost of the Services or affect the projected completion date of the Services (or portions thereof), the fee and/or completion date(s) shall be adjusted to a degree commensurate with such changes. Cancellation of Services in progress will result in a partial charge commensurate with the percentage of work completed at the time of cancellation, in addition to any approved expenses not cancelable at the time of termination which are actually incurred prior to termination, or other termination or cancellation charges described in the Services Agreement.
4. **Materials and Information.** Client will provide CREATIVE BIOLABS with sufficient amounts of Client's information and materials such as cells, compounds, samples, or other substances needed to perform the Services ("Client Materials"), as well as comprehensive data or information concerning the stability, storage

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and safety requirements of such Client Materials needed by CREATIVE BIOLABS to perform the Services. Unless otherwise agreed in writing, CREATIVE BIOLABS will use Client Materials in accordance with the Services Agreement and only in the performance of Services for the benefit of the Client. Unless otherwise requested by the Client, upon completion of the Services any remaining Client Materials will be destroyed. CREATIVE BIOLABS will not use the Client Materials, nor make chemical or genetic modification to the Client Materials, or attempt to determine the structure of Client Materials, except as may be agreed upon in a separate writing. CREATIVE BIOLABS will not transfer or provide Client Materials, in whole or in part, to any third party, other than a subcontractor, without the Client's prior written approval. CREATIVE BIOLABS shall comply to the best of its knowledge in all material respects with all applicable laws and governmental rules and regulations which are applicable to its use of the Client Materials, and with any written safety precautions as provided by the Client and accepted by CREATIVE BIOLABS.

5. **Intellectual Property.** Except as set forth otherwise in the Services Agreement, Client shall be the exclusive owner of and shall have title to all documentation, information, records, specimens or other work product supplied by Client and/or generated by CREATIVE BIOLABS as a direct result of the performance of the Services ("Data"). Unless otherwise agreed in writing, CREATIVE BIOLABS will use Data in accordance with the Services Agreement and only in the performance of Services for the benefit of the Client. Upon completion of the Services CREATIVE BIOLABS will maintain a file of the Data for a period of no less than one (1) year. CREATIVE BIOLABS and Client shall agree that all pre-existing intellectual property of either of them remains the property and the other party shall have no right or license to it ("Background IP"). Nothing herein shall be regarded as an express or implied transfer or license of a party's Background IP. CREATIVE BIOLABS is the sole and exclusive owner of all right, title and interest in and to all intellectual property claiming or covering CREATIVE BIOLABS technology.
6. **Confidentiality.** During performance of the Services and for three (3) years thereafter, CREATIVE BIOLABS will treat all Data as proprietary and confidential and will not disclose the same to any person except its employees, consultants, and subcontractors to whom it is necessary to disclose the Data for purposes of providing the Services. CREATIVE BIOLABS shall protect the Data by using the same degree of care as CREATIVE BIOLABS uses to protect its own confidential information, but in any event no less than a reasonable degree of care. Notwithstanding any other provisions herein, CREATIVE BIOLABS shall have no liability or obligation to Client for, nor be in any way restricted in, its disclosure or use of any Data which (a) is already known to CREATIVE BIOLABS without obligation of confidentiality to Client; (b) is or becomes publicly known by any means other than wrongful act of CREATIVE BIOLABS; (c) is received from a third party without such party's breach of obligation of confidentiality to Client; (d) is disclosed pursuant to an enforceable order of a court or administrative agency; and/or (e) is independently developed by or for CREATIVE BIOLABS. Client acknowledges that, notwithstanding anything to the contrary herein, CREATIVE BIOLABS shall be free to disclose Data, provided that in so doing CREATIVE BIOLABS never attributes or otherwise associates any such data with Client.
7. **Payments.** The Client shall pay CREATIVE BIOLABS according to CREATIVE BIOLABS invoice(s). CREATIVE BIOLABS shall invoice the client following payment schedules described in the quotation. The payment terms is 30 days, otherwise differently requested in the invoice. All payments due hereunder shall be made in the currency specified by CREATIVE BIOLABS in writing. If Client defaults in any payment when due, CREATIVE BIOLABS, at its option and without prejudice to its other lawful remedies, may delay performance, defer delivery, charge interest on undisputed amounts owed, and/or terminate the Services Agreement. If

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payment is not received by the due date, a service charge will be added at the rate of 1.5% per month (18% per year) or the maximum legal rate, whichever is less, to unpaid invoices from the due date hereof. If CREATIVE BIOLABS is compelled to bring suit to collect amounts due hereunder, it shall also be entitled to recover interest on amounts due as provided by law and reasonable attorney fees and costs of suit incurred in connection with the action. Client's acceptance of delivery of any Service based on this Service Agreement shall constitute a representation that Client is solvent.

8. **Indemnity.** The Client shall defend and indemnify CREATIVE BIOLABS and its affiliates, directors, officers, employees, representatives, consultants, agents and service providers (collectively, the "Company Indemnified Parties"), against any and all costs, damages, expenses (including reasonable legal fees) and losses suffered by any Company Indemnified Party in connection with any third party action, assessment, claim, demand, proceeding or suit to the extent arising or resulting from (a) the Client's negligence or willful misconduct; (b) the Client's breach of this Agreement; or (c) CREATIVE BIOLABS' use, or alleged use, in the performance of the Services in the conduct of the Project, of any Client Background Intellectual Property, Client Provided Materials or Client Provided Material Information licensed or provided by the Client to CREATIVE BIOLABS for the purpose of performing the Services in the conduct of the Project.

9. **Limited Warranty.** The Services Agreement (Quote) is a contract for services. CREATIVE BIOLABS's sole warranty with respect to the Services is that CREATIVE BIOLABS will perform all Services in accordance with the standard of performance set forth in Section 2 above. Client shall notify CREATIVE BIOLABS in writing of any claim for a breach of such warranty by CREATIVE BIOLABS within one (1) month after delivery by CREATIVE BIOLABS of the last-to-deliver Deliverable relating to such Services. The sole remedy of Client for breach of such warranty shall be to require CREATIVE BIOLABS to re-perform the Services (or such portion thereof as may reasonably be required to be re-performed), and, in such event CREATIVE BIOLABS shall diligently pursue the re-performance of the Services or portions thereof until completion, or, if CREATIVE BIOLABS cannot re-perform the Services (or such portion) in accordance with this limited warranty, then it shall refund amounts paid by the Client for the applicable Service giving rise to the breach of warranty.

TO THE MAXIMUM EXTENT PERMITTED BY APPLICABLE LAW, IN NO EVENT SHALL CREATIVE BIOLABS BE LIABLE UNDER ANY LEGAL THEORY (INCLUDING BUT NOT LIMITED TO CONTRACT, NEGLIGENCE, STRICT LIABILITY OR WARRANTY OF ANY KIND) AS A RESULT OF CREATIVE BIOLABS' FAILURE TO PERFORM THE SERVICES IN ACCORDANCE WITH THIS WARRANTY FOR ANY DIRECT, INDIRECT, SPECIAL, INCIDENTAL, CONSEQUENTIAL, OR EXEMPLARY DAMAGES, EVEN IF CREATIVE BIOLABS HAD NOTICE OF THE POSSIBILITY OF SUCH DAMAGES. TO THE MAXIMUM EXTENT PERMITTED BY APPLICABLE LAW, THE WARRANTY SET FORTH IN THIS SECTION IS IN LIEU OF ANY AND ALL OTHER WARRANTIES RELATING TO THE SERVICES, EXPRESS OR IMPLIED, INCLUDING, WITHOUT LIMITATION, ANY IMPLIED WARRANTIES OF MERCHANTABILITY OR FITNESS FOR A PARTICULAR PURPOSE, CUSTOM, TRADE, QUIET ENJOYMENT, ACCURACY OF INFORMATIONAL CONTENT, OR SYSTEM INTEGRATION, OR THAT THE USE OR SALE OF DELIVERABLES OR INFORMATION PROVIDED HEREUNDER WILL NOT INFRINGE OR MISAPPROPRIATE ANY THIRD PARTY INTELLECTUAL PROPERTY RIGHT. TO THE MAXIMUM EXTENT PERMITTED BY LAW, CREATIVE BIOLABS' LIABILITY TO CLIENT FOR BREACH OF ANY TERMS AND CONDITIONS OF THE SERVICES AGREEMENT (OTHER THAN ANY BREACH OF THE WARRANTY CONTAINED IN THIS SECTION IN RESPECT OF WHICH ANY LIABILITY SHALL BE LIMITED TO RE-PERFORMANCE OR REFUND AS SPECIFIED HEREIN) SHALL BE LIMITED TO DAMAGES (OTHER THAN INDIRECT, SPECIAL, INCIDENTAL, CONSEQUENTIAL, OR EXEMPLARY

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DAMAGES) IN AN AMOUNT NOT TO EXCEED THE FEE PAID OR TO BE PAID BY CLIENT TO CREATIVE BIOLABS IN CONNECTION WITH THE SERVICES.

10. **Termination.** CREATIVE BIOLABS may terminate the Services Agreement in the event that (a) the Client breaches or fails to comply with any material provision of the Services Agreement and, where the breach or failure is capable of being remedied, fails to remedy the breach or failure to the satisfaction of CREATIVE BIOLABS within fifteen (15) days of receiving written notice thereof; (b) in the event CREATIVE BIOLABS has agreed to procure from a third party non-standard or custom Client Materials specifically for use in the performance of Services and CREATIVE BIOLABS is unable to reach agreement with such third party on the terms and conditions of such procurement, or the third party is unwilling or unable to provide the Client Materials for reasons beyond CREATIVE BIOLABS' reasonable control; or (c) in the event that any of the following actions occur in relation to the Client: an order is made for the winding up of the Client; a receiver or receiver and manager of any property of the Client is appointed; a provisional liquidator of the Client is appointed; the Client is or is deemed by law to be unable to pay its debts; the Client makes any arrangement or compromise with its creditors or members or with any class of its members or creditors; and/or the Client ceases to carry on its business in the areas necessary for the performance of its obligations under the Services Agreement.
11. **Choice of Law and Jurisdiction.** This Quotation shall be governed by and construed in accordance with the laws of the State of NY, U.S.A. Any litigation or other dispute resolution between the parties relating to this Quotation shall take place in the U.S. District Court where Creative Biolabs is registered. The parties consent to the personal jurisdiction of and venue in the state and federal courts within the State of NY.

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