What is PolyHRP?

Why it is ultra-sensitive?

What is special in SA-PolyHRP detection?

How to handle PolyHRP conjugates?

Which requirements to other kit components have to be considered in order to successfully achieve ultra-sensitive performance with
SA-PolyHRP20, 40 & 80 conjugates?

Which reasons cause backgrounds when using SA-PolyHRP?

How an ELISA test system has to be designed in order to most effectively realize the high sensitivity detection potential of PolyHRP?

These and many other questions are addressed in our

PolyHRP Detection Users Guide:
Ultra-sensitive two-site (double antibody sandwich) ELISA systems utilizing Streptavidin-PolyHRP conjugates.

by D. Plaksin, SDT

The purpose of this communication is to provide users with an essential knowledge and correct understanding of PolyHRP detection along with related practical recommendations.

The idea of PolyHRP is very simple. PolyHRP conjugates quantitatively deliver a large number of signal-generating enzyme molecules to one bound analyte molecule. This results in multiple detection enhancement which is directly proportional to HRP polymerization range.

SA-PolyHRP conjugates are made of 5 (five) identical covalent HRP homopolymer blocks that are, also covalently, coupled to multiple streptavidin molecules. Three different homopolymers are currently used in our production process. These are PolyHRP20, PolyHRP40 and PolyHRP80. Three different specific SA-PolyHRP items follow respectively. An average total number of

HRP monomer molecules in design of SA-PolyHRP80 is 400 (80 × 5), in SA-PolyHRP40 is 200
(40 × 5), and in SA-PolyHRP20 is 100 (20 × 5). SA-PolyHRP80 is more sensitive than SA-PolyHRP40, and SA-PolyHRP40 is more sensitive than SA-PolyHRP20.

Technically detection with SA-PolyHRP is also very simple. There are no changes of principle that would affect an assay scheme (number of steps, incubation intervals). Composite kit reagents are essentially the same as in conventional ELISA using standard SA-HRP. Similarly, PolyHRP detection may be used with any of the applicable HRP (colorimetric, fluorimetric) substrate development systems including enhanced chemiluminescence. This also means perfect compatibility with existent routine and newer emerging ELISA instrumentation.

There are actually no technical limitations, neither patent restraints associated with PolyHRP. This makes PolyHRP detection very open developer-friendly item. At the same time, we would never claim that one might be able to easily achieve the best high sensitivity performance with PolyHRP by just substituting standard SA-HRP with SA-PolyHRP conjugate in existent ELISA test kit.

Such claim would disregard the fact that well developed ELISA kit is an integrate, finely balanced system. Selected assay conditions that are optima for using standard SA-HRP will not necessarily be the same with PolyHRP conjugates. Effective introducing SA-PolyHRP20, 40 or 80 into customers' own ELISA system instead of standard SA-HRP will suggest need for another level of the balancing of the system, i.e. actually a development of another, superior new kit.

Proper developmental work is worth effort anyway. Right point is to consider it as reasonable investment that will pay back with outstanding performance of an ELISA test kit exploiting unique advantages of the PolyHRP detection.

We hope to be of help to interested users trying to assist their developmental work with the given general comments and specific troubleshooting guidelines. Below are some important facts and considerations that one has to take into account when running development of an ultrasensitive ELISA with the use of the PolyHRP detection. This guide is focused primarily on double antibody sandwich ELISA for an antigen.

1. PolyHRP size.

All three items of our current product line are very large in size. Although still

soluble and stable in solutions, SA-PolyHRP20, 40 & 80 are practically not filterable through 0,2µm - 0,22µm filter units. At the same time all three conjugates can effectively be filtered through standard 0,45µm filter devices, with no loss on filtration.

Therefore, when working on stable pre-diluted and ready-to-use PolyHRP formulations developers should use 0,45µm filtration devices, but NEVER 0,2µm or 0,22µm units.

2. Biotin.

SA-PolyHRP conjugates are very sensitive to minimal biotin content potentially present in diluents developers may currently use. Endogenous (both free and bound) biotin will naturally weaken detecting activity of any SA-HRP conjugate. Due to the fact that molar HRP/SA ratio in SA-PolyHRP is largely shifted towards HRP, our polymeric conjugates are inhibited by smaller biotin concentrations apparently stronger as compared to conventional SA-HRP.

2.1. SA-PolyHRP diluents [1].

In most cases we recommend using diluents made of our filterable Casein Buffer Concentrate 1, (#CBC1), specialty developed reagent which is certified as material containing virtually no biotin. Absence of biotin in our Casein Buffer is directly proven in ELISA „use test“ with SA-PolyHRP80 diluted down to 1:20.000. If you do wish to use with SA-PolyHRP your own diluent system, we strongly recommend that you at least test your suggested formulations vs. our standard ready-to-use Universal (SA-PolyHRP) Casein Diluent/Blocker (#UCDB) and/or Streptavidin-PolyHRP Conjugate Stabilizers #SA1 and #SA2-HT taken in the capacity of biotin-free reference material.

Diluents made of ANIMAL SERA as a rule are NOT COMPATIBLE with SA-PolyHRP. Exhaustive dialysis and other methods do not completely remove bound. Bound biotin associated with medium and high molecular weight material (“conjugated” biotin) rests with animal sera diluents after dialysis and can still strongly inhibit SA-PolyHRP conjugates.

3. Backgrounds.

In sandwich ELISA with two-step biotin-streptavidin detection system, an actual background, i.e. the signal recorded in absence of analyte in the given matrix, is a result of an aggregate non-specific binding (NSB) process in which both specific detecting antibody-biotin reagent and Streptavidin conjugate

take part.

Substantially, there are three events that influence background associated with any biotin-streptavidin detection system*:

A - NSB of antibody-biotin conjugate to the surface of immunoplate coated with capture antibody;

B - direct NSB of SA-(Poly)HRP to the same (intact) immunosorbent surface;

C - indirect binding of SA-(Poly)HRP through non-specifically bound biotinylated antibody (A).

Binding C occurs only in consequence of primary binding event A. There is no C without A: when A is quantitatively zero, C is also zero.

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* background problems associated with specimen matrix are not considered here.

SA-PolyHRP conjugates alone do not cause direct (B) backgrounds, when applied in compatible diluent that effectively blocks non-specific interactions with adsorbed capture antibody. As already mentioned, PolyHRP conjugates are very large in size. This suggests that their possible NSB to immunosorbent surface will be realized as massively multivalent (multipoint) protein-protein interaction, whereas bindings between single-point reacting sites within multiple interaction are very week.

3.1. SA-PolyHRP diluents [2].

Larger, preferably colloidal, polymer reagents, such as casein and some gelatin preparations, are evidently more effective in eliminating above (massively multivalent) NSB. Biotin-free casein #CBC1 is much recommended as constituent part of SA-PolyHRP diluent systems. It performs excellent in the capacity of an effective NSB blocker.

With low/medium (1/2.000 - 1/5.000) diluted conjugates casein additionally boosts positive signals in PolyHRP detection. Along with eliminating backgrounds, this increases signal-to-noise ratio and thereby on the total improves analytical performance with PolyHRP.

0,45µm-0,2µm-filterable biotin-free Casein is also helpful in making stable diluted SA-PolyHRP formulations.

Ready-to-use Universal (SA-PolyHRP) Casein Diluent/Blocker (#UCDB) and Streptavidin-PolyHRP Conjugate Stabilizers (#SA1 and #SA2-HT) are ideal products for using with SA-PolyHRP conjugates.

At the same time, simple BSA-based diluents may be quite effective when applying SA-PolyHRP at lower concentrations (higher dilutions 1/10.000 -1/ 20.000), provided the binding antibody is not compromised as material causing backgrounds (see below). This is, again, due to the fact that SA-PolyHRP alone, in respect to purely Non-Specific Binding, is a sort of quite quiet, almost “cold” reagent.

For using with highly diluted (1/10.000-1/20.000) SA-PolyHRP, try our biotin-free #BSA1 product. Simple mixing of this reagent with PBS may give you fairly effective and economical (with the end BSA concentration in the range 1 - 0,2%) diluent that would be well applicable in research application mode (for making daily working dilutions of SA-PolyHRP, preferably from pre-diluted stabilized items).

While A and B are true non-specific protein-protein interactions, C is a two-stage process which, in its first part, is the same A and, in its second part, involves specific biotin-streptavidin interaction. One has to recall an enormous strength of biotin-to-streptavidin binding in order to get a right understanding of the real background situation with SA-(Poly)HRP, which is as follows.

Double impact of intrinsically bound events A + C influences background much stronger as compared to independent event B. Thereby in practice backgrounds are substantially due to the non-specific binding of antibody-biotin conjugate.

It is clear that, in the real ELISA, an intensity of antibody-biotin mediated background (event A) ultimately depends on the detecting activity of Streptavidin conjugate (event C). Detecting potential of PolyHRP is exceptionally high. Respectively, detection
utilizing SA-PolyHRP is extremely sensitive to NSB of antibody-biotin reagent.

In other words, the actual background in a sandwich assay will be a result of the very high detecting strength of SA-PolyHRP conjugate which is capable of "seeing" truly trace amounts of detecting biotinylated antibody, that are non-specifically bound to immunosorbent in absence of analyte and are simply not detectable when standard SA-HRP is applied. Thus, quantitatively the same level of non-specific binding of detector antibody-biotin conjugate that appears negligible in respect to actual background levels in detection with standard SA-HRP may become unpleasantly visible with SA-PolyHRP.

This is exactly the point that necessitates re-balancing of the whole system, i.e. bringing it to another performance level where higher sensitivity will not be compromised through higher backgrounds. "Trick" is how to eliminate backgrounds without loosing sensitivity.

Both "positive", specific (yielding sensitivity) and "negative", non-specific (causing background) binding processes are frequently understood as inevitably parallel processes. This is nevertheless not the case when one takes care of eliminating in particular NSBs using methods that do not affect essentially specific binding. Important is the proper understanding of the nature and the "structure" of backgrounds.

3.2. Background associated with antibodies.

Apart from possible matrix effects, NSB between adsorbed capture antibodies and biotinylated detector antibodies is actually the key event (A) that causes backgrounds. It is particularly important to eliminate said trigger NSB when aiming at really ultrasensitive performance with SA-PolyHRP.

In practice, backgrounds are predominantly associated with the presence in either binding or detecting antibody preparations aggregated or spontaneously polymerized IgG material, immune complexes, microbial impurities or possible contamination of another origin. For instance, trace amounts of Protein A or Protein G, as well as positively charged DEAE-polymer fragments (as a result of minimal resin leakage in process of IgG purification) may cause huge backgrounds.

3.2.1. Quality Control requirements to antibodies.

It is very advisable that users of the PolyHRP apply stringent quality control to both capture and detector antibody, allowing no presence of aggregated or spontaneously polymerized material with M.W. over 150-160 kDa characteristic of chromatographically pure IgG.

Biotinylation results in chemical modification of IgG surface that may cause aggregation of IgG after conjugation with biotin. Therefore, prepared detecting antibody-biotin reagent should undergo the second control. Analytical HPLC is the method of right choice in controlling both capture antibodies and detector antibody-biotin conjugate.

Aggregates, if present, must be removed by size-exclusion chromatography. At regular preparative rates, gel-penetration chromatography using column with Sephacryl S-300HR, Superdex 200 or Toyopearl HW-55 allows efficient preparation of chromatorgaphically pure IgG in production quantities.

3.2.2. Effect of biotinylation.

The assumption is that the most developers will apply most frequently used standard biotinylation protocol using NHS-(amidocaproate)-biotin ester – to – IgG molar ratio 30-33. Usually, this results in good performance. Nevertheless, functional results of specific antibody biotinylation can not be smoothly predicted in 100%. This especially concerns monoclonal antibodies.

Sometimes, even if aggregates are not present or after their removal, biotinylated antibody may still provoke unacceptably high background. This can be associated with excessive biotinylation. In this case, lowering of biotinylation degree through selecting smaller NHS-LC-biotin/IgG conjugation ratio is advisable. In difficult cases, optimum level of biotinylation has to be found in functional „chess“ titration experiment (see below).

3.2.3. Use of the Antigen-binding Antibody fragments may result in smaller NSB.

Purified smaller antigen-binding F(ab)2 and Fab fragments of specific IgG are good antibody reagents of choice. Smaller the size and the surface of the molecule (within the same charge distribution and hydrophobic/hydrophilic balance patterns), as a rule, weaker the protein-protein NSB. Besides, removal of Fc fragments is usually helpful in preventing possible (plasma/serum) matrix effects associated with heterophilic antibodies, HAMA, rheumatoid factors, components of the complement system, etc.

3.2.4. Detector antibody-biotin conjugate diluents

and

3.3. Casein Buffer as universal Blocker/Diluent eliminating NSB.

Again, background in the real two-site ELISA with biotin-streptavidin detection system is predominantly antibody-biotin mediated background. If NSB between immobilized onto the surface of immunoplate capture antibody and antibody-biotin conjugate is limited to a solely protein-protein NSB, diluents made of Casein Buffer are very effective in eliminating backgrounds. Efficiency of e.g. #UCDB is so high that it could be used as reference test material for experimental discriminating between backgrounds that are due to true NSB and backgrounds of essentially specific or other non protein-protein NSB origin.

#UCDB will effectively eliminate backgrounds resulting from true NSB, i.e. NSB recognized as unidentified protein-protein binding referred to a certain “amorphous” sum of essentially weak physicochemical interactions having no any specific substratum. Said result is clearly related to situation where both antibody reagents have applicable purity.

#UCDB will not eliminate backgrounds of not true NSB origin. Said backgrounds are most frequently originated from different impurities, e.g. traces of Protein A or Protein G, “hidden” trace microbial or polycationic contaminations. Minimal presence of such impurities may result in strong “bridge” binding between capture and detector antibody, which binding is of essentially specific or polyelectrostatic origin. Above binding manifests in particularly large, rigid and tenacious backgrounds that are practically impossible to overcome.

3.2.5./3.3.1. Casein Buffer as Antibody-biotin Diluent.

With biotinylated antibody, presence of endogenous biotin in diluent (unless it can somehow be bound or conjugated to immunosorbent during incubation) does not play any role. Therefore, our second casein product, Casein Buffer Concentrate 2 #CBC2, may be recommended for formulating antibody-biotin diluents that will effectively block NSB between antibody-biotin conjugate and immunosorbent. Related ready-to-use products, Antibody/Antigen/(PolyHRP) Conjugate Diluent/Blocker #AADB and Antibody/Antigen (PolyHRP) Conjugate Stabilizers #AA1 and #AA2-HT were specially developed for using with detector antibody-biotin conjugates and are also perfectly working reagents with diverse other antibody and antigen conjugates. Although Universal (SA-PolyHRP) Casein Diluent/Blocker (#UCDB) and Streptavidin-PolyHRP Conjugate Stabilizers #SA1 and #SA2-HT are as well applicable in the capacity of effective diluents/stabilizers for detector antibody-biotin conjugate, non biotin-free products #AADB, #AA1 and #AA2-HT are certainly reagents or more economic choice.

3.3.2. Casein Buffer as Specimen/Calibrator Diluent.

Casein Buffer products perform very well in the capacity of Sample/Specimen and Standard/Calibrator diluent, too. Through eliminating NSBs in specimen matrices they effectively level matrix effects in situation when the same analyte must be quantified in different specimens (e.g. cytokines in serum/plasma, cell culture supernatants and urine). Like situation with detector antibody-biotin conjugates, casein will target and effectively eliminate matrix effects of only true NSB origin. Matrix effects of essentially specific nature associated with presence of heterophilic antibodies, HAMA, rheumatoid factors and components of the complement system will not be eliminated by casein blocker alone. Other, specific blockers, as HAMA Blocker #HAMAB or Universal Blocker of heterophilic antibodies, rheumatoid factors, HAMA and complement #UB will need to be added in Specimen Diluent formulation to take care of adverse specific matrix effects.

3.4./3.3.3. Blocking procedure.

Finally, Casein Buffer may be used for the blocking of immunoplates coated with antibody. This procedure is used with the sense of blocking free remaining sites on the surface of the adsorbent immunoplate after adsorption of capture antibodies.

In spite of intuitive appreciation, casein (heavily colloidal polymer) as Blocker in separate procedure of blocking antibody-coated immunoplate does not cause steric hindrances to the adsorbed antibodies. Like BSA, that is perhaps the most frequently used in blocking procedure protein, adsorbed casein does not hamper specific binding of an antigen and respectively does not affect/worsen high sensitivity performance of ELISA with PolyHRP detection.

Although blocking does practically not influence (in sense of improving) performance of the detection system, in respect to background sorted out as detection system background, it can be helpful in eliminating adverse matrix effects. In addition, adsorption of casein improves stability of immobilized binding antibody, especially stability of dried antibody-coated immunoplates.

3.3.4. Casein as Stabilizer.

Thus, filterable Casein Buffer will perform very well in the capacity of Diluent/Blocker with Sample/Specimen, antigen Standard/Calibrator, detector Antibody-biotin conjugate and SA-PolyHRP conjugate (only biotin-free Casein Buffer based products). It will also work perfect as general protein Stabilizer with all assay reagents that are used in liquid ready-to-use formulation.

Streptavidin-PolyHRP Conjugate Stabilizers #SA1 and #SA2-HT and Antibody/Antigen (PolyHRP) Conjugate Stabilizers #AA1 and #AA2-HT are specialty casein based stabilizers that contain proprietary antioxidant and oxygen scavenger components.

3.2.6. Two diluent strategies. Many different antibody-biotin diluents are applicable.

Universal (SA-PolyHRP) Casein Diluent/Blocker (#UCDB) is very useful miscellaneous reagent, convenient in designing of ELISA test kits for the Life Science Research. In this marketplace manufacturers can rely on experienced users who will prepare all working reagent dilutions for ELISA themselves. That way manufacturer can benefit from supplying kit with only one universal buffer for diluting all essential test kit components like Sanquin/CLB do with HPEB (High Performance ELISA Buffer which is analog to our #UCDB) in their PeliKine ELISA kits. Although “logistically” a bit more complicated, we nevertheless suggest using also in Research ELISAs two different diluent systems:
one - Universal (SA-PolyHRP) Casein Diluent/Blocker #UCDB – for blocking plates and diluting SA-PolyHRP; and second – Antibody/Antigen/(PolyHRP) Conjugate Diluent/Blocker #AADB – for diluting Sample/Calibrator and detector antibody-biotin conjugate. Using of two separate-diluent systems is quite justified: #AADB is 30% cheaper compared to biotin-free #UCDB.

A design concept of the test kit for the routine clinical diagnostics would rather include all reagents in stabilized pre-diluted form at final working strength. Casein Buffer based products are very good candidates for designing effective ready-to-use formulations, especially our Streptavidin-PolyHRP Conjugate Stabilizers #SA1 and #SA2-HT that are undoubtedly the best products for making ready-to-use SA-PolyHRP. Antibody/Antigen (PolyHRP) Conjugate Stabilizers #AA1 and #AA2-HT will work well in ready-to-use Antigen Controls/Calibrators and ready-to-use detector antibody-biotin conjugate. At the same time with biotinylated antibody manufacturers may use different other diluents including animal sera based diluents/stabilizers that may be heavily contaminated with biotin. The same concerns Specimen/Calibrator diluent. With both Specimen/Calibrator and detecting antibody-biotin, there is no restriction on diluents containing biotin, provided the wash after incubation with biotinylated antibody is effective.

4. Choice of the solid phase: immunoplates/strips/tubes/balls, etc.

We understand that the vast majority of developers interested in achieving higher sensitivities will most probably use High Binding Capacity Immunoplates.

None less, it is important to extra emphasize that, with the PolyHRP detection, use of the products certified as High Binding Capacity material is a requirement. It is simply a MUST. This literally means that high sensitivity detection potential of PolyHRP can not be fully realized when Medium or moreover Low Binding Capacity immunoplates are used. Binding 400 ng IgG per 1 cm2 ensured as typical certification of High Binding materials by such manufacturer as Costar/Corning seems work as formal criterion in making right choice of Immunoplate.

5. Coating procedure with capture/binding antibody (as applied to standard 8×12 immunoplates).

With PolyHRP, it is particularly important to minimize concentration of capture antibody in coating procedure. Recommendation is to coat wells with binding/capture antibody taken in concentration 1 µg/ml, yielding 100 ng specific IgG per well, respective to the applied volume 100 µl per well. Applying antibody at larger concentration will decrease sensitivity of an assay. There is practically no much sense in doing optimization experiments with various coating antibody concentrations. Coating with
1 µg/ml purified capture IgG, 100 µl per well, will in the most cases yield the best analytical performance. Regular 50-100 mM Na-Carbonate-Bicarbonate Buffer (CBB) pH 9,4-9,6 is well applicable when coating with capture IgG. As an easy, effortless and convenient option, try our Binding/Coating Buffer Concentrate #BBC; 1 Volume mixed with
9 Volumes of deionized water will yield 50 mM Na-CBB pH 9,5 reliably preserved with BND at 300 ppm. Static overnight (18 hours) incubation at +4° C is preferable compared to faster coating protocols at higher temperatures. With many antibodies, coating at +4°C gives better performance compared to coating at room/ambient temperature.

When using purified antigen-binding IgG fragments, effective concentrations should be optimized in separate titration experiment. Optimum coating concentrations of F(ab)2 and Fab fragments, depending on specific system, may appear to be 1,5-2 – to - 3-4 times lower compared to the whole IgG antibody.

5.1. Blocking of the coated immunoplates.

It is advisable to introduce appropriate blocker right after removal (aspiration) of capture antibody solution, with no prior washing, i.e. avoiding contact with detergent. Larger application volume of blocker compared to capture antibody volume, e.g. 100 ml/well antibody and then 200 µl/well blocker, will secure better performance, especially with “difficult” specimens.

5.2. Drying of the antibody-coated immunoplates

Try our Drying Buffer/Stabilizers #DBS-B and #DBS-C which contain anti-microbial, general protein stabilizing, abundantly
OH-group-donating and anti-oxidizing components that reliably protect and preserve immobilized (adsorbed) antibody and antigens guaranteeing long real-rime stability of dried immunoplates at +2°C/8°C and room/ambient temperature regular storage and handling regimens.

6. Choice of specific SA-PolyHRP conjugate.

This depends on what developers desire and which other possibilities do they have. Of course, the best high sensitivity performance can be achieved with SA-PolyHRP80 rather than, for example, with SA-PolyHRP40. System utilizing PolyHRP20 will be less sensitive than system with PolyHRP40. At the same time, several customers having available high affinity antibodies have been achieving in their colorimetric (TMB) sandwich ELISAs pico-femtogram sensitivity level already with SA-PolyHRP20, typically measuring antigen in concentrations below 100-50 pg/ml, where 100-50 pg/ml is a higher, first concentration calibrator point.

With all individually optimized concentrations and other parameters, overall end-point sensitivities in two-site ELISA with SA-PolyHRP conjugates are distributed in logical agreement with HRP polymerization range. SA-PolyHRP80 is on the average two times more sensitive comparing with SA-PolyHRP40, and four times more sensitive as compared to SA-PolyHRP20. Difference between PolyHRP40 and PolyHRP20 is approximately factor two.

7. Choice of effective detector Antibody-biotin and SA-PolyHRP concentration.

and

8. Incubation time/temperature/mode and other assay conditions.

Optimum concentrations of detecting reagents should be established on the base of the data obtained in „chess“ titration experiments. At this point we would like to avoid very specific practical recommendations. Results of such optimization will depend on many factors. Individual capture/detector antibody characteristics, biotinylation degree, diluents, incubation timing scheme and mode, substrate development system are all the factors influencing ELISA performance. Developers are generally free in choosing desired parameters based on their own considerations. PolyHRP detection performance can be balanced through adjusting other kit components in

basically flexible way.

Incubation with SA-PolyHRP does of course not need to be performed at elevated temperatures. We wonder, if anyone would have reported better performance of SA-PolyHRP at 37°C in comparison with regular room (18-25° C) temperature incubation.

Shaker incubation is certainly better than static incubation mode. With PolyHRP, shaking of immunoplate in standard rotary immunoplate shaker at 600-800 rpm distinctly improves High Sensitivity performance.

Incubation interval with SA-PolyHRP is usually 30 minutes. However, it may be shortened to 15-10 minutes. The same concerns incubation with other reaction participants, if one is aiming at development faster assay protocol. PolyHRP allows to substantially reduce the time of the assay while sensitivity remains significant (basically, in lower picogram range).

In routine approx. 3-hour colorimetric ELISA (shaker mode, room temperature, TMB) with good high affinity matched MAb pair, PolyHRP will yield performance enabling quantification of antigens beginning from 20-10 pg/ml (as the first, high concentration calibrator point), down to 50-100 fg/ml. At that, with the reagent volumes of
100-200* µl per well, incubation intervals will look as follows:

Sample/Calibrator	(200 µl/well)	- 60 min.
Detecting Antibody-biotin	(100 µl/well)	- 60-30 min.
SA-PolyHRP	(100 µl/well)	- 30-20 min.
TMB substrate	(100 µl/well)	- 15-10 min.

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* We recommend 100 µl/well for all steps/reagents, except for only blocking step (in preparation of the assay) and specimen/calibrator (when running assay itself). Applying 200 µl/well sample/calibrator often yields higher sensitivity compared to 100 µl/well.

In Table 1 suggested „chess“ titration experiment is shown as (a bit excessive) illustration assuming application of the above timing scheme (60'+60'+30'+15'), use of a good performance matched antibody pair and strong colorimetric TMB substrate system, as s(HS)TMB #sTMB. The given example, as it shown in Table 1, is very generic and should not be considered as direct „follow up“ practical recommendation. It just shows logic of experimental approach while including partially improbable, extreme parameters as e.g. working dilutions of
SA-PolyHRP 1/1.000 and 1/16.000.

Users may choose their own start-up concentrations and decrement intervals. It is naturally recommended that after the first screening set of „chess“ titration experiments smaller decrements shall be applied in further fine „polishing“ optimization runs.

Again, balancing of the assay system with SA-PolyHRP (as well as with standard SA-HRP) is a flexible process. Developers have to choose effective conditions themselves, and, in practice, this would unlikely be a matter of running just one or very few „chess“ titration experiments.

Effective ultrasensitive performance with SA-PolyHRP will very probably require application of detector antibody-biotin conjugate in smaller concentration, compared to one that would typically be used with the good standard SA-HRP. With PolyHRP users should not be afraid of decreasing biotinylated antibody concentration. At still higher sensitivity level, lower reagent consumption would only result in production cost savings.

A sandwich ELISA with SA-PolyHRP will outperform an ELISA utilizing the same antibody pair and conventional SA-HRP conjugate in sensitivity, speed and/or lower consumption of specific reagents.

As more practical reiteration, guidelines on designing ultrasensitive double antibody sandwich ELISA test system using SA-PolyHRP are summarized below.

(Those who are skilled in the art would appreciate that many of suggested items are nothing but normal requirements routinely applied in well-bred R&D environment, i.e. they are essentially recommendations that would only improve performance of any other assay system. Above consideration, again, supports the claim that says: the development of ultrasensitive assay with PolyHRP detection is quite practicable task.)

1. Use only pure (<0,05 µS/cm) deionized water and high purity general chemicals of Research/Analytical Grade.

In preparation of all reagents try to avoid contamination of immunoplates and liquid assay reagents with air-borne particles.

Use with all reagents reliable anti-microbial biocide/preservative. We recommend using 5-Bromo-5-nitro-1,3-dioxane (#BND-D, #BND-W). It works perfect as preservative in 0,02% -0,03% (simple salt buffers) – 0,05% -0,06% (detergent and protein containing buffers) – 0,1% -0,12% (stabilized liquid ready-to-use formulations) concentrations at +4°C and room/ambient temperature and does not interfere ELISA performance (no one step will be affected through using BND including substrate development reaction).

Filter all working buffers through 0,1-0,2µm (semi)sterile filter devices.

When filtering, avoid foaming. Remember, dissolved oxygen will damage HRP. Therefore, although it is generally not required in ELISA techniques, it is still better/safer to degas all buffers (degassing is a MUST for stabilizing buffer used in preparation of HRP conjugates in ready-to-use format at final working strength).

2. Use only High Binding Capacity Immunoplates, e.g. Costar E.I.A./R.I.A. 8×12 microwell plates, High Binding, Cat. No. 3590.

DO NOT USE Medium or Low Binding plates.

3. When possible, use chromatographically pure specific IgG or antigen-binding antibody fragments.

Carefully control both binding and detecting antibody (the latter – both before and after biotinylation) in respect to the possible presence of aggregated/polymerized material.

Apply analytical HPLC to control capture and detector antibody-biotin preparations.

If high molecular weight contamination presents, apply reliable preparative cut-off procedure in order to remove material with M.W. larger than 150-160 kDa in case of the whole IgG antibody or with M.W. higher than one characteristic of respective pure antigen-binding antibody fragment. Classical

gel-penetration chromatography is still the best method of choice that can be recommended for preparing chromatographically pure IgG.

4. Coat immunoplates with capture antibody in concentration 1 µg/ml, yielding 100 ng specific IgG per well respective to applied volume 100 µl per well.

When using antibody fragments, establish optimum concentration in separate experiments.

Consider, that effective concentration may be smaller than 1 µg/ml, but unlikely larger than 1 µg/ml.

Use standard 50 mM Na-Carbonate/Bicarbonate Buffer pH 9.5 with 0.03% BND, 0.2µm-filtered, (1 Vol. #BBC + 9 Volumes pure water).

Incubate immunoplates filled with antibody solution 18 hours at +4°C, sealed or placed into clean humid chamber.

5. (optional blocking procedure). Right after aspiration of coating buffer, fill the wells of immunoplate with effective blocker. Recommended Blockers:

- 1% biotin-free BSA in PBS (1 Vol. #BSA1 + 1 Vol. #PBSC + 8 Volumes pure water)

- Universal (SA-PolyHRP) Casein Diluent/Blocker #UCDB

- 1/5 Casein Buffer Concentrate 1 in PBS (1 Vol. #CBC1 + 0,4 Vol. #PBSC + 3,6 Volumes pure water)

Blocker volume that is twice the volume of capture antibody, i.e. 200 µl vs. 100 µl is better than equal (100 ml) volume. Incubate 1 hour or longer at room temperature.

If you are not going to use the prepared plate on the day of blocking, seal it after 1 hour incubation at room temperature and put it in refrigerator at +4° C. Plate with adsorbed antibody, filled with one of the Blockers as suggested above, will be quite safely preserved at +4°C and can wait for an assays at least several days-weeks and sometimes, depending on your given capture antibody, - months with no visible drop in antigen-capturing activity. “Over-blocking” is a mythos and does never happen.

6. Wash antibody-coated immunoplate with PBS containing 0,05% Tween-20 (PBS-Tween) 5 times (1 Vol. #WBC + 9 Volumes pure deionized water)

Optionally dry after additional single wash step with appropriate “drying” buffer containing dry reagent stabilizers, usually high concentration oligo/poly-sugars (trehalose, dextrins, dextrans) along with natural (BSA, casein) or synthetic (PVA, PVP, etc.) “supporting” polymers.

Try our Drying Buffer/Stabilizers #DBS-B and #DBS-C, formulations that have been carefully checked and selected as formulations proven to support High Sensitivity performance in systems with SA-PolyHRP conjugates.

7. Use for diluting SA-PolyHRP conjugates only those buffer systems that are biotin-free and contain effective blocker of non-specific protein-protein binding.

Universal (SA-PolyHRP) Casein Diluent/Blocker #UCDB and Streptavidin-PolyHRP Conjugate Stabilizers #SA1 and #SA2-HT are the best recommended products that can currently be used for diluting SA-PolyHRP to final working strength in R&D mode (daily dilutions - #UCDB) and for formulating SA-PolyHRP in stable liquid pre-diluted or final working strength ready-to-use format (#SA1 and #SA2-HT).

8. Pay very careful attention to antibody-biotin reagent mediated background. Apply antibody-biotin in diluent that will effectively eliminate NSB.

Antibody/Antigen/(PolyHRP)Conjugate Diluent/Blocker #AADB and Antibody/Antigen (PolyHRP) Conjugate Stabilizers #AA1 and #AA2-HT are strongly recommended as reagents that will not only allow to eliminate backgrounds of true NSB origin but also – through excluding true NSB – help in sorting out backgrounds that are due to poor or insufficient purification of capture and/or detector, biotinylated antibody or due to “hidden” contaminations compromising one or both antibodies. Alternatively, use with biotinylated antibody other diluents made of Casein or Gelatin or proven animal sera diluents. BSA-based diluents are usually too weak diluents that are poor in NSB-eliminating potential when applied with detector antibody-biotin conjugates.

Be well prepared to decrease, if necessary, concentration of biotinylated antibody in detection system compared to concentration being used in existent ELISA test with standard SA-HRP.

9. When developmental capabilities (time/budget, etc) permit or if otherwise necessary, investigate/select an optimum degree of biotinylation. Rigidly high background signals with antibody-biotin conjugate applied in #UCDB at concentrations lower than 100 ng/ml along the entire range of the used SA-PolyHRP dilutions (or at least with SA-PolyHRP80 diluted up to 1/20.000), as resulted from „chess“ titration experiment (see infra, item 10), may necessitate such optimization – provided that other background reasons, as hidden contaminations, etc., are unequivocally excluded.

10. Run „chess“ titration experiment according to suggested general Protocol (Appendix 1) and scheme of principle (Table 1). At least three antigen Calibrator points in suggested matrix should be included in duplicates into initial screening experiments along with two different detection system control points. These are specifically the following points:

(i) zero/zero (0/0): no Ag (void matrix + diluent), no Ab-biotin, SA-PolyHRP alone

(ii) zero (0): no Ag (void matrix + diluent), Ab-biotin and SA-PolyHRP

(iii) low antigen

(iv) medium antigen

(v) high antigen

Low antigen concentration point projected as the lower end calibrator point shall determine analytical end-point sensitivity of an assay. Medium antigen concentration point must generate signal that would lay preferably in the mid of the future calibration curve. High antigen concentration point shall correspond to projected upper end calibrator point, this point should preferably generate maximum recordable signal provided by measurement instrumentation within confidently linear range.

Zero point (ii) is complete true “analytical” background resulting from non-specific binding interactions between detection system components and intact immunosorbent in absence of the bound analyte. Zero/zero point (i) helps to see which impact on background has SA-PolyHRP alone, i.e. whether SA-PolyHRP binds non-specifically to immunosorbent in absence of specific detector antibody-biotin conjugate. When running this separate (for SA-PolyHRP) control, apply diluent without Ab-biotin instead of diluted Ab-biotin as in complete control (ii).

Running zero/zero (i) point is very useful in the very beginning of the development since (Scenario 1) it gives you evidence that SA-PolyHRP as such is not responsible for background. This at once gives you a confidence in PolyHRP and reveals detector antibody-biotin conjugate as background suspect number one. If you do see background in 0/0 point (Scenario 2), it says your capture antibody does not comply with QC requirements to its purity as stated above (3.2.1).

In this case we recommend to include with 0/0 run additional “Cold Capture Antibody“ controls. These are chromatographically pure ~150 kDa Mouse IgG (monoclonal antibody to hIgG γ-chain) #CCA-M and chromatographically pure ~150 kDa Goat IgG (affinity purified polyclonal antibody to hIgG [H+L]) #CCA-P. Both are enclosed with SA-PolyHRP developer kits as reference material to be used as parallel control to your specific monoclonal or polyclonal capture antibody in 0/0 run. Do not use “Cold Capture Antibodies” #CCA-M and #CCA-P as reference material in parallel zero (0) control run with your given biotinylated antibody (it is senseless and will not give you any useful information).

Both #CCA-M and #CCA-P adsorbed from 1 µg/ml in 1/10 #BBC, 100 µl/well, 18 hours at +4°C (no blocking, wash 5 times with 1/10 #WBC) after 30-min. incubation with SA-PolyHRP40 #SP40C diluted 1/5.000 (200 ng/ml) in #UCDB at room temperature, shaker mode (wash 5 times with 1/10 #WBC) and 15-min. development with s(HS)TMB #sTMB stopped with Stop Reagent #SER, measured at 450 nm vs. 620 nm reference shall give OD < 0,020.

Background in 0/0 point with your specific capture antibody that is higher than 0,020 along with expected “must” ODs < 0,020 in parallel control points with CCA-M and/or #CCA-P (serves also as “procedural” control checking for correctness of the assay running procedure including wash steps, etc.) says your further efforts will unlikely be successful unless you change quality of your capture antibody.

Once you have convinced yourself that SA-PolyHRP alone does not produce troubling backgrounds without detector antibody-biotin (which is an expected case in situation where your capture antibody is OK), there is usually no longer need in obligatory running this control in all further screening and optimization experiments.

Point (iii) over background (ii) discrimination, iii/ii, iv/ii and v/ii signal-to-noise ratios and respective absolute signal differences are all the starter criteria for screening evaluation of wanted analytical performance.

Ultimately, all the concentration points that must be used as calibrators with the future test system should be included in the later polishing experiments. Such characteristics of generated calibration curve as shape (linearity) and declination, i.e. increment of the signal in response to analyte concentration increment within dynamic range of interest, are decisive when precision of quantification is an important factor.

Appendix 1

„Chess“ titration experiment: suggested general Protocol (as not limited example)

1. Coating: captureAntibody, 1 µg/ml in Coating Buffer (1 Volume #BBC + 9 Volumes deionized water; pH 9.5 with 0.03% BND, 0.2µm-filtered), 100 µl/well (Costar E.I.A./R.I.A. plates, High Binding, Cat. No. 3590), sealed; overnight (18 hours) static incubation at +4°C.

One-time aspiration

2. Blocking: Universal (SA-PolyHRP) Casein Diluent/Blocker (#UCDB), 200 µl/well, 1 hour, room temperature, static.

3. Wash: Wash Buffer (essentially PBS containing 0.05% BND and 0,05% Tween-20, made from 1 Volume #WBC + 9 Volumes pure deionized water), 5 times.

4. Analyte: Antigen Calibrator taken in concentrations of interest including zero point.

When possible, use as control zero point material made of the real specimen (matrix) containing no antigen (negative or artificially depleted matrix), diluted in Antibody/Antigen(PolyHRP)Conjugate Diluent/Blocker #AADB. Optionally use Universal (SA-PolyHRP) Casein Diluent/Blocker #UCDB. Apply each calibrator in duplicate,
200 µl/well, incubate1 hour on rotary microplate shaker ~700 rpm at room temperature.

5. Wash: Wash Buffer, 3 times.

6. Detection:6.1. capture Antibody-biotin conjugate, different concentrations in Antibody/Antigen/(PolyHRP) Conjugate Diluent/Blocker #AADB.

Optionally use Antibody/Antigen (PolyHRP) Conjugate Stabilizers #AA1 or #AA2-HT or Universal (SA-PolyHRP) Casein Diluent/Blocker #UCDB. In first screening run take antibody-biotin in series of concentrations beginning from 500-400 ng/ml and ending with 50-20 ng/ml (2-, 3- or 4-5-fold decrements). Extended version may include 5-6 concentration points. Minimal experiment will implicate at least 3 concentration points, e.g. 100 ng/ml - recommended as starting point in any case, and e.g. 30-40 ng/ml as arbitrary lower and 300-500 ng/ml as higher (from expected optimum) points. Apply 100 µl/well, incubate1 hour on rotary microplate shaker 700 rpm. at room temperature;

6.2. Wash with Wash Bufer, 3 times;

6.3. Streptavidin-PolyHRP conjugate of choice, different concentrations in Universal (SA-PolyHRP) Casein Diluent/Blocker #UCDB.

Optionally use Streptavidin-PolyHRP Conjugate Stabilizer #SA1 or #SA2-HT. In first screening run take SA-PolyHRP serially diluted from 1/1.000 (1 µg/ml) to 1/40.000-1/50.000. In shorter version use 1/5.000 (200 ng/ml) as starting point along with e.g. 1/2.500 (400 ng/ml) and 1/10.000 (100 ng/ml) as supporting higher and lower conc. points. In many systems 1/5.000 will already be a “saturated dilution”, although in some systems using more concentrated conjugates equivalent to 1/2.000 dilution (500 ng/ml) may yield larger sensitivity. Apply 100 µl/well, incubate 30 minutes on rotary microplate shaker ~700 rpm. at room temperature;

6.4. Wash with Wash Buffer, 5-6 times;

6.5. Substrate: 100 µg/ml s(HS)TMB (#sTMB), 15 min on rotary microplate shaker ~700 rpm, room temperature,in darkness;

6.6. Stopping: Stop Reagent (#SER), 50 µl/well, 10-15 seconds on rotary microplate shaker ~700 rpm.

6. Reading 450 nm vs. 620 nm reference filter.