Some Commonly Asked Questions about custom antibody production (Click over the Q# to see details)
Questions related to **protein antigens** and general information

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Q13A. Can ADI provide some **references** for its Custom Antibody Services. See Answer in Q21.

Questions related to **peptide antibodies**

Q14. What is the appropriate peptide length for antibody production?
Q15. Can ADI help in selecting appropriate peptides from whole gene sequences?

Q16. Should I consider adding a Cysteine in peptides for making antibodies?
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Q20 How about MAPS peptides? Can ADI make those?
Q21 Can ADI provide some **references** for its Custom peptide synthesis and Antibody Services

Q22. What happens if there are no antibodies or ELISA is -ve? see answers to Q13. above.
Q23 What is Std. 63 days projects and How to request optional Extension and termination of projects. See Answers to Q7. and Q8. above.

Q1. How much antigen is needed to make good titer antibodies?

Amounts of antigen needed to produce good titer antibodies obviously depend upon the antigenicity of a given "antigen" and host species. Bacterial/viral antigens are very often highly immunogenic than mammalian proteins. In our experience, the antigen amounts given below will normally suffice to make good titer antibodies. These estimates are for using up to 2 animals using our std. 63 days protocol (involves at least 5 injection per animals) and for ELISA.

- Purified and recombinant proteins: 500 ug-2 mg.
- Peptide-Protein Conjugates: 2-5 mg.
- Proteins in SDS-PAGE gels: A band that is clearly visible upon staining (500 ug-2 mg).
- Proteins as precipitates: 500 ug-2 mg.

In general it is a good idea to provide as much antigen as practically possible (1-3 mg or more) to assure good antibody production. It doesn’t cost more to inject more.

Q2. What concentrations and buffers are compatible with proteins for immunization
In general, most biological buffers (PBS, Tris, Borate, Phosphate, etc) in moderate molarity are OK. It is desirable to send the protein at approx 1 mg/ml or higher. In general, you should ship the antigens the way they are normally kept or send them frozen in dry ice.

Q3. What if the protein is NOT soluble in regular biological buffers and if it precipitates?

High concentrations of chaotropic agents (8M Urea, GdnHCl, SDS) should be avoided. Often some recombinant antigens will not dissolve in regular buffers and require 4-8M urea etc. Try to minimize the concentrations of strong denaturants and detergents as much as possible by dialyzing in buffers containing lower concn of these components. If all fails, then keep the concentration of protein as high as possible (5-10 mg/ml). We have made antibodies to proteins containing 6-8M urea if we keep the injecting volume to a minimum (10-50 ul). It is also possible to inject proteins as precipitates. Just send the protein in buffer containing suspension of proteins. However, these extreme measures (SDS-PAGE, high concn of urea etc, and precipitates) must be avoided and used only as the last option. Antibody titer may also be compromised.

Q4. Production of antibodies using proteins embedded in SDS-PAGE gels?
This method has been used successfully if amount of gel is kept to a minimum. Acrylamide gel, if injected in large amount, is toxic to animals. It is very important to load as much proteins possible on a PREP GEL (no lanes or wells), coomassie blue dye staining., destaining, and then cutting out the most dense portions of the protein band (One gel strip or its equivalent should be enough for the entire protocol). ADI will process your gel slices by mincing and homogenization before injections. A nominal charge of Rs2500 will be added to process the gels.

Q5. What is the best method of shipping antigens to ADI?
All antigens can be safely shipped overnight on dry ice. Some antigens such as peptide in powder forms or SDS-PAGE gels can be shipped at room temperature. Other antigen (proteins bound to Sepharose gels, precipitates, peptide/protein-conjugate solutions can be shipped overnight in cold-paks).

Q6. What Species and animal number are appropriate for antibody production?
Antigen source, its sequence conservation, and antisera volume are the primary factors in choosing a host species and their numbers. For example, antigens purified from rabbits should be injected into goat, g. pig or chicken to produce high titer antibodies. In general, peptide-protein conjugate can be reliably injected into rabbits (even if sequence of the peptide is the same in rabbit). Mammalian protein antigens, that are known to be poor immunogens, could produce high titered antibodies in non-mammalian hosts such as chicken.

Large animals (goat/sheep) are generally used if large volume (several liters) of sera is required. Rabbits are mostly suited for research uses (100-500 mls serum). Although **chicken** can give ~ 10 ml serum/bleed,

eggs can also be collected to obtain more antibodies. Antibody titer can be up to 2-3 fold higher in the egg than in serum. Egg contains **IgY** sub-type as opposed to regular IgG type in serum. In addition, Chicken antibodies will often given cleaner results in many applications because of less crossreactivity with mammalian proteins.

Polyclonal antibodies generated in common animals will generally show a variation not only in their titer but also in their quality even if all animals are injected with the same antigen and bled at the same time. Therefore, it is best to include at least 2 or more animals per antibody protocol. It is often recommended to include large number of animals if the desired antibody is expected to distinguish closely related isoforms within or across the species or has other subtle changes (**phosphorylated Vs non-phosphorylated**). We generally recommend the use of at least 2 animals per protocol in Rabbit, G. Pig, Hamster, and Chicken. Because of high cost of using goat and sheep, one may consider using just 1 animal per antigen. Up to 5-10 animals are needed when antibodies are made in mouse/rat to collect sufficient amount of serum.

Q7. What is Std. 63 Days Immunization and Bleed Protocol?
Although there are dozens of immunization and bleed schedules, we have found the std 63 days protocol to be effective for most antigens. It includes injections at 15 days intervals at multiple sites (2-4) subcutaneous and 1 intramuscular site; 20-200 ug antigen; total volume 0.5 ml; in complete Freund's adjuvant for the first injection; all subsequent injections are given in incomplete adjuvant). Bleeding is done from the marginal ear vein (rabbit), jugular vein (goat/sheep), or cardiac puncture (G. Pig/Hamsters) or Wings (chicken) or tail/ocular vein (mouse/rat). Bleed out (exsanguination) is performed after proper anesthesia.

- **Injections**: 0, 2, 4, 6, 8 weeks (continued during optional extension period at 10, 12, 14, 16 weeks, etc.)
- **Pre-immune bleed** is taken before the first injection (~ 2 ml/rabbits, ~10 ml goat/sheep, 0.5 ml from G. Pig/Hamster; and 2-5 ml from Chicken).
- **Production (immune) Bleeds**: 1st bleed (week 7) and others collected at week 9 (continued during optional extension at Weeks #11, 13, 15, etc).

Bleed Shipments: Crude antiserum is very stable as long as it contains anti-bacterial agents to prevent bacterial growth. In fact, many researchers like to heat antiserum at 65oC to inactivate the complement. All bleeds are sent as serum on cold paks and normally contain a preservative (0.1% azide or 0.02% merthiolate unless instructed other wise). For an extra charge, we can ship antisera on **dry-ice** if required.

Q8. How much sera is expected from various species during the std. 63 days immunization program?

The following information gives standard bleed volume from various species.

Animals	Pre-bleed	Immune Bleed	Bleed Out/ Exsanguination
Rabbit	2 ml	15-20 ml	50-60
Goat or Sheep	10 ml	200-300 ml	600-1200
G. Pig or Hamster	1-2 ml	2-5 ml	10-15 ml
Chicken	2-5 ml	10-20 ml	25-50 ml
Mice	0.1-0.2 ml	0.3-0.5 ml	1-3 ml
Rats	0.1-0.5 ml	0.5-2 ml	2-5 ml

Q9. What options are available after the expiration of std. 63 days protocol?

Animals on a given protocol can be extended as desired on a monthly basis only. Extension charges are determined by per diem housing, number of injections and bleeds. Please consult ADI for current charges in various species during optional extension period. For example, one rabbit can be housed for 1 month for just Rs 13500 per month per rabbit (includes housing and care, 2 injections, and 2 std. Bleeds). Animals can be **bled out** (Rs 6000 for rabbits) at any time to collect additional blood. All projects can be extended as long as desired depending upon the availability of antigen or peptide-protein conjugate. Please use form Ext-3 for all project extension.

Q10. Is it possible to Pre-screen, non-immunized animals for the presence of antibodies?

It is generally a good idea to pre-screen animals for the presence of natural antibodies if there is a reasonable chance that the antigen of interest may be found in general environments. This screening is particularly important when making antibodies to common bacterial, viral antigens or common allergens. We can arrange to send a small sample (0.5 ml/per animal) from a number of animals for a nominal charge (Rs 12500 for 5-6 animals). All animals are put on hold for about a week. We request that pre-bleed should only be requested when antigen is ready for injections and the screening should be completed within a week to avoid unnecessary holding time.

Q11. Are other alternate immunization and bleeding protocols available?

We can follow a given immunization and bleed protocol if it has proven to work for a given antigen or if there are reasons to believe that ADI’s protocol will not give a desired response. In addition, we can utilize alternative adjuvants (RIBI, Adjuvax, etc) if supplied by the researcher. Alterations in injection and bleed protocol can also be done after the expiration of 63 days protocol.

Q12. How do I know if antibodies are made to the antigen and which animal is the best?

We recommend ELISA analyses on the first bleed (week 7) to determine antibody titer. Most animals will respond by this time. If antibody titer is poor then testing is repeated on the 2nd bleed at no additional cost. A decision can then be made and the protocol modified or terminated. Antibody testing is done by ELISA using the free peptide or protein coated ELISA plates. Antibodies to the carrier proteins are not detected this way. ADI will provide a complete ELISA report indicating titer of antibody in each animal.

Q13. What happens if there are no antibodies or ELISA is -ve?

ADI recommends ELISA on the first bleed. If results are negative, testing is repeated at no additional cost on second bleed. If there are no signs of antibody by ELISA and another technique (Western, IHC), it may be necessary to terminate the project or include changes in immunization protocol. Most custom antibody work is performed on best-effort basis. ADI has Scientist with over 20 years to experience in raising and using antibodies. We do everything possible that we know or what is recommended to us by the researchers. There are number of reasons when antigens fail to induce antibody response. It may be due the poor antigenicity of an antigen, conservation of peptide sequence in a given species, and poor conjugation of peptide to the carrier protein. Many of these factors are not in our control and we can not alter the nature of antigens or antibodies. In such cases, we offer to repeat the project in another species (Chicken for example). In case of peptides, you may elect to have another peptide made and we will make antibodies at no extra cost. In this case, we will only add the cost of making the new peptide and its conjugation.

No Scientist can predict functionality of antibodies. In many cases, antibodies may not work in all techniques. A given antibody may not work in blotting and be still useful for ELISA or IHC or IP’s or vice versa. This is particularly true for anti-peptide antibodies. In some cases high titer antibodies generated against the peptide may not recognize the full-length protein in Western or fail to immunoprecipitate the antigen or may not work in immunohistochemistry. Therefore, our warranties are limited to production of reasonable titer antibodies (at least 1:1K; average titer 10K-100K) by ELISA. In case of protein antibodies,

we must be provided with sufficient antigen (500 ug-2 mg) for our warranties to be valid. When antigens are provided in gels or beads, our services will strictly be on best efforts basis as there are usually no good estimates of the amount of antigens in gels or beads.

Q14. What is the appropriate peptide length for antibody production?

In general we recommend, approx. 15-25 aa peptides if there are no constraints in selecting such peptides. Longer peptides (>25 aa) can be used but it increases the cost. We can make up to ~100 aa long peptides. It is generally not a good idea to choose peptides <10 aa unless there are valid reasons for it such as potential sequence homology with a related family member or other proteins. Shorter peptides (<10 aa) may present limited # of epitopes.

Q15. Can ADI help in selecting appropriate peptides from whole gene sequences?
ADI has analyzed thousands of sequences and assisted researchers in selecting immunogenic peptides. Whole gene sequences are analyzed by computer programs for antigenicity, hydrophilicity, accessibility, etc. to select antigenic peptides. It is very helpful to have additional information as to what regions of the protein (N or C-terminus, or a given domain) to specifically target or avoid. Any potential for crossreactivity with other closely related members of the same family should also be mentioned. A sequence alignment of closely related members is of tremendous help to select specific peptides (please fax alignments and do not email). All recommended peptides are compared for sequence homology with other proteins by BLAST searches. A final selection is then made with the user input. It is a good idea to provide us with the gene accession number of published sequences or send us the sequence by email. All info shared with us always remains confidential. It will normally take 1-3 days to complete our analyses. There are no charges for this assistance or obligation.

Click here for Additional details on this service Free Services: Selection of Antigenic Peptides, BLAST searches, Identification of Signal peptide, Transmembrane domains, Sequence Alignment, and other common structural motifs

Q16. Should I consider adding a Cysteine in peptides for making antibodies?

All small peptides must be coupled to a carrier protein (KLH, BSA, Ovalbumin, etc) in order to elicit high titer antibodies. Generally, peptides can be coupled to other proteins by utilizing a free NH2 or COOH, or a Cysteine group. Chemical conjugation using Cysteine offers a single point attachment provided there is just one Cys in the sequence (added or part of the native sequence). It is preferable to add Cys at the NH2 terminus if the peptide is internal or it represents the very C-terminus. This will keep the COOH free (non-conjugated) as it exists in native protein. For peptides representing the very NH2-terminal sequences, Cys should be added at the C-terminus of the peptide. For internal peptides, Cys can be added at either end but it is easier to synthesize peptides containing a NH2-terminal Cysteine. Cysteine can also be used to couple peptides to Sepharose for affinity purification of antibodies. Amino or COOH-conjugation chemistries should be avoided as most peptides contains several NH2 and COOH groups available in a given peptide sequence resulting into multi-point attachment and peptide distortion.

Q17. Is it necessary to use 95-98% pure peptides for antibody production?
Although, pure peptides are always better but it is not a requirement to have 90-95% pure peptides for antibody purposes. It is generally more economical to synthesize about 70-85% pure peptides than to spend lot more to purify peptides. The antibody project is not likely to fail because of the use of 70-85% pure peptide as opposed to >90% or better purity.

Q18. How about coupling peptides to 2 different carriers (KLH and BSA)?
For most anti-peptide antibody purpose, coupling of peptides to just one carrier protein (KLH or BSA) should suffice for immunization. Many small peptides will not coat well on ELISA plates. Coupling of peptides to another protein is done to facilitate coating of peptide on ELISA plates. ADI uses free peptide for coating and screening antibodies by ELISA. We have developed special technique to coat most peptides to ELISA plates. Therefore, we do not recommend coupling peptides to more than one protein and hence save these expenses.

Q19. What should I consider when making antibodies to phosphorylated peptides or other modified peptdies (sulfated etc)

ADI has made many antibodies to phosphorylated peptides. Pleaseclick here for additional details.

Q20. How about MAPS peptides? Can ADI make those?
MAPS or Multi-Antigenic Peptide System is a polymer (symmetric or asymmetric) of 8-16 lysines, and each one of those can take a peptide during peptide synthesis, thereby increasing the length of the polymer. This is done to eliminate the coupling of peptides to KLH. It cost approx. Rs 20,000 more to make MAPS, the same as conjugation. In our limited comparisons, we have seen more failure to MAPS than conventional KLH conjugation. In addition, there is no free peptide produced when making MAPS, making it difficult to remove MAPS-core directed antibodies. We do not recommend MAPS but we can make them if requested.

Q21. Can ADI Provide some **references** for its Custom peptide synthesis and Antibody Services?

ADI is truly a global company providing custom peptide and antibody services throughout the US and many international institutions. ADI's services have been referenced in most top-ranked scientific journals.