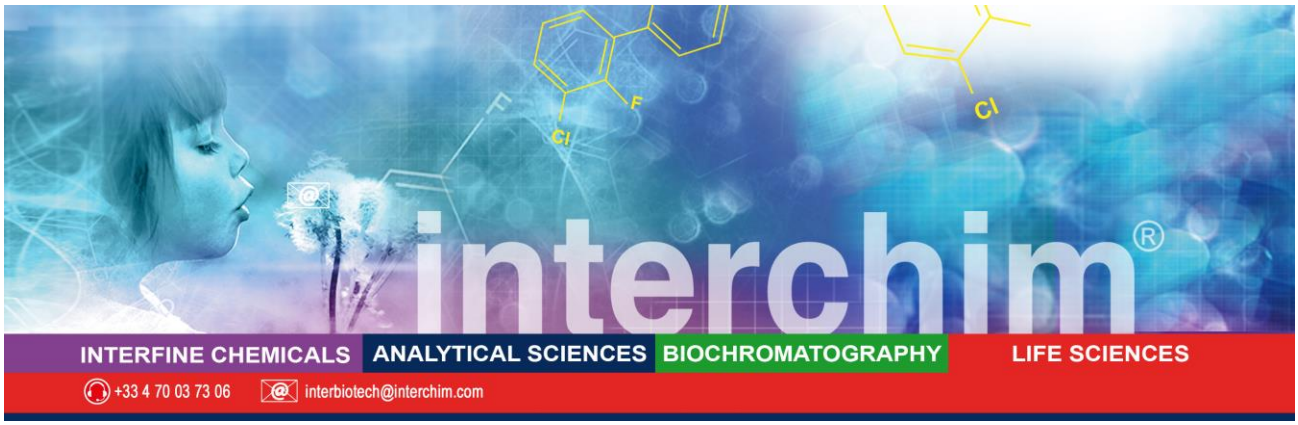

PolyHRP Detection Users Guide

PolyHRP conjugates for amplified (ultra-sensitive) detection of biotinylated compounds in ImmunoAssays

The purpose of this guide is to provide users with an essential knowledge and correct understanding of PolyHRP detection along with related practical recommendations, especially for ultra-sensitive two-site (sandwich) ELISA systems.

Used products examples:

StreptAvidin-PolyHRP conjugates (FT-CV3681): made of covalent HRP homopolymer blocks coupled to multiple streptavidin molecules.

Summary :

Introduction: what is polyHRP?

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 enhancements, which is directly proportional to HRP polymerization range.

Detection with SA-PolyHRP is very simple. There are no changes of principle that 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, using colorimetric, fluorimetric, as well enhanced chemiluminiscence substrates. This also means perfect compatibility with existing routine ELISA instrumentation, as well as blotting, microarrays, or certain cell assays.

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 by just substituting standard SA-HRP with our PolyHRP conjugates. Such claim would disregard the fact that a good ELISA kit is an integrated well-balanced system. Selected assay conditions that are optima for using standard SA-HRP will not necessarily be the same with PolyHRP conjugates. Effective introduction of SA-PolyHRP20, 40 or 80 into customers' own ELISA instead of standard SA-HRP will mean need for another level of the balancing of the system, i.e. actually a development of another, new kit. Proper developmental work is worth effort anyway. Right point is to consider it as reasonable time/effort investment that will pay back with outstanding performance of an ELISA kit using unique advantages of the PolyHRP detection.

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. We hope to be of help to interested users trying to assist their developmental work with the given general comments and specific troubleshooting guidelines.

1. PolyHRP size.

All three items SA-PolyHRP20, 40 & 80 are very big in size. They are still soluble and stable in solutions, and can effectively be filtered through standard 0.45µm filter devices, with no loss on filtration. However they are practically not filterable through 0.2µm – 0.22µm filter units.

Therefore, when working on stable 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. Endogeneous (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, true biotin free, (CBC 5.5% #13.14.8), 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 OEM customers wish to develop their own SA-PolyHRP diluents, we would strongly recommend that they at least test their suggested formulations vs. our standard CBC #13.14.8 taken in the capacity of biotin-free reference material (or the ready to use version cat.#CV373D, 1.375% casein)
Diluents made of animal sera are not compatible with SA-PolyHRP. Exhaustive dialysis and other methods do not completely remove bound biotin associated with medium and high molecular weight material ("conjugated" biotin).Compatibility of different diluent systems with SA-PolyHRP along with their efficiency in the capacity of miscellaneous Blockers/Stabilizers is shown in the Table 1.

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

SA-PolyHRP conjugates alone do not usually show direct (B) backgrounds, if applied in compatible diluent that effectively blocks non-specific interactions with adsorbed/immobilized binding antibody. As already mentioned,

PolyHRP conjugates are very large in size. This suggests that their NSB to immunosorbent surface can very probably be realized as massively multivalent (multipoint) protein-protein interaction.

3.1. SA-PolyHRP diluents [2] .

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

With low/medium diluted (1/1 000 - 1/8 000) conjugates casein additionally boosts PolyHRP detection. Along with eliminating background, this increases signal-to-noise ratio and thereby improves the entire analytical performance of an assay. Specialty Casein is also helpful in making stable diluted SA-PolyHRP formulations.

At the same time, applying of highly diluted (1/10 000 -1/20 000) SA-PolyHRP in simple BSA-based diluents may be quite effective, if the binding antibody is not compromised as material provoking backgrounds (see below). This supports the fact that, in respect to purely Non-Specific Binding, PolyHRP itself is never a "bad substance".

While A and B are true non-specific, primarily 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 the unique efficacy 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 mostly due to the antibody-biotin component contribution.

It is clear that, in the real ELISA, antibody-biotin mediated background ultimately depends on the detecting activity of Streptavidin conjugate. 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" very tracer amounts of detecting biotinylated antibodies, that are non-specifically bound to immunosorbent in absence of analyte and are usually not detectable with standard SA-HRP.

3.2. Background associated with antibodies.

Apart from possible matrix effects, NSB between binding/capture, adsorbed, antibodies and detecting, biotinylated, antibodies is actually the core 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, tracer amounts of protein A or G, as well as positively charged DEAE-polymer fragments (as a result of possible 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 binding and detecting 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.

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.

3.2.2. Effect of biotinylation.

The assumption is that the most developers will apply most frequently used standard biotinylation protocol using NHS-(aminocaproate)-biotin - to - IgG molar ratio 30-33. Usually, this results in good performance.

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NHS-PEOx-Biotins (see related products) are recommended, providing higher hydrophilicity and longer spacer to yield more efficient binding with SA-Poly-HRP, and more stable complexes.

Nevertheless, functional results of concrete 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 show unacceptably high background. This can be associated with excessive biotinylation. In this case, lowering of biotinylation degree through selecting smaller NHS-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 (with no alteration of charge/p.i. and hydrophobic potential), 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, rheumatoid factors, components of the complement system, etc.

3.2.4. Detecting Antibody-biotin diluents / 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 the surface of immunoplate binding antibody and antibody-biotin conjugate is limited to a solely protein-protein NSB (i.e. no strange contamination, interactions of essentially specific or polyelectrostatic origin, etc. resulting in abnormally strong binding), diluents made of Casein Buffer are very effective.

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 #18.15.2, certified as miscellaneous reagent containing very low biotin, may be recommended for formulating antibody-biotin diluents that will effectively block NSB between antibody-biotin reagent and immunosorbent. Product #18.15.2 may also be successfully used as SA-PolyHRP diluent with low diluted conjugates (1/1 000 - 1/5 000), just because concentration of biotin is really very low.

Similarly, true biotin-free CBC product #13.14.8 (5.5% concentrate), specially recommended as diluent for SA-PolyHRP, may also be effectively used in the capacity of Diluent/Blocker with biotinylated antibody (or see cat#CV373D Ready to use formulation 1.375% casein)

3.3.2. Casein Buffer as Specimen/Calibrator Diluent.

Casein Buffer performs very well in the capacity of Sample/Specimen and Standard/Calibrator diluent, too. It levels matrix effects in situation when the same analyte must be quantified in different specimens (e.g. cytokines in serum/plasma, cell culture fluid and urine).

3.4./3.3.3. Blocking procedure.

Finally, Casein Buffer may be used for the blocking of immunoplates with coated antibody. This procedure is used with the sense of blocking free remaining sites on the surface of the adsorbent immunoplate after adsorption of binding 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, biotin free, will perform very well in the capacity of universal Diluent/Blocker with Sample/Specimen, antigen Standard/Calibrator, Antibody-biotin and SA-PolyHRP conjugate. It will also work perfect as Stabilizer with all the components that are used in liquid ready-to-use formulations.

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

In the capacity of universal Diluent/Blocker/Stabilizer, Casein Buffer is very useful miscellaneous reagent, convenient in rapid designing of diagnostic ELISA 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 manufacturers benefit from supplying the kit with only one universal buffer for diluting all essential test kit components/reactants.

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. Although Casein Buffer remains a good candidate for designing effective ready-to-use formulations (especially with SA-PolyHRP), manufacturers with biotinylated antibody may use any diluent they like or used to use, including animal sera diluents that may heavily be 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 washing 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 immunoplates.

Noneless, it is important to note that, with the PolyHRP detection, use of the products certified as High Binding Capacity material is a requirement. This literally means that high sensitivity detection potential of PolyHRP can not be fully realized when using Medium and Low Binding Capacity immunoplates. Binding of 400 ng IgG per 1 cm^2 ensured as typical certification of High Binding materials by such manufacturer as Costar/Corning does seem sufficient for the effective PolyHRP detection.

5. Coating procedure with capture/binding antibody (as applied to standard 8 X 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 not higher than 1 µg/ml, yielding not more than 50-200 ng specific IgG per well, respective to the applied volumes of 50-200 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 50-100mM Na-Carbonate-Bicarbonate Buffer (CBB) pH 9.4-9.6 is well applicable when coating with capture IgG. Static overnight (18 hours +/- couple of hours) incubation is preferable compared to possible shorter coating protocols. Depending on antibody, coating by +4°C may result in essentially better performance than coating by 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 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. contact with detergent. Larger application volume of blocker compared to capture antibody volume, e.g. 100 ul/well antibody and then 200 µl/well blocker, will secure better performance, especially with difficult specimens.

6. Choice of specific SA-PolyHRP conjugate item.

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-PolyHRP20. 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. PolyHRP40 may achieve an intermediate and reasonnable performance. The performance should alos be balanced by cost/use.

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 detecting Antibody-biotin reagent and SA-PolyHRP concentration.

The concentration of primary antibody, biotinylated (secondary) antibody should be calibrated to give minimal background, along with the concentration of SA-PolyHRP.

8. Incubation time/temperature/mode and other performance 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, because the results of such an optimization strongly depend on many factors. Individual binding/detecting antibody characteristics, biotinylation degree, diluents, incubation timing scheme and mode and 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 can be balanced with other kit components in generally flexible way.

Temperature: 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 (18-24°C) temperature incubation.

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

Incubation **time** 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 aims at development shorter assay. PolyHRP allows to substantially reduce the time of an 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 allowing 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) - 1 hour
Detecting Antibody-biotin (100 µl/well) - 1 hour
SA-PolyHRP (100 µl/well) - 30 min.
TMB substrate (100 µl/well) - 15-30 min.

* We usually recommend 100 µl/well for all steps/reagents, except for only blocking step (in preparation) and specimen/calibrator. Applying 200 µl/well sample often gives higher sensitivity compared to 100 µl/well.

In Tables 2 and 3 suggested "chess" titration experiment is shown as illustration assuming application of the above timing scheme, use of a good performance matched antibody pair and colorimetric TMB development. The given example is very broad and not limited to the "follow up" practical instruction. 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 matter of running just one or very few "chess" titration experiments.

9. Antibody-biotin concentration

Effective ultrasensitive performance with SA-PolyHRP will very probably require application of antibody-biotin reagent in smaller concentration, than it would typically be done with the good standard SA-HRP. With PolyHRP users should not be afraid of decreasing biotinylated antibody concentration. At still higher sensitivity level or at least with no loss in desired sensitivity, 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.

Guide lines for use for ELISA with SA-PolyHRP

As more practical reiteration, guidelines on effective and correct designing of primarily ultrasensitive sandwich ELISA test system using SA-PolyHRP are summarized below.
Note: 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. are essentially the recommendations that would only improve performance of any other assay system. Above consideration, again, supports simple statement that designing of ultrasensitive assay with PolyHRP detection is quite practicable task.

1. **Biochemicals**
Use in development only pure (<0,05 µS/cm) deionized water and high purity general chemicals
In preparation of all reagents try to avoid contamination of immunoplates and liquid assay kit constituents with air-borne particles.

Use with all reagents reliable anti-microbial/preservative. We recommend 5-Bromo-5-nitro-1,3-dioxane (BND, Microcide I #GN0955). It works well as preservative in 0,02% (simple salt buffers) - 0,05%-0,06% (high protein buffers) - 0,1%-0,12% (stabilizers) concentrations at +4°C and room/ambient temperature and does not interfere ELISA performance including substrate development reaction(s).

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

When filtering, avoid foaming. Remember, solubilized 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 conjugates in ready-to-use format at final working strength).

2. **Microplates**
Use only High Binding Capacity Immunoplates, e.g E.I.A./R.I.A. 8X12 microwell plates, High Binding [r].
Do not use Medium or Low Binding plates.

3. **Antibodies**
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.

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

4. **Coating**
Coat immunoplates with binding/capture antibody in concentration not higher than 1 µg/ml, yielding 50-200 ng specific IgG per well with the used volumes of 50-200 µl per well.

Universal specific protocol with application exactly 1 µg/ml IgG, 100 µl/well, will work fine in the most cases.

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 e.g. 50mM Na-Carbonate/Bicarbonate buffer pH 9.4-9.6 with 0,03% BND, 0.1/0.2µm-filtered, as coating buffer.

Incubate immunoplates filled with antibody solution 15-18 hours, preferably at +4°C, sealed or placed into clean humid chamber.

5 (optional **blocking procedure**).
Right after aspiration, fill the wells of immunoplate with 1-2% BSA or other applicable blocker (specific reagent should previously be tested) in e.g. 0,12M NaCl / 0,03M Na-Phosphate Buffer pH 7,3 containing 0,03% BND, 0.1/0.2µm-filtered (0,15M PBS-BND), or Casein Buffer Concentrate (either #13.14.8 or #18.15.2) diluted 1/4 - 1/5 in PBS-BND.

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Use larger than with capture antibody volume. Blocker volume that is twice the volume of capture antibody, i.e. 200 µl vs. 100 µl is O.K.. Incubate 1 hour or longer at room temperature, preferably onto ELISA staker.

6. **Wash** antibody-coated immunoplate with e.g. PBS containing 0,05% Tween-20 (PBS-Tween) 5 times. Optionally dry after additional washing with appropriate buffer containing dry reagent stabilizers, usually high concentration simple sugars, oligosugars and natural (the same BSA, casein) or synthetic (PVA, PVP, etc.).

7'. **Antibody incubations**
Pay very careful attention to antibody-biotin reagent mediated background. Apply antibody-biotin in diluent that will effectively eliminate NSB.

Try with biotinylated antibody diluents made of Casein or Gelatin. Alternatively, use proven animal sera diluents. Be well prepared to decrease, if necessary, concentration of biotinylated antibody in detection system compared to one used in existent ELISA with standard SA-HRP.

8'. **Optimisation**
Optimization (investigate/select an optimum degree of biotinylation using NHS-PEOx-Biotin #UPR20278 and #BZ0971) may be required :
-When developmental capabilities (time/budget, etc) permit
-When observing rigidly high background signals with antibody-biotin reagent applied in casein diluent 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 below, item 10).

9'. **SA-PolyHRP incubations**
Use for diluting SA-PolyHRP conjugates buffer systems that are biotin-free and contain reagents effectively eliminating protein-protein NSB, as e.g. diluents made of specially developed for the PolyHRP detection Casein Buffer Concentrate #13.14.8, true biotin-free, or (with conjugates diluted not higher than 1/5.000) Casein Buffer Concentrate #18.15.2, very low biotin.

Select effective Casein concentration as factor that reflects the dilution of Casein Buffer Concentrate (containing approx. 5,5% m/v phosphoprotein) in your own (assumed biotin-free!) diluent or simple PBS. Casein Buffer Concentrate (CBC) in the capacity of Blocker additive may be diluted very flexibly, starting from 1/2 (1Vol. CBC+1Vol. PBS) down to 1/5 - 1/10 - 1/20 (1 Vol. CBC + 4-9-19 Vols PBS). This depends on desired working strength which affects chosen conjugate dilution (see table 1). Conjugate dilution largely determines sensitivity of the assay.

Check performance of your own suggested diluent against Casein Buffer Concentrate #13.14.8, true biotin-free (or ready to use cat#CV373D)

When obtaining of ready-to-use (diluted at final working strength) conjugate formulation that shall provide long-term stability with consistent performance over long time interval (typically 12 months) is required, try our SA-PolyHRP Stabilizers.

SA-PolyHRP Stabilizer, ready-to-use, #21.17.4 $250.00/500ml, $438.00/1 Liter) will provide real-time stability for ready-to-use SA-PolyHRP conjugates during at least 18 months at +2°C/+8°C and over 8 months at +18°C/+22°C (room temperature).

This product will not support strongly diluted ready-to use SA-PolyHRP conjugates at elevated temperatures (+37°C and 45°C) in time intervals longer than 72 hours at +37°C and 24 hours at +45°C which is adequate for "heat shock in traffic jam" simulation. Therefore this product is not suitable for running accelerated ("short and dirty") stability studies which will not provide extrapolation on real time stability at regular storage temperature regimens.

SA-PolyHRP Stabilizer HT (High Temperature), ready-to-use, #23.31.7 will provide real-time stability for ready-to-use SA-PolyHRP conjugates at +2°C/+8°C and +18°C/+22°C (room temperature) storage regimens that is comparable or better than stability with product #21.17.4. This product will secure longer stability at higher temperatures compared to product #21.17.4.

10. **Run "Chess" titration experiment: (see following suggested general Protocol)**

See suggested general Protocol and Scheme of principle (see Appendix 1 and Table 1 for Protocol and Tables 2 and 3 for Scheme). At least three antigen Calibrator points in suggested matrix should be included in duplicates into initial screening experiments along with two "void" (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
(ii) zero (0) no Ag (void matrix + diluent), Ab-biotin, 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 somewhere 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 true "analytical" background resulting from possible non-specific binding interactions between complete detection system and intact immunosorbent in absence of 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 without specific detecting antibody-biotin conjugate. When running this (separate for SA-PolyHRP) control, apply diluent without Ab-biotin instead of diluted Ab-biotin as per complete control (ii).

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 suggested/wanted analytical performance.

Ultimately, all the concentration points that must be used as calibrators with the future test system kit 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

1. **Coating**: capture Antibody, 1 µg/ml in coating 0,05M Carbonate-Bicarbonate Buffer pH 9,5 containing 0,03% BND, 0.2µm-filtered, 100 µl/well (E.I.A./R.I.A. plates, High Binding), sealed; overnight (15-18 hours) static incubation at +4°C.
One-time aspiration

2. **Blocking**: Casein Buffer Concentrate #13.14.8, true biotin-free, diluted 1/4 in 0,12M NaCl / 0,03M Na-Phosphate Buffer pH 7,3 containing 0,05% BND, 0.2µm-filtered (1 Vol. CBC +3 Vol. PBS-BND), 200 µl/well, 1 hour on rotary microplate shaker ~700 rpm, room temperature.

The same blocking buffer made of CBC will work well as universal diluent for specimen/calibrator, Antibody-biotin and SA-PolyHRP conjugates. Applicable abbreviation is HPEB (High Performance ELISA Buffer).

3. **Washing**: PBS-BND containing 0,05% Tween-20 (PBS-Tween), 5 times.

4. **Analyte**: your Antigen Calibrator taken in concentrations of interest including zero point. When possible, made as material diluted from the real sample matrix containing no antigen (depleted matrix), diluted in Sample/Calibrator diluent of choice, e.g. HPEB, each point in duplicate, 200 µl/well, 1 hour on rotary microplate shaker ~700 rpm, room temperature.

5. **Washing**: PBS-Tween, 3 times.

6. **Detection**: 6.1. detecting Antibody-biotin reagent, different concentrations in appropriate diluent of choice, e.g. HPEB, 100 µl/well, 1 hour on rotary microplate shaker 700 rpm, room temperature;

6.2. Wash with PBS-Tween, 3 times;

6.3. Streptavidin-PolyHRP conjugate of choice, different concentrations in HPEB or SA-PolyHRP Stabilizer (product #21.17.4 or #23.31.7), 100 µl/well, 30 minutes on rotary microplate shaker ~700 rpm, room temperature;

6.4. Wash with PBS-Tween, 5 or (better) 6 times;

6.5. Substrate: 100 µg/ml TMB + 0,006% H2O2 in 0,1M Na-Acetate/Citrate Buffer pH 5,5 containing 0,02% BND, 100 µl/well, 30-15 minutes on rotary microplate shaker ~700 rpm, room temperature, in darkness;

6.6. Stopping: 10-25% H2SO4, 50 µl/well, 10-15 seconds on rotary microplate shaker ~700 rpm.

6. **Reading** 450 nm vs. 620 nm (or, if available on your ELISA reader, longer wavelength) reference optical filter.

Related / associated products and documents

(strept)avidin-HRP #51558C
CBC biotin free casein diluent #CV373D
BSA 30% solution #UP90010 see other saturating agents for immunoassays (page A352+)
NHS-PEOx-Biotin #UPR20278 and #BZ0971
5-Bromo-5-nitro-1,3-dioxane #GN0955: Preservative agent, does not contain azide nor mercury; replaces Thimerosal, Azide, Xylenols and other Hazardous Preservatives

 Rev.R12E-R07E