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Elisa troubleshooting

Wells are insufficiently washed Wash wells are per protocol recommendations. Be sure to follow the above. High Signal may occur for a number of reasons including insufficient plate washing, not stopping the reaction or adding too much detection reagent. Use multichannel pipettes without touching the reagents on the plate. Ensure substrate is not exposed to light—store in a dark place. Adhere to the protocol that comes with your assay Improper calculations of standard curve Check calculations, make new standard curve & use internal controls Avoid contact with the bottom of the well during pipetting. Incorrect standard curve dilutionsprepared Check your pipetting technique. Care should be taken when using the same pipette tips used for reagent additions. At the end of each washing step, invert plate on absorbent tissue and allow to completely drain, tapping forcefully if necessary to remove any residual fluid.Inconsistent incubation temperatureManufactured kits have optimized protocols. Verify the wavelength and read the plate again Plate washing is too vigorous Check the correct pressure in the automatic plate washer. Ensure correct preparation and incubation time for both coating and blocking steps.Not enough detector antibody usedManufactured kits have optimized protocols. Be sure to remove any residual liquid.Alternatively, a squirt bottle or automated plate washer may be used. This will prevent wells from contaminating each other.Incorrect dilutions preparedCheck pipetting technique—see below—and double-check calculations.Longer incubation times than recommendedManufactured kits have optimized protocols. In: M.H.V. Van Regenmortel, ed. Ensure all wells are washed correctly, use a ELISA plate washer where possible Reagents not at room temperature All reagents should at room temperature from the start of the assay. Data with high variation can skew real results and cause inconsistencies in your data. Incorrect dilutions prepared Check pipetting technique—see below—and double-check calculations. If developing ELISA using antibody pairs you may need to optimize the assay. High antibody concentration Try different dilutions for optimal results. Do not allow wells to become dry once the assay has started. Samples contain analyte concentrations higher than the highest standard point Samples may require further dilution Use appropriate washing procedure—see below. Limit exposure to light while running assay.Longer incubation times than recommendedManufactured kits have optimized protocols. Wells are insufficiently washed Wash wells as per protocol recommendations. At the end of each washing step, invert plate on absorbent tissue and allow to completely drain, tapping forcefully if necessary to remove any residual fluid.Capture antibody didn’t bind to plateEnsure that you are using an ELISA plate, not a tissue culture plate. Detection antibody cross-reacting with coating antibody. A poor standard curve will prove unpublishable results if not prepared correctly. Longer incubation times thanrecommended Make sure your incubation times are correct and adhere to the protocol provided with the technical manual. Humana Press, a part of Springer Science + Business Media, LLC 2009.Butler J.E. The Behavior of Antigens and Antibodies Immobilized on a Solid Phase. Cover the plate using sealing film or tape for all incubations. Ensure wells are pre-processed to prevent nonspecific attachment Use an affinity purified antibody, preferably pre-absorbed Concentration of conjugated second antibody too high Perform dilutions to determine optimal working concentration. Or change the detection method Buffer containing FCS used to reconstitute antibodies Re-evaluate reagents used. Increase number of washes. Slow colour development of enzymatic reactions Prepare substrate solution immediately before use. Ensure plate reader is set accurately for type of substrate being used.Possible CauseSolutionInsufficient washingUse appropriate washing procedure—see below. Have a read through the reasons below to avoid these problems. Add 30 second soak step in-between washes. Include a sample that the assay is known to detect the positive control Recognition of epitope impeded by absorption plate To enhance the detection of a peptide by direct or indirect ELISA, conjugate peptide to a large carrier protein before coating onto a microtiter plate Assay buffer incompatibility Ensure assay buffer is compatible with the target of interest (e.g. enzymatic activity retained, protein interactions retained.) Not enough detection reagent Increase concentration of amount of detection reagent following manufacturer guidelines Sample prepared incorrectly Ensure proper sample preparation/dilution. Our 101 ELISA troubleshooting tips is designed to help researchers improve and troubleshoot a range of common problems that they may face with their ELISA kits when performing assays. High background may result in false positive/negative data and affect your results. See ELISA Development and Optimization for more information. This 72-page guide provides detailed information about different tools for protein and RNA quantitation. Be aware of fluctuations in temperature due to environmental conditions.Plate sealers not used or reusedDuring incubations, cover assay plates with plate sealers. In this ELISA troubleshooting guide we have detailed the 6 … Check with technical support for appropriate protocol modifications. Colour will keep developing if the substrate reaction is not stopped. Ensure that pipette tips do not touch the reagents on the plate. Ensure correct preparation and incubation time for both coating and blocking steps. Mixing or Substituting reagents from different kits Avoid this as it can affect the quality of your assay Find troubleshooting solutions to common ELISA problems like weak or too much signal, poor standard curves. See ELISA Development and Optimization for more information.Incorrect standard curve dilutions preparedCheck pipetting technique—see below—and double-check calculations.Possible CauseSolutionIncorrect standard curve dilutions preparedCheck pipetting technique—see below—and double-check calculations.Capture antibody didn’t bind to plateEnsure that you are using an ELISA plate, not a tissue culture plate. Plate left too long before reading on the plate reader Colour will keep developing (though at a slower rate if stop solution has been added) Contaminants from laboratory glassware Ensure reagents are fresh and prepared in clean glassware Substrate incubation carried out in the light Substrate incubation should be carried out in the dark. Salt concentration of incubation/wash buffers Increasing salt concentrations may reduce non-specific and/or weak off-target interactions. Automated plate washers may need to be calibrated so tips don’t touch bottom of wells.Plate read at incorrect wavelengthManufactured kits have optimized protocols. Precipitate formed in wells upon substrate addition Increase dilution factor of sample or decrease concentration of substrate Clean the bottom of the plate with a wipe No signal in your ELISA assay may occur due to your wash buffer containing azide, failing to add avidin-HRP or your desired target levels being below the detection range of the assay. Use a clean V bottom container prior to pipetting substrate solution into wells. Place a humidifying water tray (bottled clean/sterile water) in the bottom of the incubator. If 37oC incubation is indicated make sure plate is in the center of incubator.EvaporationSeal the plate completely with a plate sealer during incubations.Stacked platesAvoid stacking plates during incubation. Boca Raton, FL: CRC Press, 1992: 209-259. Make sure to follow recommended incubation temperatures. Longer incubation times thanrecommended Manufactured kits have optimized protocols. Plate sealers not used or reused During incubations, cover assay plates with plate sealers. To avoid this use fresh plate sealer and reagent reservoir for each step. Make sure to follow recommended incubation times. Blocking buffer ineffective (e.g. detection reagent binds blocker; wells not completely blocked) Try different blocking reagent and/or add blocking reagent to wash buffer. Download this valuable technical resource that covers technologies useful for cancer and inflammation research, immunology, neurology and more. This can result in a loss of data due to negative or no results. Always make fresh buffers. Room temperature should be reached following 15–20 minutes on the bench. Plates stacked during the incubations Stacking of plates does not allow even distribution of temperature across the wells of the plates. Add 30 seconds each time you let wash buffer soak. Ensure lids are left on the plates at all times when incubating. Plate sealers or reagent reservoirs resused, resulting in presence of residual HRP. Waiting too long to read plate after addition of stop solution Read plate immediately after adding stop solution High antibody concentration Try different dilutions of optimal results Substrate incubation is carried out in light Substrate incubations should be carried out in the dark or as recommended by manufacturer. Precipitate formed in wells upon substrate addition Increase dilution factor of sample or decrease concentration of substrate. Increase soaking time between washes prior to addition of substrate solution. Note: Take care not to scratch the inside of the well.Fill the wells with at least 400 µL of diluted wash bufferLet soak for 15 to 30 secondsAspirate wash buffer from wellsRepeat as directed in protocol (usually 3–4 times)After washing is complete, invert plate and tap (forcefully, if necessary) dry on absorbent tissue. TMB Substrate Solution was contaminated Use fresh TMB substrate solution which should be clear and colorless prior to addition to wells. Standard was incompletely reconstituted or was incorrectly stored Reconstitute standard according to the protocol provide and follow storage instructions Reagents were added to the wells at incorrect concentrations Check for pipetting errors and correct the reagent volume Incubations done at incorrect temperature Follow protocol for storage, incubation and agitation Wells not completely aspirated Completely aspirate between steps, use plate wash where possible Plates stacked during incubation Check dilution steps according to protocol Make sure to mix reagents thoroughly Poor or variable adsorption of reagents to plate Check choice of coating buffer, usually PBS with a pH of 7.4 or carbonate bicarbonate buffer pH 9.6. Try extending this incubation time or consider using different plates Check that standard was stored correctly Try plotting use different scales, e.g. log-log, 5 parameter logistic curve fit Check pipettes and calibrate Capture antibody didn’t bind to the plate Ensure that you are using and ELISA plate, not a tissue culture plate. Calibration of pipettes might be required. Explore different ELISA troubleshooting issues and potential solutions Possible CauseSolutionReagents not at room temperature at start of assayIt is recommended that all reagents be at room temperature before starting the assay. Reuse of plate sealers may lead to the presence of residual HRP, leading to non- specific colour change of TMB. Run appropriate controls. Aim the pipette tip to the side of the well to avoid disrupting the bottom High background may result from inadequate washing steps, cross reactivity of samples or contamination. Second Edition. Out of Range issues may arise due to your samples, insufficient washing or incorrect dilution preparation. Optimising your ELISA and removing mistakes can dramatically improve results and the sensitivity of your ELISA assays. Confirm all reagents are removed complete in all wash steps Thoroughly mix samples before pipetting Samples may have high particular matter Remove the particulate matter by centrifugation Insufficient plate agitation The plate should be agitated during all incubation steps using an ELISA plate shaker at a speed where solutions in wells are within constant motion without splashing When reusing plate sealers check that no reagent has touched the sealer. Increasing duration of soak steps may also help. Use an automated plate washer if available. Samples contain no or below detectable levels of analyte If samples are below detectable levels, it may be possible to use high sample volume. This will prevent wells from contaminating each other.Incorrect dilutions preparedCheck pipetting technique—see below—and double-check calculations.Possible CauseSolutionUneven temperatureSeal the plate completely with a plate sealer during incubations. "Enzyme immunoassay (EIA)/enzyme-linked immunosorbent assay (ELISA)." Clinical chemistry 51.12 (2005): 2415-2418.For Research Use Only. Ensure correct preparation and incubation time for both coating and blocking steps.Possible CauseSolutionInsufficient washingUse appropriate washing procedure—see below. See ELISA Development and Optimization for more information.Wells scratched with pipette or washing tipsUse caution when dispensing and aspirating into and out of wells. No signal may mean no results from precious samples. Ensure correct preparation and incubation time for both coating and blocking steps.Plate sealers not used or reusedDuring incubations, cover assay plates with plate sealers. Take particular care when diluting down the plate and watch to make sure the pipette tips are all picking up and releasing the correct amount of liquid. Ensure pipette tips are pushed on far enough to create a good seal. Increase number of washes. Pipette wash buffer gently if washes are done manually. At the end of each washing step, invert plate on absorbent tissue and allow to completely drain, tapping forcefully if necessary to remove any residual fluid.Substrate exposed to light prior to useEnsure substrate is not exposed to light—store in a dark place. At the end of each washing step, invert plate on absorbent tissue and allow to completely drain, tapping forcefully if necessary to remove any residual fluid. Antibody dilutions/reagents are not well mixed To ensure a consistent concentration across all wells, ensure all reagents and samples are mixed before pipetting onto the plates. Incorrect assay temperature Check that the incubation temperature did not exceed 37°C Ensure all wells are filling with wash buffer and are being aspirated completely. Also find protocols, tips and tricks for washing and pipetting. Contaminating enzymes present in sample Test sample with substrate alone to check for contaminating enzyme activity. Ensure pipettes are working correctly and are calibrated. This will prevent wells from contaminating each other.Possible CauseSolutionInsufficient washingUse appropriate washing procedure—see below. This will turn the TMB blue non-specifically. Lengthen incubation times or increase temperature. Reasons for a poor standard curve include inadequate mixing of reagents, degraded standard or pipetting errors. Clean the bottom of the plate. If 37oC incubation is indicated make sure plate is in the center of incubator.EvaporationSeal the plate completely with a plate sealer during incubations.Stacked platesAvoid stacking plates during incubation.Possible CauseSolutionReagents not at room temperature at start of assayIt is recommended that all reagents be at room temperature before starting the assay. Allow longer incubation. Incorrect or no detection antibody was added Add appropriate detection antibody and continue Add avidin-HRP according to protocol and continue Substrate solution was not added Add substrate solution and continue Wash buffer contains azide Avoid sodium azide in the wash buffer Incubation time too short Incubate samples overnight at 4˚C or follow manufacturers guidelines Target present below detection limits of assay Decrease dilution factor or concentrate samples Detection may be reduced or absent in untested samples types. Ensure all reagents used are within date Assay format not sensitive enough Switch to a more sensitive assay type (e.g. direct ELISA to sandwich ELISA). Most kits need to be stored at 2–8oC.Expired reagentsConfirm expiration dates on all reagents. See ELISA Development and Optimization for more information.Possible CauseSolutionInsufficient washingUse appropriate washing procedure—see below. This will prevent wells from contaminating each other. Avoid stacking. All reagents including plate should be at room temperature or as recommended by the manufacturer before proceeding. Substrate solution mixed too early and turned blue Substrate solution should be mixed and used immediately Too much streptavidin-HRP Check dilution, titrate if necessary Plate sealers or reagent reservoirs resused, resulting in presence of residual HRP. Learn more about how antibody pairs, ELISA kits, and multiplex kits for the Invitrogen Luminex platform may help advance your research.Download the Biomarker quantitation assay guideJohn R. In this ELISA troubleshooting guide we have detailed the 6 most common areas where researchers encounter problems with their ELISA. Too much detection reagent added Ensure the reagent has been diluted properly or decrease the recommended concentration of detection reagent. Dilute antibody in PBS. Structure of Antigens. Tips on pipetting and washing techniques for ELISA Pipetting techniqueUse the correct pipette that is within the range suggested by manufacturerConfirm tip is firmly seated on the pipetteConfirm there are no air bubbles while pipettingChange tips between each standard, sample, or reagentUse different reservoirs for each reagentPipette sample into the side of wells to avoid splashingAlways run samples/standards in replicateWashing procedureCompletely aspirate liquid from all wells by gently lowering an aspiration tip into the bottom of each well. At the end of each washing step, invert plate on absorbent tissue and allow to completely drain, tapping forcefully if necessary to remove any residual fluid.Plate sealers not used or reusedDuring incubations, cover assay plates with plate sealers. This will turn the TMB blue non-specifically Use fresh plate sealer and reagent reservoir for each step Buffers contaminated with metals or HRP Pipette errors, mistakes during sample preparation and insufficient plate agitation may all lead to High Variation. Matrix used has endogenous analyte or interference Check the matrix ingredients for cross reacting components (e.g. interleukin modified tissue culture medium). Use a fresh sealer each time the plate is opened. If developing ELISA using an Antibody Pair Kit you may need to optimize the assay. Make sure to follow recommended antibody dilutions. Non-specific binding of antibodies Ensure a block step is included and a suitable blocking buffer is being used. Multichannel pipette errors Plate washing was not adequate or uniform Make sure pipette tips are tightly secured. Limit exposure to light while running assay. Do not use reagents that are past the expiration date.Reagents added/prepared incorrectlyCheck protocol, ensure reagents were added in the proper order and prepared to correct dilution.Incorrect dilutions preparedCheck pipetting technique—see below—and double check calculations.Capture antibody didn’t bind to plateIf using a ready-to-use kit: Manufactured kits come with plates pre-coated with capture antibody.If coating your own plate with an Antibody Pair Kit: Ensure that you are using an ELISA plate, not a tissue culture plate. Not for use in diagnostic procedures. Samples may be incompatible with microtiter plate assay format Try different concentrations/dilutions of antibodies Incubation temperature is too low Ensure the incubations are carried out at the correct temperature. Not enough detection antibody Check dilution, titrate if necessary Incorrect calculation of standard curve dilution Check your calculations and make a new curve. Make sure to use recommended wavelength/filter. Allow reagents to sit on bench for 15–20 minutes to reach room temperature.Incorrect storage of componentsDouble check storage conditions on kit label. Too much detection reagent Ensure the reagent has been diluted properly or decrease the recommended concentration of detection reagent Blocking buffer ineffective Try different blocking buffer reagent and/or add blocking reagent to wash buffer Salt concentrations of incubation/wash buffers Increasing salt concentrations may reduce non-specific and/or weak off target interactions. Background wells were contaminated Avoid cross-well contamination by using the sealer appropriately. Vol.1, 209; CRC Press, Inc.Lequin, Rudolf M. Bottom of the plate is dirty Clean the bottom of the plate carefully before re-reading the plates Ensure no bubbles present prior to reading the plate Ensure the plate and all reagents are at room temperature Ensure reagents and samples are stored are correct temperature Capture antibody didn’t bind to the plate Ensure that you are using an ELISA plate, not a tissue culture plate. If you have a high signal this can result in false positives and incorrect data. Ensure the stock solution has not expired and is not contaminated. Crowther, Methods in Molecular Biology, the ELISA Guidebook. We recommend using 5 to 10% serum from the same species of the secondary antibody, or bovine serum.