February 2006

LiquiChip® Applications Handbook

For protein-based suspension arrays using
xMAP™ technology

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Kit Contents

Cat. no.	922500	922501	922503	922505
LiquiChip Ni-NTA Beads	0.25 ml, bead code 52	4 x 0.25 ml, bead codes 50, 52, 54, and 58	4 x 0.25 ml, bead codes 32, 34, 36, and 38	4 x 0.25 ml, bead codes 24, 26, 28, and 30
Cat. no.	**922520**	**922521**	**922523**	**922525**
LiquiChip Penta·His Beads	0.25 ml, bead code 81	4 x 0.25 ml, bead codes 75, 77, 79, and 81	4 x 0.25 ml, bead codes 51, 53, 55, and 57	4 x 0.25 ml, bead codes 6, 8, 17, and 19
Cat. no.	**922540**	**922541**	**922543**	
LiquiChip Activated Beads	0.5 ml, bead code 61	4 x 0.5 ml, bead codes 61, 62, 63, and 64	4 x 0.5 ml, bead codes 65, 66, 67, and 68	
Cat. no.	**922400**	**922402**	**922404**	**922406**
LiquiChip Carboxy Beads	1 ml, bead code 21	4 x 1 ml, bead codes 11, 13, 15, and 21	4 x 1 ml, bead codes 31, 33, 35, and 37	4 x 1 ml, bead codes 40, 42, 44, and 46

Storage

All LiquiChip beads and fluorescently labeled detection reagents are light-sensitive and should be protected from light during all stages of storage and use. When pipetting LiquiChip Beads, work quickly to minimize their exposure to light. Protect the beads from light by wrapping reaction vessels in aluminum foil or by placing reactions in a cupboard, a drawer, or under a box. If the bead-classification fluorophores are exposed to light they become photobleached and can no longer be classified by the instrument, and any attendant reporter fluorescence will not be measured.

LiquiChip beads should be stored at 2–8°C in the dark. DO NOT FREEZE LiquiChip beads!

Streptavidin–R-PE should be stored at 2–8°C in the dark. DO NOT FREEZE Streptavidin–R-PE!

Penta·His Alexa Fluor® Conjugates and **Penta·His Biotin Conjugates** should be stored at 2–8°C.

LiquiChip System Fluid should be stored at room temperature.

Quality Control

In accordance with QIAGEN's ISO-certified Total Quality Management System, LiquiChip products are tested against predetermined specifications to ensure consistent product quality.

Safety Information

When working with chemicals, always wear a suitable lab coat, disposable gloves, and protective goggles. For more information, please consult the appropriate material safety data sheets (MSDSs). These are available online in convenient and compact PDF format at www.qiagen.com/ts/msds.asp where you can find, view, and print the MSDS for each QIAGEN kit and kit component.

24-hour emergency information

Emergency medical information in English, French, and German can be obtained 24 hours a day from:

Poison Information Center Mainz, Germany, Tel: +49-6131-19240

Product Use Limitations

LiquiChip products are developed, designed, and sold for research purposes only. They are not to be used for human diagnostic or drug purposes or to be administered to humans unless expressly cleared for that purpose by the Food and Drug Administration in the USA or the appropriate regulatory authorities in the country of use. All due care and attention should be exercised in the handling of many of the materials described in this text.

Product Warranty and Satisfaction Guarantee

QIAGEN guarantees the performance of all products in the manner described in our product literature. The purchaser must determine the suitability of the product for its particular use. Should any product fail to perform satisfactorily due to any reason other than misuse, QIAGEN will replace it free of charge or refund the purchase price. We reserve the right to change, alter, or modify any product to enhance its performance and design. If a QIAGEN product does not meet your expectations, simply call your local Technical Service Department or distributor. We will credit your account or exchange the product — as you wish.

A copy of QIAGEN terms and conditions can be obtained on request, and is also provided on the back of our invoices. If you have questions about product specifications or performance, please call QIAGEN Technical Services or your local distributor (see inside back cover).

Technical Assistance

At QIAGEN we pride ourselves on the quality and availability of our technical support. Our Technical Service Departments are staffed by experienced scientists with extensive practical and theoretical expertise in molecular biology and the use of QIAGEN® products. If you have any questions or experience any difficulties regarding LiquiChip products or QIAGEN products in general, please do not hesitate to contact us.

QIAGEN customers are a major source of information regarding advanced or specialized uses of our products. This information is helpful to other scientists as well as to the researchers at QIAGEN. We therefore encourage you to contact us if you have any suggestions about product performance or new applications and techniques.

For technical assistance and more information please call one of the QIAGEN Technical Service Departments or local distributors (see inside back cover).

Introduction

The LiquiChip Suspension Array System

The QIAGEN LiquiChip System is a flexible, bead-based system for suspension arrays. A wide variety of assay types, such as immunoassays, kinase enzyme assays, and interaction assays are performed in an aqueous, homogeneous format, both quickly and efficiently. Multiplexing of assays offers the potential for the simultaneous detection and quantification of up to 100 different analytes within a single sample.

The LiquiChip principle

LiquiChip assays involve the interaction of immobilized, bead-bound assay components with reaction partners in solution. In the most basic type of detection assay, a capture-molecule–coated bead is added to an assay and reacts with an analyte. The analyte is detected using a fluorescently labeled reporter molecule specific for the analyte (Figure 1). The reporter molecule that binds to the analyte is used to quantify the interaction between the assay reactants. Capture molecules can be attached to the surface of beads using Ni-NTA– and Penta·His Antibody–6xHis-tag interactions or covalent immobilization (see page 16). By offering a range of surface chemistries, the LiquiChip System allows high flexibility in the immobilization of proteins, peptides, antigens, and other biomolecules, and therefore in assay setup and design. Ready-to-use LiquiChip Kits for measuring cytokines and a range of kinases are also available. Using this simple assay set up, a typical LiquiChip assay takes place in a "mix-and-measure" procedure in which beads, reagents, and detection reagents are sequentially or simultaneously added to a reaction vessel, mixed, incubated, and measured. The LiquiChip System is a highly flexible assay platform, which enables you to perform a wide range of assay types. By allowing you to use or adapt many of your lab's existing reagents and protocols, assay development time can be significantly reduced.

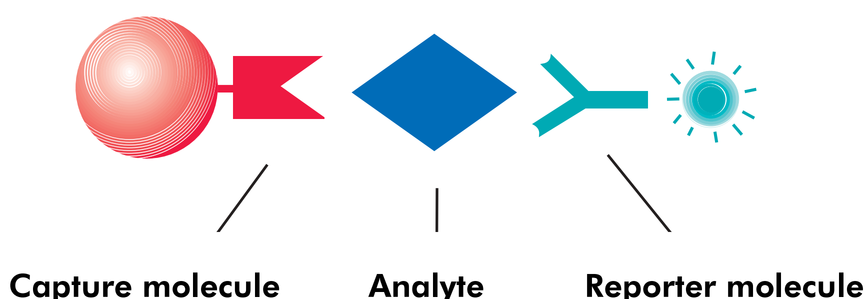

Figure 1 The LiquiChip assay principle. Analytes free in solution interact with bead-bound capture molecules and are detected using reporter molecules specific for the analyte.

Assays adaptable to the LiquiChip System include:

- Immunoassays (e.g., ELISA)
- Protein-protein interaction assays (e.g., interaction mapping)
- Enzyme assays (e.g., kinase, phosphatase, and protease assays)
- Receptor–ligand assays
- Protein–nucleic acid assays
- Nucleic-acid hybridization assays

Using competitive assay techniques even unlabeled interaction partners can be screened by measuring the decrease of signal. In addition to the protocols supplied in this handbook and with LiquiChip Assay Kits, supplementary protocols (e.g., for the immobilization of nucleic acids on LiquiChip Carboxy Beads) are available from QIAGEN Technical Service Departments (see back cover).

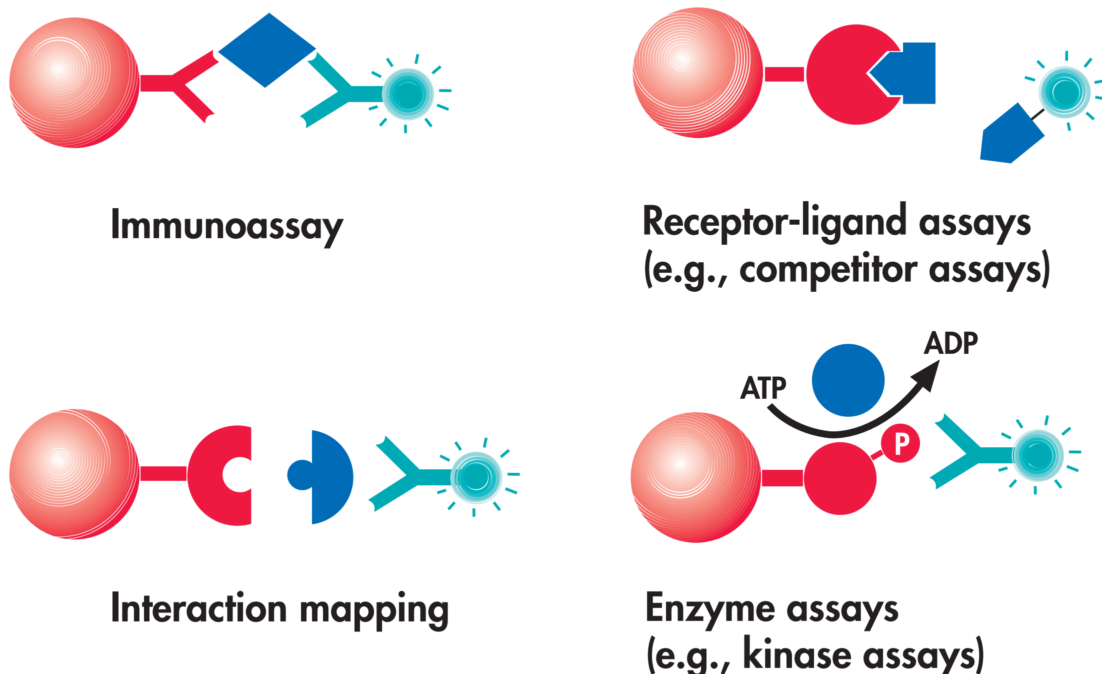

Figure 2 Adaptation of protein assays to the LiquiChip System.

Assigning a color code (classification code) to each set of beads enables multiplexing of assays. The color code is derived from a precisely controlled mixture of two red fluorescent dyes within the beads and serves to identify each reaction set throughout the test procedure (Figure 3 and 4, page 10). Reporter molecules carry a green fluorescent dye spectrally distinct from those used for bead color-coding, which allows discrete simultaneous measurements of red color-code and green reporter-molecule fluorescence. The multiplexing capacity significantly reduces the volumes of both capture and detection molecules required per assay.

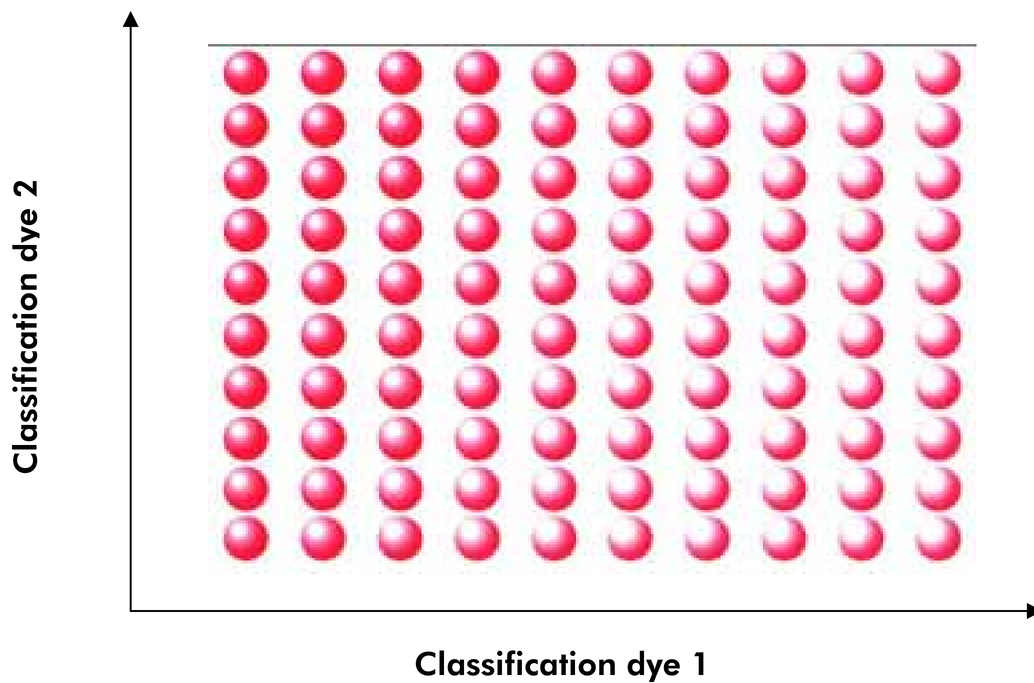

Figure 3 By using precisely controlled ratios of two fluorescent dyes, a matrix of 100 spectrally distinct bead sets can be created.

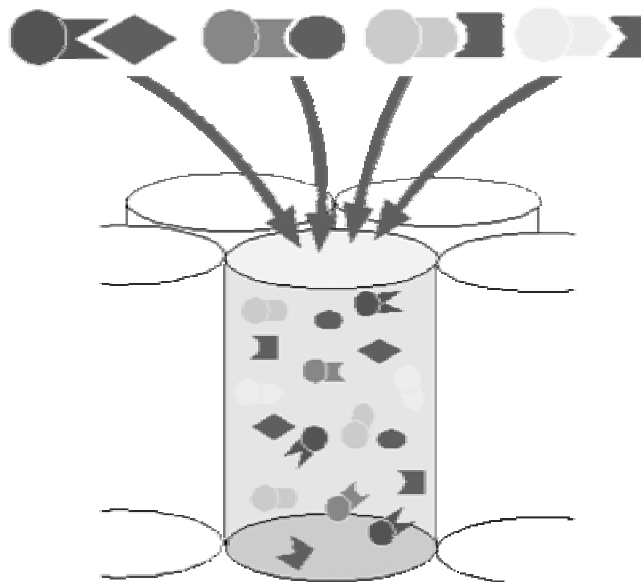

Figure 4 Multiplexing of LiquiChip assays. Bead sets with different color codes and capture molecules are added to an assay where they react with different analytes. The color code unique to each bead set allows the discrete quantification of each analyte in the LiquiChip Reader.

Reporter molecule fluorescence is only recorded in conjunction with color-code fluorescence, which means that signals from free, unbound reporter molecules are not recorded. This feature enables a homogeneous assay, in which there is no need to separate assay phases or carry out wash steps, as in ELISA or RIA procedures (Figure 5).

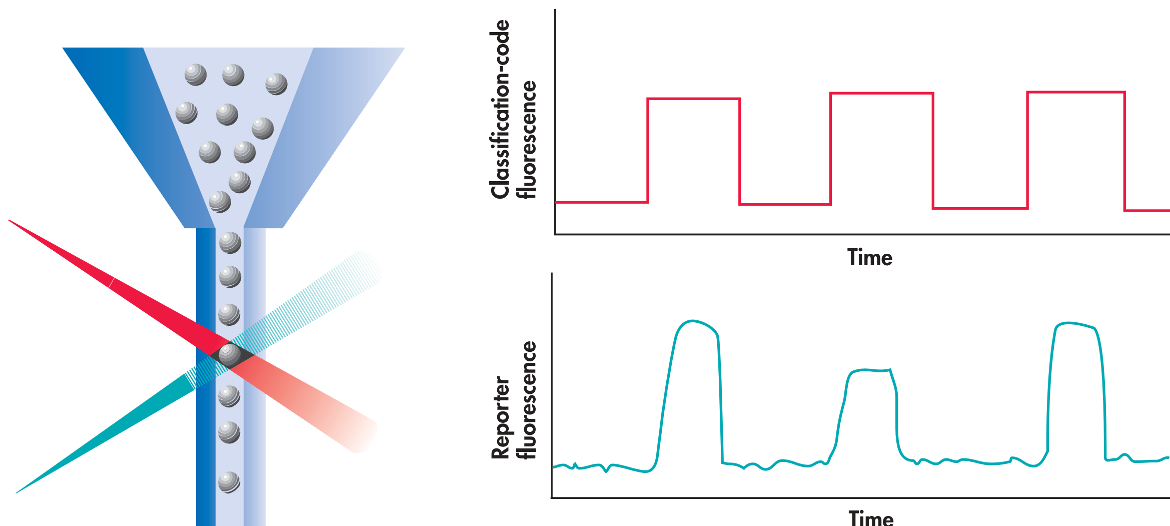

Figure 5 Simultaneous detection of classification-code fluorescence (upper trace) and reporter fluorescence (lower trace). Only reporter fluorescence that is associated with a valid classification signal is recorded.

The high sensitivity of the LiquiChip System extends the detection limit of assays. For example, the typical detection limit of an immunoassay is around 100 pg of antigen. In contrast, the typical detection limit for an analogous LiquiChip Ni-NTA Bead-based assay is around 1 pg (Figure 6).

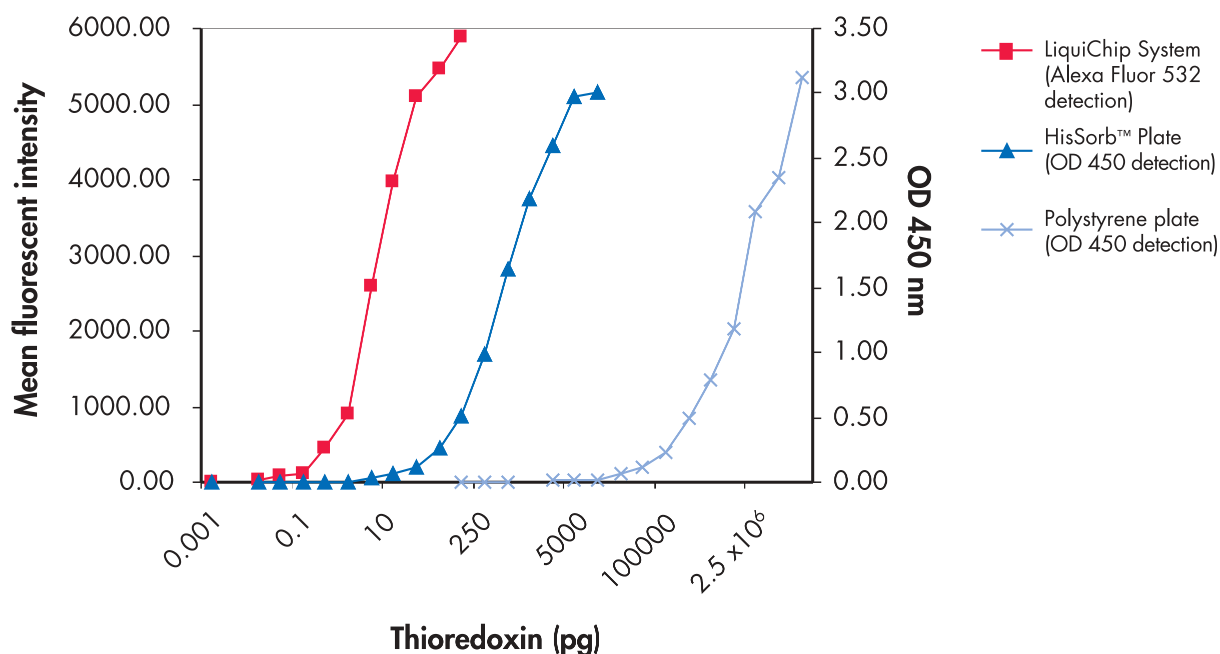

Figure 6 Higher assay sensitivity using the LiquiChip System. Varying amounts of 6xHis-tagged thioredoxin were assayed using a conventional ELISA technique (▲ and X) or were immobilized using LiquiChip Ni-NTA Beads and added to a LiquiChip assay (■). Detection was performed by measuring the OD 450 (ELISA) or by measuring the mean fluorescence intensity of an Alexa Fluor 532 reporter molecule (LiquiChip assay).

The QIA*express*® System

The QIA*express* System is a powerful and versatile tool for working with recombinant proteins and other biomolecules containing a 6xHis affinity tag. The system provides materials for expression, purification, detection, and assay of 6xHis-tagged proteins and is based on the remarkable selectivity of Ni-NTA for proteins or peptides carrying the 6xHis tag. The system uses both the specificity of the interaction between the histidine residues and immobilized nickel ions and the strength with which these ions are held to the NTA resin. Biomolecules carrying a 6xHis tag can be immobilized in fully active conformations and usually offer optimal access of binding domains to potentially interacting partners. In contrast to other affinity-tag systems, the 6xHis tag–Ni-NTA interaction is not dependent on three-dimensional conformation and can be utilized under native or denaturing conditions. The small size of the 6xHis tag does not usually interfere with protein structure or function, and the tag can be used as a highly selective system to immobilize functional enzymes and ligands. The adaptation of the QIA*express* System to LiquiChip technology allows one chemistry to be used for the handling of 6xHis-tagged proteins at all stages of the purification and assay process. This increases standardization and reproducibility, simplifies assay development, and minimizes the need for optimization trials. LiquiChip beads coated with Ni-NTA moieties or Penta·His anti-His tag antibodies enable the directed immobilization of biologically active 6xHis-tagged biomolecules; enhancing assay sensitivity, minimizing nonspecific binding, and increasing reproducibility and signal-to-noise ratios (Figure 7).

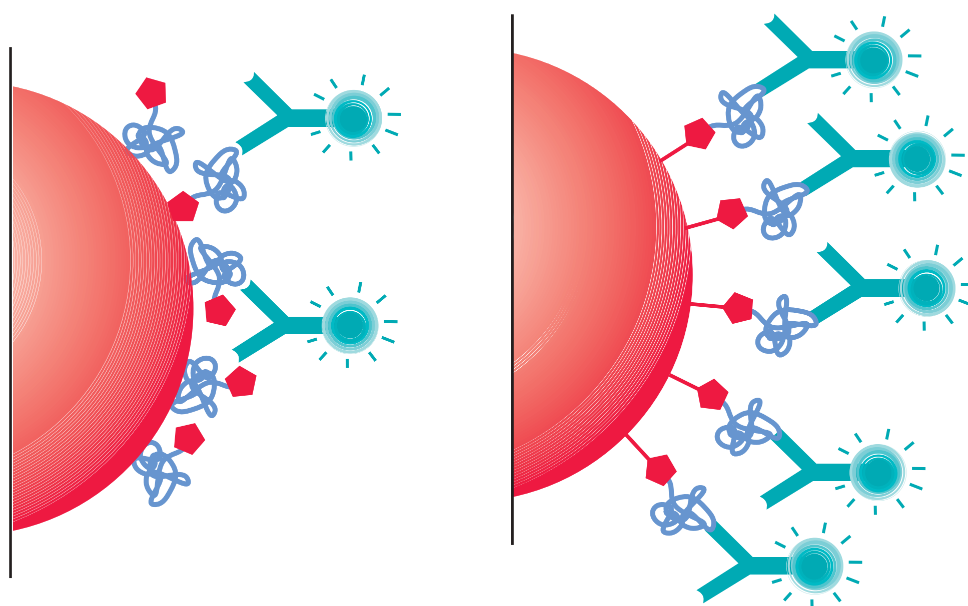

Figure 7 Directed immobilization of 6xHis-tagged proteins on LiquiChip Ni-NTA Beads provides vastly improved accessibility compared with randomly immobilized proteins.

Features of the LiquiChip System

Bead-based solid-phase techniques

Solid-phase assay techniques — in which reagents are immobilized on a support matrix — have several advantages over other analytical methods. These include higher reproducibility, sensitivity, and signal strength, and the ability to perform experiments with small amounts of reagents. In addition, bead-based solid-phase techniques have several advantages over flat-surface–based solid-phase techniques (e.g., the coating of wells in plastic plates). The large reaction surface area of a sphere and concentration of reactants on the surface of beads leads to increased signal intensity. The intrinsic high sensitivity of fluorescent detection increases total assay sensitivity still further, allowing detection of a few hundred reporter fluorophores per bead. Because classification and reporter fluorescence signals are measured simultaneously, the measurement focus can be reduced to such a size (i.e., the size of a bead), that only bead-associated fluorescence is measured. This enhanced signal intensity increases the sensitivity of protein assays to the femto– picomolar (10^{-15}–10^{-12} M) range, and allows smaller amounts of reagents to be used in assays. Assays are generally easy to implement, adapt, and automate. In addition, if different bead populations can be discriminated (as is the case with LiquiChip bead sets), multiplexing of assays is possible.

Performing multiplex assays

Using precise ratios of red classification fluorophores, a spectral array can be created encompassing 100 different bead sets, each with its own distinct spectral address. Since each bead set can be recognized by its spectral address, sets can be combined, giving the potential for simultaneous measurement (multiplexing) of up to 100 different analytes in a single vessel. This feature allows e.g., screening of antibodies against several different antigens within a single blood sample.

Carrying out multiplex assays in solution has many advantages. Reaction rates in a suspension are faster than in a fixed solid-phase system, such as a microarray chip. Through immobilization of reactants on the surface of beads, the reaction surface is greatly enlarged, which also raises reaction rates. A further important advantage of bead-based suspension arrays is their flexibility. In contrast to a microarray chip, measurement parameters, and the number of tests that are carried out simultaneously, can be altered whenever desired. The makeup of the suspension array reflects the makeup of the test-specific bead sets. Simply changing the bead sets added to a reaction allows the modification of the array.

Homogeneous assay setup

Measurement and registration of reporter fluorescence only takes place when the measured bead can be recognized as belonging to a defined bead set and having a defined size. As a result, you can be sure that measured reporter fluorescence is derived from a bead, and not from unbound fluorophores free in solution. This allows homogeneous assays, with no requirement to separate free fluorophores from the fluorophores bound to beads. This saves time and reagents, and increases sensitivity. In addition, the equilibrium of the system is not affected by removal of unbound reactants.

Soluble background fluorescence signals at 575 nm (from reporter molecules free in solution) are automatically subtracted from median fluorescence intensity (MFI) values.

Statistical processing of raw data

The bead suspension in a LiquiChip assay provides near liquid-phase reaction kinetics. Theoretically, each bead binds an equal number of reporter molecules. This results in a statistically even distribution of reporter molecules on each bead within a bead set. Therefore, each single bead represents a replicate of a given experiment. During the measurement, multiple beads from each bead set are analyzed, increasing the robustness of the test procedure. The average fluorescence intensity of 100 single beads per bead code is usually sufficient for statistically meaningful results.

For each bead set, a median fluorescence intensity (MFI) value is reported in the "Median" mode of the "Acq. Detail tab" of the IS Software. This value is the median signal intensity measured per bead code. It gives a direct statistical average of each bead analyzed in a sample run.

Evaluating more than 100 beads will not increase the statistical relevance of the result. Measuring fewer than 30 beads is not recommended. During a measurement you can follow the number of beads analyzed per bead set either as bar graphs in the "Diagnostics" tab or in the "Count" mode of the "Acq. Detail" tab of the IS Software. The day-to-day and assay-to-assay coefficient of variation (CV) for LiquiChip assays is around 4%.

Assay sensitivity and dynamic range

The reporter channel of the LiquiChip Reader has a dynamic range of 3.5 orders of magnitude (MFI = 5 to 35,000). Fluorescence from less than 1000 R-phycoerythrin (R-PE) fluorophore molecules per bead can be detected. This level of sensitivity allows proteins to be detected in picogram amounts per milliliter. Careful selection of classification and reporter fluorescence profiles and bandwidths of the classification and reporter channels, ensures that no overlap of signal occurs.

Assay throughput

Using the LiquiChip System, thousands of beads can be interrogated in seconds, allowing a 96-well microplate to be measured in 35–60 minutes, depending on assay set up and degree of multiplexing. Using a 100plex assay, up to 9600 data points can be measured in 1 hour.

Assay analysis

In order to quantify analytes within a sample, a standard curve is generated using known/defined standard concentrations. The measured fluorescence intensity values of the unknown samples are compared to those in the standard curve and the amount of analyte is calculated.

All points used to generate a standard curve should fit the curve closely. The quality of fitting can be checked by performing a regression analysis. In the linear region of a standard curve, points are connected using a linear regression curve which has a general formula $y = mx + c$ where (m = slope, x = concentration, c = intersection of the y-axis when $x = 0$) In a conventional assay setup, standard curves are linear and standard-curve points generated using high concentrations of the analyte that lie within the saturation area are not used. In general, quantification of analytes will be more precise as the slope of the curve becomes steeper. To find the formula that best fits your standard points check actual and calculated values of your standards from the regression curve and choose the curve that fits best.

For more information on LiquiChip assay analysis, please refer to the *LiquiChip Analyzer Software Basic User Guide*.

LiquiChip Bead Sets

The surface chemistry of each type of LiquiChip Bead dictates the form of assays that can be carried out using them. Ni-NTA beads and Penta·His Beads are developed for the universal directed immobilization of 6xHis-tagged proteins. LiquiChip Activated Beads are used to immobilize biomolecules via thiol groups. Carboxyl groups on the surface of LiquiChip Carboxy Beads allow covalent attachment of proteins and other reactants using N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide (EDC) and N-hydroxysulfosuccinimide (S-NHS). The same bead lot is used to produce all of the spectrally distinct sets. The classification dyes are held within the beads. This means that the bead surface — upon which the coupling and subsequent reactions occur — is identical for all bead sets for a given bead type.

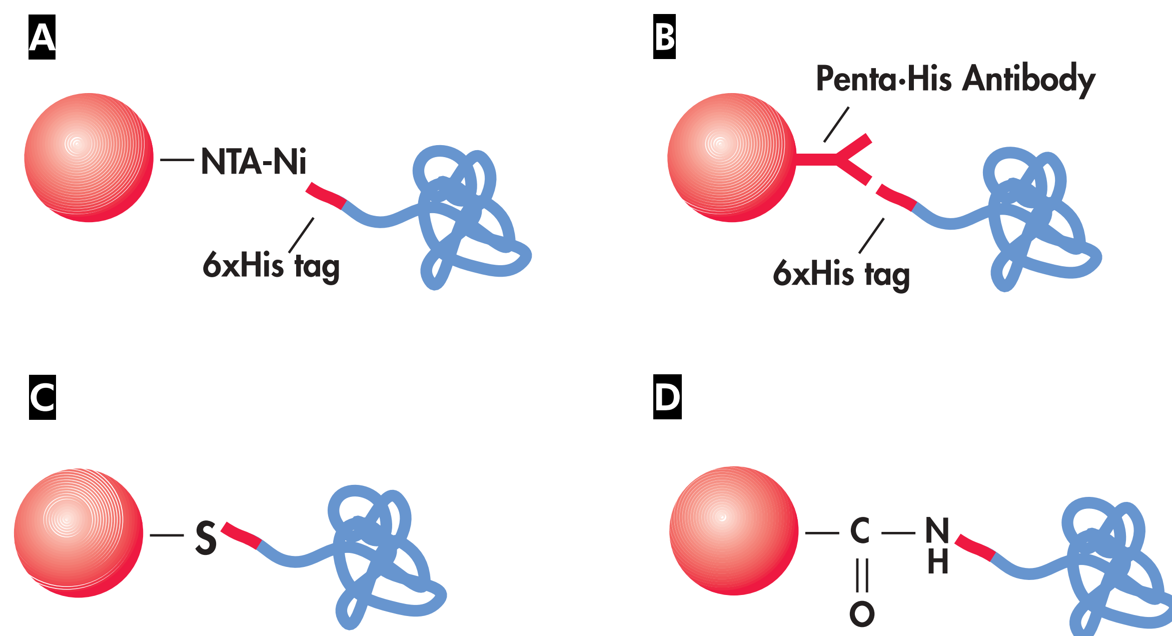

Figure 8 Schematic representation (not to scale) of immobilization of protein on **A** LiquiChip Ni-NTA Beads, **B** LiquiChip Penta·His Beads, **C** LiquiChip Activated Beads, and **D** LiquiChip Carboxy Beads.

	Ni-NTA Beads	Penta·His Beads	Activated Beads	Carboxy Beads
Immobilized capture-molecule	6xHis-tagged proteins and peptides	6xHis-tagged proteins and peptides	Proteins, peptides, oligos, and other biomolecules with a free thiol group	Proteins, peptides, nucleic acids, and other biomolecules with a free amino group
Binding chemistry	Ni-NTA–6xHis-tag interaction	Antigen (6xHis tag)–antibody interaction	Covalent binding via thiol group	Covalent binding through EDC/NHS (see page 57)
Amount of protein per assay data point	<5 ng	<5 ng	10–25 ng	10–25 ng
Advantages	Ready-to-use; universal coupling protocol for directed immobilization of all 6xHis-tagged proteins; protease resistant; high binding capacity	Ready-to-use; universal coupling protocol for directed immobilization of all 6xHis-tagged proteins; resistant to most chemicals interfering with the Ni-NTA–6xHis tag interaction	Ready-to-use; allow immobilization of any biomolecule with a free thiol group; direct coupling of purified antibodies	Allow immobilization of any molecule containing a free amino group; direct detection of 6xHis-tagged analytes using Penta·His Alexa Fluor 532 Conjugates
Restrictions on use	Not compatible with buffer components that may disturb the Ni-NTA–6xHis tag interaction (e.g., imidazole)	Not protease resistant	Proteins must contain a free, reduced thiol group	Optimization needed, relatively large amounts of capture molecule required

LiquiChip Ni-NTA Beads

LiquiChip Ni-NTA Beads combine all the benefits of the Ni-NTA–6xHis-tag system with the convenience and speed of LiquiChip technology. NTA is coupled to the surface of the beads via a spacer that provides optimal accessibility, minimal steric hindrance, and a high binding capacity for capture molecules. Ni-NTA beads allow analysis of protein–protein and protein–nucleic acid interactions, and also immunoassays that require higher binding capacities for the biomolecule under analysis. The high binding capacity of Ni-NTA beads enables less sensitive detection procedures to be used for analysis of bound constituents. The signal-to-noise ratio for low-affinity complexes, (e.g., receptor–ligand interactions) is increased by immobilizing larger amounts of the 6xHis-tagged capture molecule. Immunoassays can be conveniently performed and can be used for detection of low-affinity antibodies or interacting proteins, using protein-specific antibodies (see protocols). The protein binding capacity of the 0.5 μl Ni-NTA bead suspension used per assay data point is approximately 2 ng (depending on the specific protein).

LiquiChip Penta·His Beads

LiquiChip Penta·His Beads can be used for assays involving 6xHis-tagged proteins where a higher level of specificity is required. The Penta·His Antibodies coated on the surface of the beads recognize and bind to a five consecutive histidine residue epitope, regardless of the amino acids surrounding the motif. Therefore, Penta·His Antibodies can bind to partially hidden 6xHis tags. Penta·His beads are therefore highly suited for the directed immobilization of 6xHis-tagged proteins. An advantage of using Penta·His beads is the higher specificity of the Penta·His Antibody for 6xHis tags compared to Ni-NTA. Use of Penta·His beads is recommended when low concentrations of 6xHis-tagged proteins can be added to or detected in an assay. As the Penta·His bead–6xHis-tagged protein interaction is an antibody–antigen interaction, it is immune to chemicals that affect the Ni-NTA–6xHis-tag interaction (e.g., imidazole, hemoglobin, or EDTA) or salt concentration effects. The protein binding capacity of the 0.5 μl Penta·His bead suspension used per assay data point is approximately 1 ng (depending on the specific protein).

Penta·His Antibodies are mouse monoclonal IgG1 antibodies that have a high affinity and specificity for five consecutive histidine residues. The antibodies are obtained from serum-free hybridoma cell cultures, ensuring preparations free of viruses, mycoplasmas, and contaminating immunoglobulins. Purification by adsorption chromatography is performed without using Protein A or Protein G, and entirely at physiological pH, to yield preparations with the highest purity and activity. At the beads' surface, antibodies are immobilized in a directed manner through a spacer via their Fc domain.

As LiquiChip Penta·His Beads are coated with the comparatively larger antibody molecules, the binding capacity for 6xHis-tagged proteins of Penta·His beads is lower than that of Ni-NTA beads. For this reason, use of the detection reagent Streptavidin–R-PE is recommended in combination with a biotin-conjugated secondary antibody. Alternatively Alexa Fluor 532 labeled secondary antibodies can be used. Another point to bear in mind is that anti-mouse secondary antibodies cannot be used, as they will react with the bead-bound Penta·His Antibodies.

LiquiChip Activated Beads

LiquiChip Activated Beads offer a fast and convenient "chemistry-free" method for the covalent immobilization of capture molecules, especially antibodies, on LiquiChip beads. The simple coupling procedure does not require tedious pre-activation, multiple wash steps, or chemical reactions (e.g., EDC/S-NHS) to immobilize biomolecules. Capture molecules are simply incubated with beads in a one-tube procedure and are ready for use after a single, short wash step. Capture molecules are immobilized through thiol groups, allowing easy and efficient immobilization of antibodies, and any other proteins containing cysteine residues (e.g., GST fusion proteins). LiquiChip Activated Beads are highly suited for use in ELISA sandwich assays, and once immobilized, antibodies are stable for up to 6 months.

LiquiChip Carboxy Beads

LiquiChip Carboxy Beads have carboxyl (COOH) groups on their surfaces. Through activation by EDC/S-NHS, all molecules containing a free amino group can be covalently linked and immobilized on the beads. This chemistry can be used when it is not possible to immobilize a capture molecule using a 6xHis tag or a cysteine residue, or when detection will be carried out through a Streptavidin–Biotin system. A typical application is a sandwich ELISA, in which a capture antibody is used for immobilization of the target.

In contrast to the efficient and directed immobilization offered by the 6xHis-tag system, LiquiChip Carboxy Beads require an immobilization protocol that must be optimized for each protein. In addition, the proteins are not immobilized in a directed manner, leading to losses in sensitivity and an increased requirement for protein. By immobilizing biomolecules through free amino groups on their surface, their structure may be altered.

Assay Development Using the LiquiChip System

The first step in a LiquiChip assay is the immobilization of capture molecules on LiquiChip beads. The nature of the capture molecule influences to some extent the choice of LiquiChip bead. For instance, if the capture molecule is a 6xHis-tagged protein, LiquiChip Ni-NTA or Penta·His Beads can be used for immobilization.

For the bead types listed above, 2 ng protein is incubated with 0.5 µl bead suspension for each assay point. Capture-molecule–coated beads can then often be added directly to LiquiChip Assays.

If the capture molecule does not possess a 6xHis tag, it can be immobilized using LiquiChip Activated or Carboxy Beads. Capture molecules are covalently bound to the surface of beads via free thiol or amino groups respectively. As these are chemical processes, the immobilization procedure requires larger amounts of capture molecule (10–25 ng per assay point).

Using a 6xHis-tagged protein as a capture molecule

If the capture molecule is a recombinant protein, it is recommended that you express it as a 6xHis-tagged protein. In addition to simplifying the purification process, the presence of the 6xHis tag offers several advantages in LiquiChip assays:

- Even, reproducible immobilization
- The protein retains functionality, as it is not chemically coupled
- The protein is bound in a directed manner, allowing optimal access to interaction partners
- Directed immobilization leads to higher signal intensities, which in turn means that less protein is required per assay

LiquiChip Ni-NTA or Penta·His Beads can be used for immobilization of 6xHis-tagged capture molecules. Monoclonal antibodies on the surface of LiquiChip Penta·His Beads recognize and bind tightly to an epitope of five consecutive histidine residues. As this interaction is an antibody–antigen interaction, it is resistant to most chemical or salt concentration effects that may interfere with the Ni-NTA–6xHis-tag interaction, such as the presence of imidazole, serum, hemoglobin, or EDTA. Therefore, LiquiChip Penta·His Beads are recommended for use in systems where such chemicals are present.

Using a non-tagged capture molecule

Capture molecules without a 6xHis tag can be quickly and efficiently immobilized via their free thiol groups on LiquiChip Activated Beads. The one-tube procedure used to immobilize biomolecules on LiquiChip Activated Beads reduces loss of beads that may occur during the multiple handling steps required for immobilization on LiquiChip Carboxy Beads, and therefore LiquiChip Activated Beads are recommended for immobilization of non-tagged molecules. If capture molecules do not contain free thiol groups, they can be immobilized via their free amino groups on LiquiChip Carboxy Beads using N-(3-dimethylaminopropyl)- N'-ethylcarbodiimide/N-hydroxysulfosuccinimide (EDC/S-NHS) chemistry. Carboxyl groups on the surface of the beads are activated using EDC and S-NHS to form S-NHS esters. Amine groups on the biomolecule displace S-NHS, leading to covalent attachment via peptide bonds (see Appendix, page 57).

Coupling antibodies to LiquiChip beads

In general, we recommend coupling monoclonal antibodies to LiquiChip beads because of their high antigen specificity, which is needed for multiplex experiments. However, polyclonal antibodies can be used following an affinity-purification step.

Adding beads to the assay

LiquiChip beads are provided at a standard concentration (2.5×10^6 beads/ml). A 0.5 µl aliquot of the stock bead suspension is sufficient for 1 assay data point. However, if beads are washed after coupling, the yield may be lower than the starting concentration due to loss of beads during wash steps. Bead loss can vary according the properties of coupled reactants, and volume of the coupling reaction. For reproducible assays, it is important to add the same number of beads to each reaction. If wash steps are used after a coupling protocol, the concentration of the bead suspension should be determined before adding beads to an assay (see protocol on page 38).

RCAT signal amplification

Rolling circle amplification technology (RCAT) provides signal amplification in xMAP assays; greatly increasing assay sensitivity. The assay is based on a sandwich immunoassay. LiquiChip beads are coated with target-protein specific antibodies and added to an assay. During incubation, target molecules bind to the antibodies. A second target-protein specific biotinylated antibody is added to the assay. A third anti-biotin antibody is then added. This antibody carries a short DNA sequence that anneals to a single-stranded DNA circle included in the antibody solution (Figure 9A). The RCAT reaction is initiated by adding a

reaction mix containing dNTPs and RCAT DNA Polymerase, which extends the double-stranded annealed sequence (Figure 9B). By including biotinylated dNTPs in the reaction, numerous biotin moieties are incorporated into the growing DNA strand. The biotin moieties in the DNA strand bind the Streptavidin-Phycoerythrin that is added to the assay in the final detection step, providing a signal amplification effect and greatly increasing the sensitivity of the assay.

RCAT Technology for Amplification of LiquiChip Assay Signals

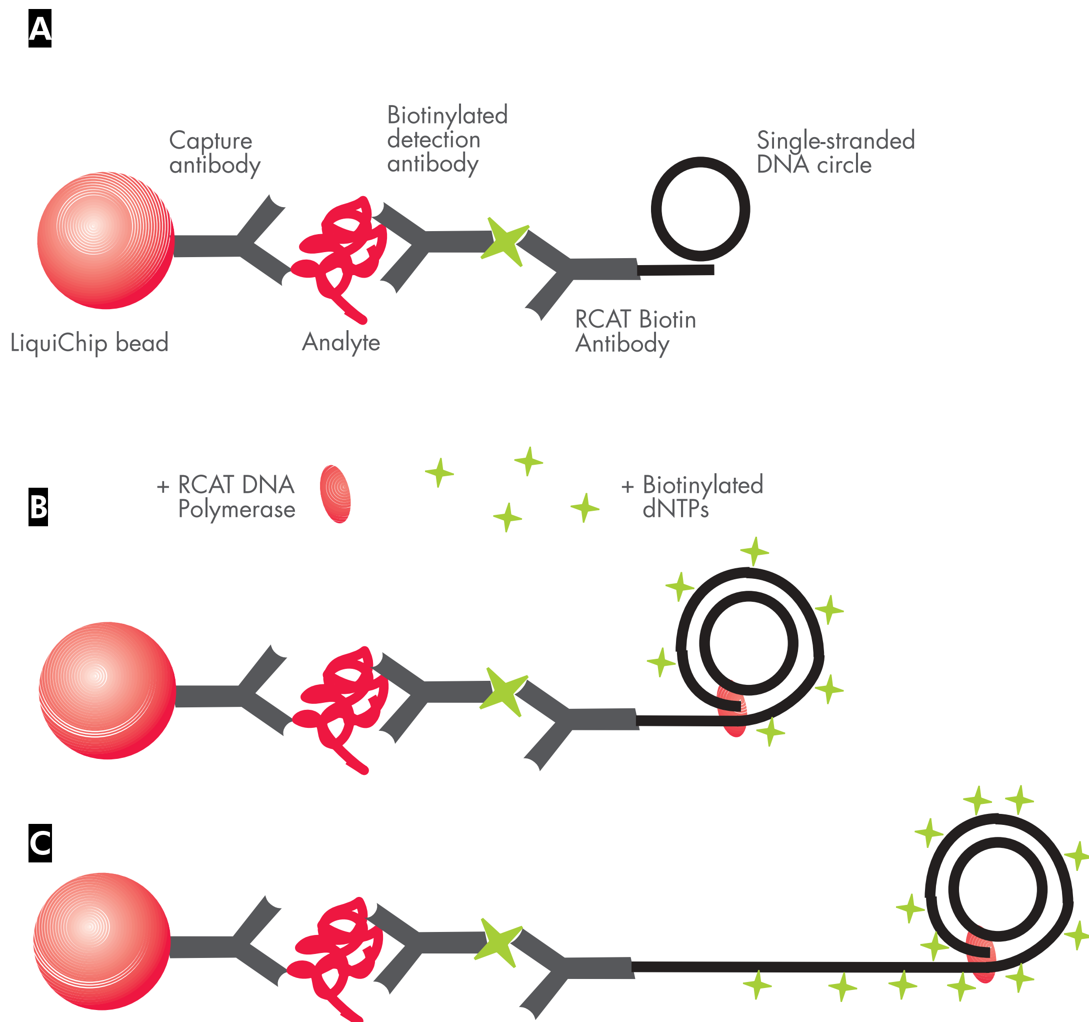

Figure 9 RCAT LiquiChip assay signal amplification technology. **A** The anti-biotin DNA conjugate binds to biotin and the DNA circle anneals to the DNA strand. **B** RCAT DNA Polymerase is added and extends the DNA strand attached to the anti-biotin antibody, using the single-stranded circle as a template. Once the polymerase reaches the origin of duplication, its strand-displacement activity enables the polymerase to extend the DNA strand indefinitely. **C** Biotinylated dNTPs are incorporated into the growing DNA strand.

Detection of Analytes

Analytes bound to capture molecules can be detected using either direct or indirect methods. Analyte-specific reporter molecules that carry a green fluorophore suitable for use with the LiquiChip system (e.g., R-PE, Alexa Fluor 532, or Cy3) can be used for direct detection. Fluorescently labeled secondary antibodies that recognize analyte-specific primary antibodies can be used for indirect detection in a process analogous to ELISA. Using indirect detection methods, higher sensitivity can be obtained through signal amplification. A signal can be amplified by using multiple labeled antibodies, each specific for a single analyte, or by using a detection reagent carrying several biotin moieties which in turn binds multiple copies of a fluorescently labeled avidin molecule.

Detection reagents can either be added simultaneously to save time or sequentially, which generally results in higher assay sensitivity.

In addition to direct and indirect assays, competitor assays are also possible where labeled interaction partners are displaced by unlabeled reactants. This allows the utilization and/or identification of unlabeled reactants, saving both time and costs.

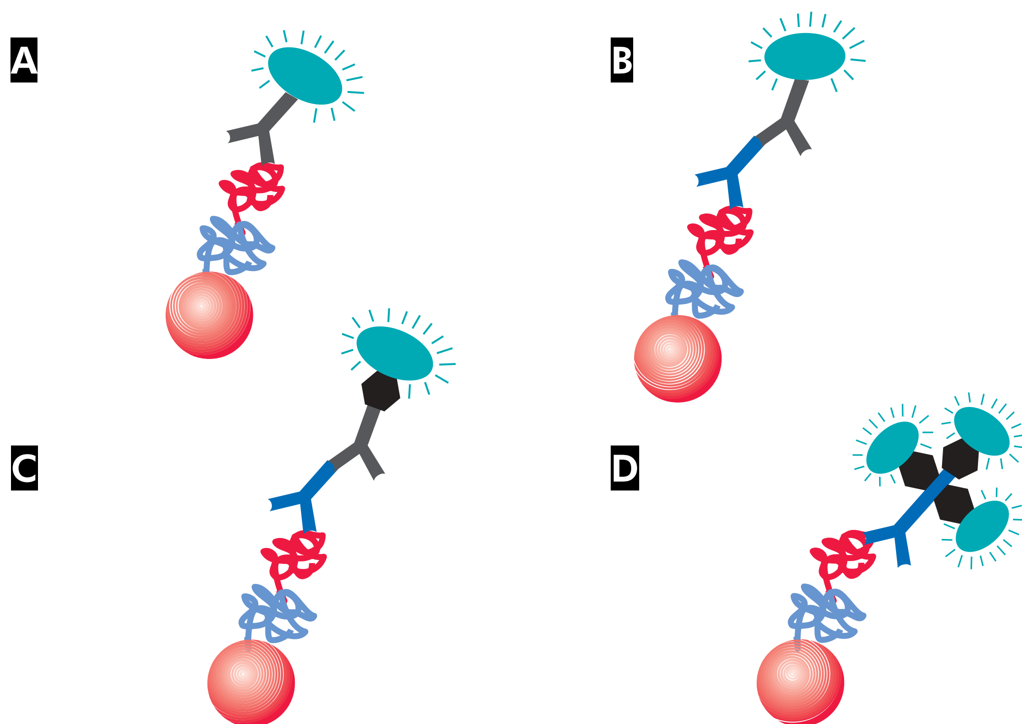

Figure 10 Direct and indirect assay of analytes in LiquiChip assays. **A** Direct detection of an analyte using a fluorescently labeled protein-specific antibody. **B** Indirect detection using a fluorescently labeled secondary antibody. **C** Indirect detection using a biotinylated secondary antibody and a fluorescently labeled avidin conjugate. **D** Direct detection using a biotinylated primary antibody and Streptavidin R-PE.

LiquiChip Detection Reagents

Wavelengths of classification and reporter fluorescence have been carefully chosen to ensure that no cross-contamination of signal can take place. Reporter signal is obtained by stimulation using a green laser at 532 nm, and recorded using the bandwidth of 565–585 nm. Due to this required specificity, only fluorophores that are excited at 532 nm and emit between 565 and 585 nm can be used. The optimal fluorophores for use with this system are phycoerythrins, especially R-phycoerythrin (R-PE), Alexa Fluor 532, and Cy®3. Used in conjunction with the LiquiChip instrument these fluorophores deliver the highest fluorescence yields and do not emit in the classification wavelengths. On a molecule to molecule basis Alexa Fluor 532 displays 28% of the fluorescence intensity delivered by R-PE. However, as R-PE is a much larger molecule (240 kDa), detection reagents can be labeled with a much higher density using Alexa Fluor 532 and therefore the difference in fluorescence intensity obtained when using R-PE or Alexa Fluor 532 is often insignificant. Cy3 usually yields around 50% of the signal intensity obtained using R-PE or Alexa Fluor 532.

Streptavidin–R-PE

Streptavidin–R-PE comprises streptavidin conjugated to the protein fluorophore R-phycoerythrin (R-PE), and binds biotinylated molecules with high affinity. The absorption and emission profiles of R-PE are ideally suited for its use as a reporter molecule for detection and quantification of biotinylated analytes or primary antibodies, such as the Penta·His Biotin Conjugate, in LiquiChip assays. Phycoerythrin is among the best dyes currently available for applications that require high sensitivity and has a quantum yield of 0.82 and an extinction coefficient of 2×10^6. Using the LiquiChip System, the detection limit of R-PE is less than 1000 molecules per bead.

Penta·His Alexa Fluor 532 Conjugate

The Alexa Fluor dyes from Molecular Probes are a series of superior fluorescent dyes. Benefits of the Alexa Fluor dyes and their conjugates are more intense fluorescence than other spectrally similar conjugates, pH insensitivity, and water solubility. Penta·His Alexa Fluor 532 Conjugates comprise Penta·His Antibodies conjugated to the fluorophore Alexa Fluor 532, and can be used for highly sensitive and specific detection of 6xHis-tagged proteins without the need for a secondary antibody. The absorption and emission profiles of the conjugated dye Alexa Fluor 532 are ideally suited for its use as a reporter molecule in LiquiChip assays. The green laser in the LiquiChip instrument emits light of a wavelength that corresponds exactly to the absorption maximum of Alexa Fluor 532. Alexa Fluor 532-labeled detection reagents and protein labeling kits are available from Molecular Probes, Inc.

Penta·His Biotin Conjugate

Penta·His Biotin Conjugates comprise Penta·His Antibodies conjugated to biotin, and bind to 6xHis-tagged proteins with high affinity and specificity. By taking advantage of the high-affinity interaction between biotin and avidin, Penta·His Biotin Conjugates can be in turn detected with high sensitivity using avidin or streptavidin conjugates (e.g., Streptavidin–R-PE).

Assay range and detection limits

The range over which the analyte can be assayed is highly dependent on the analyte binding capacity of the capture-molecule–coated beads, the affinity of the analyte for the capture molecule, and on the detection method.

Typically, LiquiChip assays have a dynamic range of up to three orders of magnitude. However, the dynamic range of a given assay is dependent on the quality of the detection reagents used and on possible steric hindrance at the beads' surface, and may therefore be lower.

The four types of LiquiChip bead display different capture-molecule binding capacities. In general, LiquiChip Ni-NTA Beads are able to bind approximately twice the number of capture molecules per bead than Penta·His Beads, which in turn bind more than Activated and Carboxy Beads.

Ni-NTA > Penta·His > Activated and Carboxy Beads

Analytes with a high affinity for their capture molecule, can be detected at lower levels (i.e., with higher sensitivity) than analytes that bind weakly to capture molecules.

Homogeneous assay systems

Because the LiquiChip Reader measures only bead-bound fluorescence, it is not necessary to remove unbound detection reagent in a wash step. This not only makes the assay quicker, but also increases assay sensitivity, as the reaction equilibrium is not disturbed during measurement. This allows the assay of interaction partners whose affinities for each other are too low to allow measurement using conventional ELISA techniques (Figure 6, page 11).

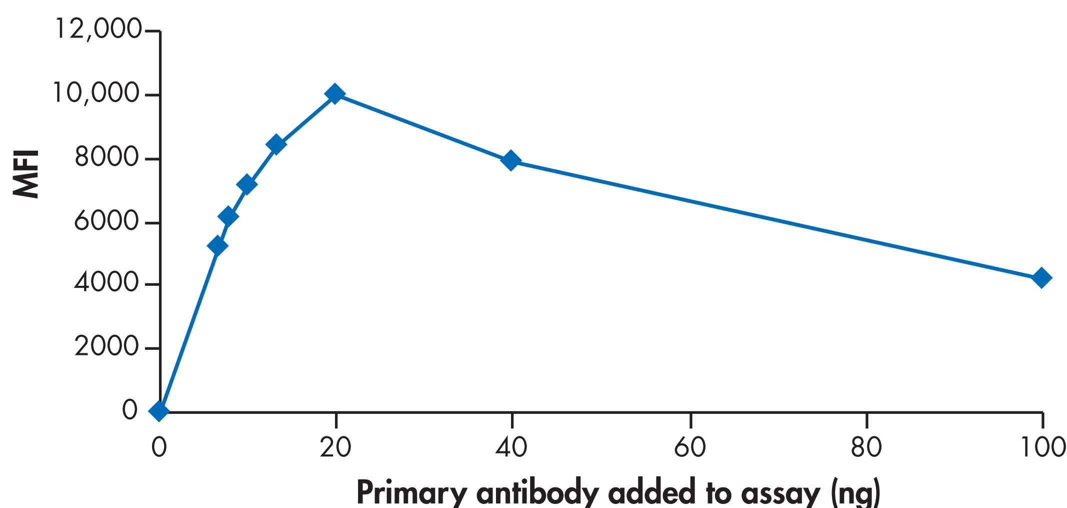

Figure 11 The effect of primary to secondary antibody ratio on signal strength. Aliquots of LiquiChip Ni-NTA Beads (equivalent to 0.5 µl stock bead suspension) were incubated with 1 ng 6xHis-tagged thioredoxin for 3 h at room temperature. The indicated amount of mouse polyclonal anti-thioredoxin antibody (10 mg IgG/ml) was added, and the samples incubated for a further 1 h. Secondary antibody (250 ng Alexa Fluor 548-labeled goat anti-mouse) was added to each well and the samples incubated for a further 1 h, before measurement using the LiquiChip Reader.

Points to note when performing homogeneous assays

In principle, almost any assay can be performed in homogeneous format, but the following points should be considered. Because neither excess analyte nor excess primary detection reagent is removed in a wash step, free complexes may compete for labeled secondary detection reagents. For example, such a problem may arise when an antigen is coupled to beads, and antigen-specific antibodies in a mouse serum sample are assayed using a labeled anti-mouse secondary antibody. As all antibodies in the sample will react with the secondary antibody, enough labeled antibody must be added to the sample to ensure that all antibodies, including the antigen-specific antibody, are bound (Figure 11). To avoid such problems when using indirect detection methods, ensure that the amount of primary detection reagent never exceeds the amount of secondary detection reagent. We recommend using a 5–20-fold excess of secondary detection reagent. Optimization trials, in which secondary antibodies are titrated against primary antibodies, are recommended to achieve optimal performance. As a starting point for ELISA procedures using the LiquiChip System, use 10 ng primary antibody and 200 ng labeled secondary antibody or 100 ng Streptavidin–R-PE.

Homogeneous assays are not possible when high concentrations of reagents that affect the detection process are present. For example, when measuring analytes in samples containing RPMI medium, the high concentration of biotin in the medium make assay using an avidin-based detection reagent impossible. In such cases, a wash step must be built into the assay procedure.

Assay volume

The assay volume is dependent on the type of microplate used. Table 1 gives guidelines for sample volumes. In round-bottom and half-area–bottom plates a minimum assay volume of 60 µl is required. In flat- and conical-bottom plates, a minimum volume of 100 µl is required. A minimum of 25 µl is aspirated by the LiquiChip Reader for each assay. The extra volume is required to ensure that no air is drawn into the system. If the volume of your assay is less than the figures quoted above, the reaction can be performed in the smaller volume, but must be diluted before measurement.

Performing assays in smaller volumes has the advantage of increasing the concentration of beads in the sample, which increases reaction kinetics and speeds up measurement in the LiquiChip Reader.

Table 1. LiquiChip Assay Volumes and Aspirated Volumes.

Type of microplate	Minimum assay volume (µl)	Maximum aspirated volume (µl)	Aspirated volume (µl)
Round-bottom	60	Total assay volume –20	25–40
Half-area–bottom	60	Total assay volume –20	25–40
Flat-bottom	100	Total assay volume –25	25–75
Conical-bottom	100	Total assay volume –25	25–75

Multiplexing

The bead classification feature of the LiquiChip System allows the simultaneous assay of multiple analytes in a single sample. For each analyte, couple the respective capture molecule to a bead set carrying a different bead code, for example, bead codes 50 and 52. Mix the bead suspensions, add your sample containing both analytes, add your detection reagent, and incubate. The sample is aspirated into the LiquiChip Reader, measured, and the mean fluorescent intensity for each different bead code (and therefore analyte) calculated. This multiplexing feature gives the potential to assay up to 100 different analytes in a single sample. Beads with differing surface chemistries can be used in the same assay. For example, you can immobilize a 6xHis-tagged molecule using LiquiChip Ni-NTA Beads carrying one bead code, and an amino-group-containing protein using LiquiChip Carboxy Beads carrying a different bead code. Both sets of beads can be added to the same assay. The bead codes of LiquiChip beads have been carefully chosen to ensure that no bead code is repeated. It is planned to introduce further bead codes in the future.

Cross-reactions in multiplex assays

The binding of capture molecules to LiquiChip beads is very stable, and therefore migration of capture molecules between beads does not take place. In a multiplex assay, it is advisable to check whether the detection reagent(s) used lead to cross-reactions. This can be done by comparing standard curves performed in both single and multiplex assay formats.

Multiplexing of assays does not automatically lead to an increase in sample volume. Bead suspensions and detection reagents can be added to assays as a concentrated "master mix".

Adaptation of existing assay procedures to LiquiChip technology

Existing assay procedures, e.g., ELISA, receptor–ligand assays, or competitive assays, can be easily and quickly adapted to LiquiChip technology. For example, to adapt an ELISA procedure, the relevant beads are coated with capture molecule, and titrated against the relevant detection reagent. This handbook contains protocols for immunoassay and subsequent detection using both direct and indirect methods. For antibody-mediated direct detection, use of 10–50 ng labeled antibody per assay point is recommended. For indirect detection, 10 ng primary antibody and 50–200 ng labeled secondary antibody per assay point deliver optimal results.

Stability of coupled beads

The reagent immobilized on the beads' surface will determine the shelf life of the preparation. The stability of a capture-molecule–coated bead preparation in solution is affected by aseptic processing conditions, storage buffer, preservative, etc. Alternatively coupled beads may be dried or lyophilized.

Labeling proteins and antibodies

Kits are commercially available for labeling proteins and antibodies with reagents used in LiquiChip assays. PhycoLink® SMCC-Activated R-Phycoerythrin (ProZyme®, San Leandro, CA, USA, www.prozyme.com) and the Alexa Fluor 532 Protein Labeling Kit (Molecular Probes, Eugene, OR, USA, www.probes.com) can be used to label detection reagents. EZ-Link™ Biotinylation Kits (Perbio Science, www.perbio.com) can be used for the biotinylation of assay components.

Preparation of Beads

LiquiChip beads have a density of 1.05 g/ml. Therefore, over time, they will settle out of solution. Depending on their surface chemistries, some beads may show a tendency to aggregate. For reproducibility of reactions, beads in the stock suspension should be homogeneously suspended before use. Beads should be vortexed for at least 30 seconds to obtain a homogeneous suspension. This suspension will remain homogeneous for approximately 2 hours. Before addition of an assay component, briefly shake the tube or microplate.

LiquiChip beads are internally dyed with fluorophores to distinguish between the different beads' codes. Loss of fluorescence may lead to a misclassification of the bead sets. The bead-code fluorophores are light-sensitive and exposure to daylight should be kept to a minimum. For incubation times longer than 5 minutes, place the reaction vessel in a cupboard or drawer, or cover with aluminum foil.

Even though LiquiChip bead stock suspensions are delivered in opaque tubes, the tubes should be protected from light. Beads coated with protein are only as stable (and as sterile) as the coating protein itself. They are usually stable for some weeks at 4°C.

Coupling Proteins to LiquiChip Beads

The highest level of sensitivity in LiquiChip assays is obtained by coupling proteins to beads using a protocol without washing steps, (i.e., a homogeneous protocol, page 30). If the buffer used in the assay is not compatible with the coupling buffer, or the amount of protein in your sample is very difficult to estimate, it is advisable to wash excess protein or buffer away.

LiquiChip beads are provided at standard concentrations. 0.5 µl of the stock bead suspension is sufficient for 1 assay data point. However, if beads are coupled using wash procedures, the yield after a coupling process may be lower than the starting concentration due to loss of beads during wash steps. If wash steps are carried out during the preparation of beads, use 1 µl bead suspension per assay point and determine the number of beads in your capture-molecule–coated bead suspension by counting (see protocol on page 38). Bead loss can vary according the properties of coupled reactants, and scale of coupling.

Protocol: Homogeneous Coupling of 6xHis-tagged Proteins to LiquiChip Ni-NTA or Penta·His beads

This protocol generates sufficient capture-molecule–coated LiquiChip Ni-NTA or Penta·His beads for 100 assay data points (1 microplate). If more or fewer data points are required the protocol can be scaled up or down accordingly.

For composition of buffers, see Appendix, page 51.

Equipment to be supplied by user

- Vortex mixer
- Pipets (1–1000 µl)
- Microcentrifuge and microcentrifuge tubes*
- PBS/1% BSA, PBS/0.1% BSA, and PBS

Note: Text marked with a ■ refers to Penta·His Beads. Text marked with a ▲ refers to Ni-NTA Beads.

Procedure

1. **Vortex the LiquiChip Ni-NTA or Penta·His Bead stock suspension for 30 s at full speed.**
2. **Pipet 50 µl of the bead suspension into a 1.5 ml microcentrifuge tube.***
3. **Dilute your 6xHis-tagged protein to 4 µg/ml in ■ PBS/0.1% BSA or ▲ PBS buffer.**
4. **Add 50 µl of your 6xHis-tagged protein dilution (4 µg/ml)† to the 50 µl LiquiChip bead suspension.**
5. **Incubate at 4°C in the dark for 2 hours or, for higher assay sensitivity, overnight.**
6. **Add 900 µl ■ PBS/1% BSA or ▲ PBS/0.1% BSA buffer to the protein-coupled LiquiChip Bead suspension, adding 0.05% azide as preservative, if desired. Use 10 µl of the diluted protein-coupled LiquiChip Bead suspension for each LiquiChip assay data point.**

   If your protein is stable, the protein-coupled beads can be stored for some weeks at 4°C in the dark, allowing batch preparation of LiquiChip beads for routine assays. If using LiquiChip Penta·His Beads, store beads in PBS/1% BSA.

* We recommend using microcentrifuge tubes made from polypropylene copolymer (e.g., Fisher Scientific Cat. No. #3544350 (EU), 05408-10 (US) or Fisher Brand SB56075 (EU), 1415-2500 (US) because they show the lowest interaction with protein coupled beads.

† This amount of protein corresponds to 2 ng protein per 0.5 µl bead suspension.

Protocol: Protein-Coupling with Wash Steps for LiquiChip Ni-NTA, Penta·His, or Antibody-Coated Beads

This protocol generates sufficient capture-molecule–coated LiquiChip Ni-NTA, Penta·His, or antibody-coated Activated or Carboxy Beads* for at least 100 assay data points (1 microplate). If more or fewer data points are required the protocol can be scaled up or down accordingly.

For composition of buffers, see Appendix, page 51.

Equipment and reagents to be supplied by user

- Vortex mixer
- Pipets (1–1000 µl)
- Microcentrifuge and microcentrifuge tubes†
- PBS/1% BSA, PBS/0.1% BSA, and PBS

Note: Text marked with a ■ refers to Penta·His Beads and antibody-coated beads. Text marked with a ▲ refers to Ni-NTA Beads.

Procedure

1. **Vortex the LiquiChip bead stock suspension (2.5 x 10^6 beads/ml) for 30 s at full speed and pipet 100 µl bead suspension into a 1.5 ml microcentrifuge tube.†**
2. **Dilute your 6xHis-tagged or biotinylated protein solution to 10 µg/ml using ■ PBS/0.1% BSA or ▲ PBS buffer.**
3. **Add 100 µl of your protein dilution to the 100 µl LiquiChip Bead suspension, and vortex for 30 s at full speed.**
4. **Incubate at 4°C in the dark for 2 hours or, for higher assay sensitivity, overnight.**
5. **Add 1 ml ■ PBS/0.1% BSA or ▲ PBS buffer to the beads and centrifuge at 10,000 x *g* for 2 min.**
6. **Carefully remove the supernatant using a 200 µl pipet. The pellet containing the capture-molecule–coated LiquiChip beads is very small, and may be difficult to see.**
7. **Repeat steps 5 through 6 twice.**

* See protocols on pages 33 and 35.

† We recommend using microcentrifuge tubes made from polypropylene copolymer (e.g., Fisher Scientific Cat. No. #3544350 (EU), 05408-10 (US) or Fisher Brand SB56075 (EU), 1415-2500 (US) because they show the lowest interaction with protein coupled beads.

8. **Resuspend the beads in 400 µl ■ PBS/1% BSA or ▲ PBS/0.1% BSA, adding 0.05% azide as preservative, if desired.**
9. **Calculate the concentration of beads in your suspension using the LiquiChip Reader or a cell-counting chamber (see protocol on page 38).**
10. **Adjust the concentration of the bead suspension to 125 beads/µl and use 10 µl per assay data point.**

    If your protein is stable, the protein-coupled beads can be stored for some weeks at 4°C in the dark, allowing batch preparation of LiquiChip Beads for routine assays. If using LiquiChip Penta·His or antibody-coated beads, store beads in PBS/1% BSA.

Protocol: Coupling Peptides, Antibodies, or Proteins to LiquiChip Activated Beads

LiquiChip Activated Beads are ready to use for direct coupling of proteins. Antibodies, or any other cysteine-containing proteins/peptides (e.g., GST fusion proteins), can be covalently immobilized on LiquiChip Activated Beads via their thiol groups. Pre-activation of beads is not necessary. The protein solution should not contain buffer components with free thiol groups (e.g., β-mercapto-ethanol, DTT, or DTE). A 100 µl aliquot of LiquiChip Activated Beads is sufficient for at least 100 assay data points. Using aliquots of less than 100 µl Activated Beads for coupling is not recommended, as the relative loss of beads increases as the size of the aliquot decreases. This protocol describes the coupling of 10 µg protein to a 100 µl aliquot of beads. If coupling protein to aliquots larger than 100 µl, increase the amount of protein accordingly. Coupled beads are stable at 4°C for at least 3 months.

For composition of buffers, see Appendix, page 51.

Equipment and reagents to be supplied by user

- LiquiChip Activated Beads
- Reaction tubes for bead coupling
- Coupling buffer (50 mM MES, pH 6.5)
- PBS
- PBS/1% BSA
- Vortex mixer
- Protein stock solution

Procedure

1. **Vortex the LiquiChip Activated Beads stock suspension for 30 s at full speed. Agitate the tube for at least another 30 min in the dark to completely resuspend the beads.**

   Agitation should be performed using an end-over-end shaker or a vortex mixer with a shaker adapter (e.g., Roth, cat. no. 509.1 and 510.1). Tubes should be wrapped in aluminum foil to exclude light.

2. **Pipet 100 µl LiquiChip Activated Beads suspension into one of the 1.5 ml reaction microtubes supplied with the beads.**

   If further tubes are required, we recommend 1.5 ml microcentrifuge tubes made from polypropylene copolymer (e.g., Fisher Scientific, cat. no. 3544350), as these tubes show the lowest level of interaction with beads.

3. **Dilute the protein stock solution with coupling buffer to a concentration of 0.2 mg/ml in a volume of 50 µl (10 µg protein).**

   LiquiChip Activated Beads are supplied in 50 mM MES, pH 6.5, the recommended buffer for coupling proteins. Other buffers (50 mM HEPES, pH 7.4, or 20 mM MOPS, pH 7.2)* can be tested to optimize protein immobilization. To change the buffer that the beads are suspended in, centrifuge the bead suspension for 3 min at 10,000 x *g*, carefully remove the supernatant, and resuspend the beads in 100 µl of the desired buffer.

4. **Pipet the 50 µl diluted protein solution from step 3 into the tube containing the LiquiChip Activated Beads.**

   Usually, coupling 10 µg protein to 100 µl LiquiChip Activated Beads produces good results. To determine the optimal amount of protein, couple various amounts of protein within the range of 2–30 µg (10–150 µl stock solution) to 100 µl LiquiChip Activated Beads.

5. **Agitate the tube containing LiquiChip Activated Beads and protein solution for 2 h in the dark at room temperature (15–25°C).**

   Agitation should be performed using an end-over-end shaker, a vortex mixer with a shaker adapter (e.g., Roth, cat. no. 509.1 and 510.1) at lowest speed, or a plate shaker at 900 rpm. For temperature-sensitive proteins, incubate overnight at 4°C to ensure efficient coupling.

6. **Centrifuge the bead suspension for 3 min at 10,000 x *g*. Carefully remove the supernatant in 50 µl aliquots until almost no supernatant remains.**

   To minimize bead loss, it is important to remove the supernatant stepwise in small aliquots and to leave approximately 10 µl liquid in the tube.

7. **Pipet 500 µl PBS into the tube and resuspend the bead pellet by gently vortexing.**

8. **Centrifuge the bead suspension for 3 min at 10,000 x *g* and carefully remove the supernatant in 100 µl aliquots until almost no supernatant remains.**

9. **Pipet 200 µl PBS/1% BSA into the tube and resuspend the bead pellet by gently vortexing.**

10. **Determine the concentration of beads in the suspension by using the LiquiChip Reader or a cell-counting chamber (see protocol on page 38).**

11. **Adjust the concentration of beads to 1.25 x10^5 beads/ml using PBS/1% BSA and store at 4°C in the dark.**

    For LiquiChip assays, use 10 µl of this bead suspension per assay data point (equivalent to 1250 beads per well).

* See Appendix, page 51.

Protocol: Coupling Untagged Proteins to LiquiChip Carboxy Beads

Untagged proteins are immobilized on LiquiChip Carboxy Beads via the amine groups in lysine side-chains. Before coupling protein to LiquiChip Carboxy Beads, the beads must first be activated using EDC/S-NHS. Figure 12, page 53, shows the chemical reaction that takes place at the beads' surface, leading to covalent immobilization of protein.

A 1 ml aliquot of LiquiChip Carboxy Beads is sufficient for at least 500 assay data points. The total number of assay data points depends on the degree of bead loss experienced during handling (e.g., during wash steps). If you use bead aliquots of less than 1 ml for coupling, reduce the suggested amount of protein by the relevant factor. We do not recommend using LiquiChip Carboxy Bead aliquots of less than 500 µl for coupling, since the relative loss of beads is greater, the smaller the aliquot treated. **Note**: The protein stock solution should not contain foreign protein, azide, glycine, Tris, or any other reagent containing primary amine groups. If any of these reagents are present, remove them by gel-filtration chromatography or dialysis. For preparation of buffers, see Appendix, page 51.

Equipment and reagents to be supplied by user

- Reaction microcentrifuge tubes* (if coupling protein to less than 1 ml beads)
- Vortex mixer and microplate shaker
- Activation buffer (100 mM NaH_2PO_4, pH 6.3)
- Coupling buffer† (50 mM HEPES, pH 7.4)
- PBS
- PBS/1% BSA
- EDC (N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide) (e.g., Fluka, cat. no. 03449)
- S-NHS (N-hydroxysulfosuccinimide) (e.g., Fluka, cat. no. 56485)
- Protein stock solution

* It is difficult to see the bead pellet in the brown tube. If desired, the beads can be transferred to a different tube. We recommend using microcentrifuge tubes made from polypropylene copolymer (e.g., Fisher Scientific cat. no. #3544350 (EU), 05408-10 (US) or Fisher Brand SB56075 (EU), 1415-2500 (US) because they show the lowest interaction with protein coupled beads.

† We recommend 50 mM HEPES, pH 7.4, which is the coupling buffer for antibodies. To optimize the coupling reaction, try the following alternative coupling buffers: 50 mM MES, pH 6.5; 20 mM MOPS, pH 7.2; or PBS. For preparation of buffers, see Appendix, page 51.

Procedure

Bead activation

1. **Vortex the LiquiChip Carboxy Beads stock suspension for at least 3 min at full speed to yield a homogeneous bead suspension.**
2. **Weigh approximately 10 mg each of EDC and S-NHS into 2 microcentrifuge tubes*.**
3. **Dissolve in deionized water to obtain solutions of 50 mg/ml each.**
4. **Centrifuge the 1 ml bead suspension for 3 min at 10,000 x *g*. Carefully remove and discard the supernatant using a 200 µl pipet.**
5. **Resuspend the beads in 80 µl activation buffer.**
6. **Add 10 µl of S-NHS solution (50 mg/ml) and 10 µl of EDC solution (50 mg/ml) to the bead suspension. Incubate with agitation for 20 min at room temperature (15–25°C) in the dark.**

   Agitation should be performed using an end-over-end shaker, a vortex mixer with a shaker adapter (e.g., Roth, cat. no. 509.1 and 510.1) at lowest speed, or a plate shaker at 900 rpm.

Coupling of protein to activated LiquiChip Carboxy Beads

7. **Dilute your protein stock solution with coupling buffer to a concentration of 0.1 mg/ml in a volume of 500 µl.**

   The optimal concentration for coupling a given protein can be determined by coupling at various concentrations within the range of 25–250 µg/ml.

   Note: The protein stock solution should not contain foreign protein, azide, glycine, Tris, or any other reagent containing primary amine groups. If any of these reagents are present, remove them by dialysis or gel-filtration chromatography.
8. **Centrifuge the beads for 3 min at 10,000 x *g*, and carefully remove and discard the supernatant.**
9. **Add the diluted protein solution (500 µl) from step 7.**
10. **Agitate the tube with activated beads and protein solution for 2 h in the dark at room temperature (15–25°C).**

    Agitation should be performed using an end-over-end shaker, a vortex mixer with a shaker adapter (e.g., Roth, cat. no. 509.1 and 510.1) at lowest speed, or a plate shaker at 900 rpm. Tubes should be wrapped in aluminum foil to exclude light. For temperature-sensitive proteins, incubate overnight at 4°C to ensure efficient coupling.

* Minimize the exposure of EDC and S-NHS to air, and close containers tightly. Use fresh aliquots for each coupling reaction and discard after use.

Washing and storage of coated LiquiChip Carboxy Beads

11. Centrifuge the beads for 3 min at 10,000 x *g*, and carefully remove and discard the supernatant.
12. Add 500 µl PBS and resuspend the bead pellet. Centrifuge the beads for 3 min at 10,000 x *g*, and remove and discard the supernatant.
13. Resuspend the bead pellet in 1 ml PBS/1% BSA.
14. Determine the bead concentration of the suspension using the LiquiChip Reader or a cell-counting chamber (see protocol on page 38).
15. Adjust the concentration of the bead suspension to 1.25×10^6 beads/ml. Use 10 µl per assay data point (equivalent to 1250 beads/well).

Protocol: Counting the Number of LiquiChip Beads in a Suspension

Using the LiquiChip Workstation

Refer to the LiquiChip Workstation User Manual for details about operating the LiquiChip Workstation and IS Software.

Materials and reagents to be supplied by the user

- PBS TBN
- 96-well microplate with round-bottom wells

For composition of buffers, see Appendix, page 51.

Procedure

1. **In the IS Software select the "Acq. Detail" tab and create a new advanced batch with the following settings:**

"General" tab

Sample size: 50 µl

Timeout: 80 s

"Bead Set" tab

Select the bead IDs of the bead sets that are present in the bead suspension

Select "Total Beads" and enter a value of 10,000

"Plate Layout" tab

Assign the "Acquire Data" command to 2 wells on the microplate

2. **Agitate the bead suspension for at least 30 min in the dark. Vortex the bead suspension for 30 s at full speed.**

   Agitation should be performed using an end-over-end shaker, a vortex mixer with a shaker adapter (e.g., Roth, cat. no. 509.1 and 510.1) at lowest speed, or a plate shaker at 900 rpm.

3. **Pipet 90 µl PBS-TBN into 2 wells of the microplate.**

   Be sure that these wells correspond to the wells selected in step 1.

4. **Pipet 10 µl of the bead suspension into the wells containing PBS-TBN.**
5. **Shake the microplate on a plate shaker for 10 s at 900 rpm.**
6. **Load the microplate into the LiquiChip Microplate Handler.**
7. **Click the "Start" button in the "Acq. Detail" toolbar.**

8. Select "Count" in the "Statistic" drop-down list in the "Acq. Detail" tab to see the number of beads counted.

The LiquiChip Reader will count beads throughout the period of 80 s because the advanced batch is set up to count a very high number of beads.

9. Calculate the bead concentration as follows.

The contents of each well are:

Total sample volume:	100 µl/well
Aspirated volume:	50 µl
Dead volume:	20 µl
Actual sample volume:	30 µl

The number of beads per ml in the original stock suspension

= Mean number of beads counted x (1000/30) x 10 (dilution factor)

= Mean number of beads counted x 333

The mean number of beads counted should be at least 500.

Ideally, the concentration of beads should be 125,000 beads per ml. We recommend diluting the bead suspension to this concentration before use in LiquiChip assays (use 10 µl of this suspension per assay).

Using a cell-counting chamber or Coulter counter

Procedure

Follow the instructions supplied with your chamber/counter. Usually 10 µl bead suspension is pipetted into the chamber and the beads are counted within four 16-squares. The mean is then multiplied by 10^4 (i.e., the chamber factor) and, if necessary, the bead dilution factor to obtain the number of beads per ml.

Protocol: Titration of Specific Antibodies or Epitope Screening

By mixing precoupled beads, this type of assay can be carried out in a multiplex format. Multiplexing allows the simultaneous analysis of multiple epitope recognition sites or determines the cross-reactivity of one antibody to several antigens. Beads can be prepared using the bead-coupling protocols on pages 30, 31, 33, or 35.

For composition of buffers, see Appendix, page 51.

Equipment and reagents to be supplied by user

- LiquiChip beads precoupled with a protein against which the antibodies under investigation (primary antibodies) are directed
- Fluorescently labeled secondary antibodies directed against the primary antibody
- PBS/0.1% BSA buffer
- Round-bottom 96-well microplate

Procedure

1. **Vortex the bead suspension for 10 s.**
2. **Pipet into each assay well:***

   40 μl PBS/0.1% BSA buffer

   10 μl of the diluted protein-coupled LiquiChip Bead suspension (equivalent to 0.5 μl bead stock suspension)

   10 μl antibody sample or standard of known concentration
3. **Mix for 10 s at full speed on a microplate shaker.**
4. **Incubate for 90 min in the dark.**
5. **Add 200 ng labeled secondary antibody in a volume of 10 μl.**
6. **Mix for 10 s on a microplate shaker.**
7. **Incubate for 90 min in the dark.**
8. **Mix for 10 s on a microplate shaker.**
9. **Click the "Eject" button in the Integrated System Software.**
10. **Insert the assay plate.**
11. **Click the "Retract" button in the Integrated System Software.**
12. **Assay your plate on the LiquiChip Reader using your assay-specific template.**

* Volumes given apply to round-bottom microplates. See Table 1, page 27 for sample volume requirements using other types of microplate.

Protocol: Direct Quantification of 6xHis-tagged Proteins in Solution

This is a quick method for the quantification of 6xHis-tagged proteins using a protein-specific, fluorescently labeled primary antibody.

If you do not have a protein-specific labeled antibody, use the protocol on page 43.

In order to quantify a protein, a standard curve, created using the protein to be assayed, is required. We recommend generating a standard curve ranging from 0.001–1 μg/ml. If higher assay sensitivity is required, use the RCAT protocol on page 47. This assay type is not suitable for multiplexing, because Ni-NTA-Beads or Penta·His Beads will bind every 6xHis-tagged protein.

Equipment and reagents to be supplied by user

- LiquiChip Ni-NTA or Penta·His Beads (0.5 μl of stock suspension is required per assay point)
- Fluorescently labeled primary antibodies directed against the 6xHis-tagged protein under investigation

  Note: If using LiquiChip Ni-NTA or Penta·His Beads DO NOT USE Anti·His Antibodies for secondary detection. The proteins' 6xHis tags will be bound to Ni-NTA and therefore not accessible to Anti·His Antibodies.

  If using LiquiChip Penta·His Beads DO NOT USE anti-mouse antibodies for secondary detection. The antibody will bind to the mouse monoclonal Penta·His Antibodies on the surface of the LiquiChip Beads.
- PBS/0.1% BSA buffer
- Round-bottom 96-well microplate

For composition of buffers, see Appendix, page 51.

Procedure

1. **Prepare standard curve samples by serial 1:3 dilution of a 1 μg/ml solution to give samples containing 1000, 333, 111, 37, 12, 4.7, 1.3, and 0.46 ng/ml.**
2. **Vortex the LiquiChip bead stock suspension for 30 s at full speed.**
3. **Pipet an aliquot of LiquiChip bead stock suspension sufficient for the number of assay points into a microcentrifuge tube. Dilute the LiquiChip bead suspension 1/10 using PBS/0.1% BSA buffer.**

4. **Pipet into each well that will be used for an assay data point:***

   40 µl PBS/0.1% BSA buffer

   10 µl diluted Ni-NTA or Penta·His Bead suspension (equivalent to 0.5 µl bead stock suspension)

   10 µl unknown or standard curve sample
5. **Mix for 10 s on a microplate shaker at full speed.**
6. **Incubate for 90 min in the dark.**
7. **Add 50 ng of a protein-specific, Alexa Fluor 532 or R-PE-conjugated antibody per well, in a volume of 10 µl.**
8. **Mix for 10 s on a microplate shaker**
9. **Incubate for 90 min in the dark.**
10. **Mix the assay plate for 10 s on a microplate shaker.**
11. **Click the "Eject" button in the Integrated System Software.**
12. **Insert the assay plate.**
13. **Click the "Retract" button in the Integrated System Software.**
14. **Assay your plate on the LiquiChip Reader using your assay-specific template.**

* Volumes given apply to round-bottom microplates. See Table 1, page 27 for sample volume requirements using other types of microplate.

Protocol: Indirect Quantification of Proteins in Solution

This protocol requires a protein-specific primary antibody and a fluorescently labeled secondary antibody, or a biotin-labeled protein-specific primary antibody and Streptavidin–R-PE.

The advantage of this protocol over the protocol on page 41 is that the protein specific antibody does not need to be fluorescently labeled. In addition, due to the signal amplification delivered by the secondary antibody/Streptavidin R-PE system, this assay protocol yields higher sensitivity.

In order to quantify a protein, a standard curve, created using the protein to be assayed, is required. We recommend generating a standard curve ranging from 0.01–50 ng/ml.

For composition of buffers, see Appendix, page 51.

Equipment and reagents to be supplied by user

- LiquiChip Ni-NTA, Penta·His, or antibody-coated Activated or Carboxy Bead stock solution (0.5 μl of stock suspension is required per assay point)
- Primary antibodies directed against the protein under investigation

  If using LiquiChip Ni-NTA or Penta·His Beads DO NOT USE Anti·His Antibodies for detection. The proteins' 6xHis tags will be bound to Ni-NTA or Penta·His antibodies and therefore not accessible to Anti·His Antibodies.
- Secondary antibodies conjugated to a fluorescent dye or biotin

  If you are using Penta·His Beads DO NOT use anti-mouse antibodies. The antibody will bind to the mouse monoclonal Penta·His Antibodies on the surface of the LiquiChip Beads.
- Streptavidin-R-PE (if biotin conjugated secondary antibody is used)
- PBS/0.1% BSA buffer
- Round-bottom 96-well microplate

Procedure

1. **Prepare samples for the standard curve.**
2. **Vortex the LiquiChip bead stock suspension for 30 s at full speed.**
3. **Pipet an aliquot of LiquiChip bead stock suspension sufficient for the number of assay points into a microcentrifuge tube. Dilute the LiquiChip bead suspension 1/20 using PBS/0.1% BSA buffer.**

4. **Pipet into each well that will be used for an assay data point:***

   30 µl PBS/0.1% BSA buffer

   10 µl diluted LiquiChip Bead suspension (equivalent to 0.5 µl bead stock suspension)

   10 µl sample or standard curve solution

5. **Mix for 10 s on a microplate shaker at full speed.**
6. **Incubate for 90 min in the dark.**
7. **Add 10 ng of protein-specific primary antibody, in a volume of 10 µl per well.**
8. **Mix for 10 s on a microplate shaker.**
9. **Incubate for 90 min in the dark.**
10. **Add 200 ng of secondary antibody labeled with a fluorophore or biotin in a volume of 10 µl, or 100 ng Streptavidin–R-PE in a volume of 10 µl, if you have used a biotinylated primary antibody.**

    IMPORTANT: If using Penta·His Beads DO NOT use an anti-mouse antibody. The antibody will bind to the mouse monoclonal Penta·His Antibodies on the surface of the LiquiChip beads.

11. **Incubate for 90 min in the dark.**
12. **Mix the assay plate for 10 s on a microplate shaker.**
13. **Click the “Eject” button in the Integrated System Software.**
14. **Insert the assay plate.**
15. **Click the “Retract” button in the Integrated System Software.**
16. **Assay your plate on the LiquiChip Reader using your assay-specific template.**

* Volumes given apply to round-bottom microplates. See Table 3, page 31 for sample volume requirements using other types of microplate.

Protocol: Quantification of 6xHis-tagged Proteins in Solution Using a Competitive Assay

Using this protocol, the relative concentration of a 6xHis-tagged protein is determined by a competition assay using a fluorescently labeled 6xHis-tagged protein. If your protein to be assayed is available in a purified form, a standard curve can be created to give an absolute value of protein concentration. This assay can be used for quick quantification of 6xHis-tagged proteins in a cleared lysate.

For composition of buffers, see Appendix, page 51.

Equipment and reagents to be supplied by user

- LiquiChip Ni-NTA or Penta·His Bead stock solution (0.5 µl of stock suspension is required per assay point)
- Round-bottom 96-well microplate
- Alexa Fluor 532-labeled 6xHis-tagged protein, stock solution (100 µg/ml)
- PBS/0.1% BSA buffer
- (For standard curve only) 6xHis-tagged protein, stock solution (1 mg/ml)

Procedure

1. **To create a standard curve, prepare 1 ml of a 1/100 dilution of the unlabeled 6xHis-tagged protein solution (i.e., pipet 10 µl of stock solution into a microcentrifuge tube and add 990 µl PBS/0.1% BSA buffer).**

   The final concentration of this dilution is 10 µg/ml.
2. **From this solution, prepare further dilutions containing 5, 2.5, 1.0, 0.5, 0.25, 0.125, and 0.0625 µg/ml.**
3. **Prepare a 1/200 dilution of the Alexa Fluor 532-labeled 6xHis-tagged protein, stock solution (i.e., pipet 5 µl of stock solution into a microcentrifuge tube and add 995 µl PBS/0.1% BSA buffer).**
4. **Vortex the LiquiChip Ni-NTA Beads stock solution for 30 s.**
5. **Prepare a 1/20 dilution of the LiquiChip Ni-NTA Beads stock solution.**

   For each well, 10 µl of this dilution will be used. Prepare 10% more than you require for the assay to allow for pipetting errors.
6. **Vortex the diluted bead suspension and store in the dark.**

7. **Add to each assay well:***

   40 μl PBS/0.1% BSA

   10 μl Alexa Fluor 532-labeled 6xHis-tagged protein (0.5 μg/ml)

   10 μl 6xHis-tagged protein standard curve sample or unknown sample
8. **Shake for 10 s on a microplate shaker at full speed.**
9. **Vortex the LiquiChip Ni-NTA bead suspension and add 10 μl to each well.**
10. **Shake briefly on a microplate shaker.**
11. **Incubate for 1 h in the dark.**
12. **Shake briefly on a microplate shaker.**
13. **Click the "Eject" button in the Integrated System Software.**
14. **Insert the assay plate.**
15. **Click the "Retract" button in the Integrated System Software.**
16. **Assay your plate on the LiquiChip Reader using your assay-specific template.**

* Volumes given apply to round-bottom microplates. See Table 1, page 27 for sample volume requirements using other types of microplate.

Protocol: RCAT Signal Amplification of xMAP Assay Signals

This protocol provides a general-use procedure for xMAP assay signal amplification using the LiquiChip RCAT Booster Kit (Biotin) (Cat. No. 922203). Recommendations for buffer components can be found in the *LiquiChip Applications Handbook*, which can be downloaded in convenient PDF form from www.qiagen.com . The RCAT procedure can also be integrated into existing protocols, where it replaces the steps in which Streptavidin-R-PE is added to the reaction.

Buffer compositions are provided in the Appendix on page 51.

Materials and reagents to be supplied by the user

- PBS-T buffer
- Streptavidin-Phycoerythrin, 1 mg/ml (e.g., QIAGEN Streptavidin-R-PE, cat. no. 922721)
- Vortex mixer
- Repeating pipet (e.g., Eppendorf® Repeater® pipet)
- Incubator set to 37°C
- Orbital shaker
- Vacuum manifold for 96-well plate (e.g., QIAvac 96, QIAGEN cat. no. 19504)
- Aluminum foil
- Tape sheet for sealing unused wells (e.g., Tape Pads, QIAGEN cat. no. 19570)

Important points before starting

- The volumes given are for 96 assay points/microplate wells. If performing fewer than 96 assay points, reduce the volumes of buffers and reagents accordingly.
- When using a repeating pipet, always wipe the tip after filling and pipet the first two dispensed aliquots back into the solution reservoir. When dispensing liquid, hold the pipet vertically a short distance over the center of the well to avoid splashing. To avoid the build-up of drops at the pipet tip, liquid dispensing strokes should be made using the same pressure and without unnecessary pauses.
- Allow orbital shaker to come to a complete stop after each incubation before removing filter plate.

- Always wear gloves when handling reagents.
- For wash steps apply a vacuum of between 20 and 100 mbar below atmospheric pressure.
- To prevent the filter plate leaking, keep the filter plate membrane clear of the ground by using a LiquiChip Filter Plate Adapter (cat. no. 9238368) or use the filter plate lid as a support during pipetting steps.

Things to do during the protocol

- During incubation of the samples with biotinylated primary detection antibody, remove RCAT Biotin Antibody and RCAT Reaction Mix from storage and bring to room temperature shortly before use. During step 3, remove RCAT DNA polymerase from storage and thaw on ice.

Procedure

1. **Perform xMAP assay procedure up to and including the step where biotinylated primary detection antibodies are added.**

   During incubation of the samples with biotinylated primary detection antibodies, remove RCAT Biotin Antibody and RCAT Reaction Mix from storage and bring to room temperature shortly before use.
2. **Wash beads twice using LiquiChip Buffer 3 (supplied in the LiquiChip RCAT Cell Signaling Kit) or PBS-T Buffer.**
3. **Pipet 50 µl RCAT Biotin Antibody into each assay well. Cover plate, and incubate samples in the dark for 30 min at room temperature (15–25°C) on a microplate shaker set to rotate at 900 rpm.**
4. **Wash beads twice using LiquiChip Buffer 4.**
5. **Pipet 10 µl RCAT DNA Polymerase directly into the tube containing 5.25 ml RCAT Reaction Mix and vortex gently.**

   If performing fewer than 96 assay points adjust volumes accordingly and aliquot unused RCAT DNA Polymerase to prevent multiple freeze–thaw cycles. Unused Reaction Mix containing RCAT DNA Polymerase should be discarded.
6. **Pipet 50 µl of the RCAT Reaction Mix/DNA Polymerase prepared in step 5 into each assay well. Cover plate, and incubate samples in the dark for 30 s at room temperature (15–25°C) on a microplate shaker set to rotate at 900 rpm.**
7. **Incubate the filter plate for 1 h in the dark at 37°C without shaking.**
8. **Wash beads twice using LiquiChip Buffer 3 (supplied in the LiquiChip RCAT Cell Signaling Kit) or PBS-T Buffer.**

9. **Add Streptavidin–Phycoerythrin according to your usual detection step.**

   We recommend adding at least 1 µg Streptavidin-Phycoerythrin per well.

10. **Cover plate and incubate for 30 min in the dark at room temperature (15–25°C) on a microplate shaker set to rotate at 900 rpm.**
11. **Wash beads twice using LiquiChip Buffer 3 (supplied in the LiquiChip RCAT Cell Signaling Kit) or PBS-T Buffer.**
12. **Add 100 µl PBS-T or your usual stop solution to each well.**
13. **Proceed with assay measurement according to your usual protocol.**

Troubleshooting Guide

This troubleshooting guide may be helpful in solving any problems that may arise. The scientists in QIAGEN Technical Services are always happy to answer any questions you may have about either the information and protocols in this handbook or molecular biology applications (see back page for contact information).

	Comments and suggestions
Measurement is slow or "Sample Empty" error message appears	
a) Sampling probe is not properly adjusted	Adjusting the sampling probe will greatly improve the speed and efficiency of your sample acquisition. The steps for sampling probe adjustment are listed in the *LiquiChip Workstation User Manual*. The sampling probe should be adjusted for each type of 96-well plate or filter plate used.
b) Sampling probe clogged	Remove sampling probe (see *LiquiChip User Manual*) and sonicate. Replace sampling probe.
c) Air has entered the system	Always fill your wells with a volume at least 25 µl greater than the volume sampled by the LiquiChip sampling probe (i.e., if you select 50 µl as your aspirated volume, ensure the volume of sample in the well is at least 75 µl). This additional volume prevents air from entering the system, which can increase sample acquisition time. Perform a 70% alcohol flush and then wash the system with 70% alcohol. Wash the system with LiquiChip System Fluid and prime.
d) Low pressure in the system	Tighten the connections to the LiquiChip System Fluid and waste containers.
e) Beads have settled to the bottom of the well	Try placing your 96-well plate on a microplate shaker for 30 seconds to resuspend your sample.

	Comments and suggestions
f) Bead master-mix not homogenous	Before use, mix the bead master-mix thoroughly by vortexing or by sonication. Check the homogeneity and number of beads in the master mix by pipetting 10 μl of master mix and 100 μl LiquiChip Wash Buffer into a well and measuring using your assay template.
g) Doublet Discriminator Gate incorrectly set	Check that the Doublet Discriminator Gate is set to 7500 "Low Limit" and 15,000 "High Limit".
Lower than expected readings for standard	
a) Samples containing high levels of biotin were not processed using a wash protocol	Repeat assay using wash protocol.
b) LiquiChip Reader not calibrated	Perform calibration using LiquiChip Calibration and Control beads.
Bead pattern is diffuse and outside white oval target	
Build-up of precipitates in system	Drain the system and perform a "Backflush".
High background signal	
a) Incorrect buffer used for dilution of samples or standards	When processing serum and plasma samples, ensure that LiquiChip Human Serum Dilution Buffer is used to dilute samples and LiquiChip Human Serum Standard Diluent is used to dilute standards.
b) Reagents have passed expiration date	Ensure reagents have not passed expiration date. Repeat assays using new or unexpired components.
c) Standards carried over to blank wells	Pipet carefully and use a new pipet tip for each sample.
d) Plate incubated with Streptavidin–PE for too long	Incubate assays for the recommended time. If the plate will not be read immediately after incubation, cover it with aluminum foil and store at 4°C in the dark.

	Comments and suggestions
Poor recovery of samples	
a) Reagents have passed expiration date	Ensure reagents have not passed expiration date. Repeat assays using new or unexpired components.
b) Incorrect amounts of assay components were used	Check all calculations (e.g., dilutions) and pipet calibration.
c) Samples and standards loaded into wells at different times	Samples and standards must be loaded into wells at the same time.
d) Microplate shaker set to rotate at the wrong speed	Ensure that the microplate shaker rotates at the correct speed. Shaking too fast may lead to splashing and cross-contamination.
e) Poor pipetting technique	Always pipet carefully, especially when using multi-channel pipets. Ensure that the pipet is calibrated. Change pipet tips after each sample.
High coefficient of variation (CV) of duplicate samples or standards	
a) Standards and samples were not kept on ice during assay setup	Because some analytes are temperature-sensitive, ensure standards and samples are kept on ice during pipetting steps.
b) Bottom of filter plate was not blotted after vacuum procedures	Always blot the bottom of filter plates on absorbent towels after vacuum procedures to prevent cross-contamination.
c) Tape used to seal plates was reused	Use a new sheet of plate-sealing tape for each assay to prevent cross-contamination.
d) Wells run dry	Ensure bottom of filter plate is blotted after vacuum procedures. Ensure that membranes do not contact other surfaces during processing.
e) Poor pipetting technique	Always pipet carefully, especially when using multi-channel pipets. Ensure that the pipet is calibrated. Change pipet tips after each sample.

	Comments and suggestions
f) Samples contaminated through splashing of wash buffer during wash steps	Ensure that wash buffer does not splash between wells. During vacuum procedures, ensure that all liquid has passed through the membrane before releasing vacuum. Ensure that the speed of the microplate shaker is not above 600 rpm.
Low signals or sensitivity	
a) Standards and samples were not kept on ice during assay setup	Ensure standards and samples are kept on ice during pipetting steps.
b) Incorrect buffer used for dilution of standards	When processing serum and plasma samples, ensure that LiquiChip Human Serum Standard Diluent is used to dilute standards.
c) Incorrect dilution of antibody or Streptavidin–PE	Check calculations and ensure that correct dilutions were used.
d) Reagents have passed expiration date	Ensure reagents have not passed expiration date. Repeat assays using new or unexpired components.
e) Assay plate was not shaken thoroughly before and during assay incubations	Always follow the recommended shaking speeds and incubation times given in the protocols.
Low bead counts	
a) Beads exposed to light and become bleached	Always store LiquiChip beads in the dark. Ensure plate is covered with foil during incubations and minimize exposure to light.
b) Assay plate was not shaken thoroughly before and during assay incubations	Always follow the recommended shaking speeds and incubation times given in the protocols.
c) System is clogged	Refer to the Troubleshooting Guide in the *LiquiChip User Manual*.
d) Wrong bead dilution used	Check calculations and ensure that correct dilutions were used.

	Comments and suggestions
e) Beads form clumps in LiquiChip bead vials	Vortex vials at medium speed for 15–20 seconds before removing aliquots.
f) Vacuum pressure too high	Check vacuum pressure and use the recommended pressure and vacuum apparatus.
g) Vacuum applied for too long	Do not apply vacuum for more than 10 seconds after all liquid has passed through the membrane.

Filter plate tears/leaks or samples do not pass evenly through membrane

a) Filter membrane tears	Reduce vacuum pressure.
b) Filter membrane leaks	Ensure that filter plate is blotted onto absorbent paper after vacuum procedures to remove hanging drops. Keep the filter plate membrane clear of the ground by using a LiquiChip Filter Plate Adapter (cat. no. 9238368) or the LiquiChip Filter Plate lid as a support during pipetting steps.
c) Some samples do not pass through membrane under vacuum	Precipitates in samples can lead to clogging of filter-plate membranes. Clear all samples by centrifugation before processing, and use only the supernatant.
d) Filter plate incubated overnight at an angle	Ensure that the assay plate is flat during incubations.
e) Filter plate not level	Ensure that the filter plate is level on the vacuum manifold. Press the filter plate gently into place to ensure a uniform vacuum pressure across the entire plate.
f) Rubber seal is worn/needs replacing	Inspect the seal and replace if required.
g) Air bubbles in well	Using a clean pipet tip, carefully aspirate the contents of the well and re-pipet into the well, ensuring that no bubbles are introduced.

Appendix: Buffers Used in LiquiChip Procedures

PBS (1 liter)

10 mM NaH_2PO_4	1.4 g $NaH_2PO_4 \cdot H_2O$ (MW 137.99 g/mol)
150 mM NaCl	8.77 g NaCl (MW 58.44 g/mol)

Dissolve NaCl and $NaH_2PO_4 \cdot H_2O$ in 900 ml distilled water and adjust pH to 7.4 using NaOH. Adjust volume to 1 liter. Before use, filter using a 0.45 µm filter.

PBS/0.1% BSA (1 liter)

10 mM NaH_2PO_4	1.4 g $NaH_2PO_4 \cdot H_2O$ (MW 137.99 g/mol)
150 mM NaCl	8.77 g NaCl (MW 58.44 g/mol)
0.1% (w/v) BSA	1 g BSA
0.05% (w/v) sodium azide	0.5 g sodium azide

Dissolve NaCl and $NaH_2PO_4 \cdot H_2O$ in 900 ml distilled water and adjust pH to 7.4 using NaOH. Add and dissolve BSA and sodium azide, and adjust volume to 1 liter. Before use, filter using a 0.45 µm filter.

PBS/1% BSA (1 liter)

10 mM NaH_2PO_4	1.4 g $NaH_2PO_4 \cdot H_2O$ (MW 137.99 g/mol)
150 mM NaCl	8.77 g NaCl (MW 58.44 g/mol)
1% (w/v) BSA	10 g BSA
0.05% (w/v) sodium azide	0.5 g sodium azide

Dissolve NaCl and $NaH_2PO_4 \cdot H_2O$ in 900 ml distilled water and adjust pH to 7.4 using NaOH. Add and dissolve BSA and sodium azide, and adjust volume to 1 liter. Before use, filter using a 0.45 µm filter.

PBS-TBN (1 liter)

10 mM NaH_2PO_4	1.4 g $NaH_2PO_4 \cdot H_2O$ (MW 137.99 g/mol)
150 mM NaCl	8.77 g NaCl (MW 58.44 g/mol)
0.1% (w/v) BSA	1 g BSA
0.02% (v/v) Tween 20	200 µl Tween 20

Dissolve NaCl and $NaH_2PO_4 \cdot H_2O$ in 900 ml distilled water and adjust pH to 7.4 using NaOH. Add and dissolve BSA and Tween 20, and adjust volume to 1 liter. Before use, filter using a 0.45 µm filter.

PBS-T Buffer (1 liter)

10 mM Na_2HPO_4	1.4 g $NaH_2PO_4 \cdot H_2O$ (MW 137.99 g/mol)
1.7 mM KH_2PO_4	0.23 g KH_2PO_4 (MW 136.1)
154 mM NaCl	8.77 g NaCl (MW 58.44 g/mol)
0.05% ProClin® 300	500 µl ProClin 300
0.05% Tween 20	500 µl Tween 20

pH should be 7.5. Store buffer at 4°C.

Coupling buffers (1 liter)

50 mM MES, pH 6.5	11.67 g MES (2-[N-morpholino]ethanesulfonic acid, potassium salt, MW 233.2 g/mol)

Dissolve in 900 ml distilled water and adjust pH to 6.5 using HCl. Adjust volume to 1 liter. Before use, filter using a 0.45 µm filter.

50 mM HEPES, pH 7.4	11.9 g HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, MW 238.31 g/mol)

Dissolve in 900 ml distilled water and adjust to pH 7.4 using KOH. Adjust volume to 1 liter. Before use, filter using a 0.45 µm filter.

20 mM MOPS, pH 7.2	4.18 g MOPS (3-(N-morpholino)-propanesulfonic acid, MW 209.3 g/mol)

Dissolve in 900 ml distilled water and adjust pH to 7.2 using KOH and volume to 1 liter. Before use, filter using a 0.45 µm filter.

Activation Buffer (1 liter)

100 mM NaH_2PO_4	13.8 g $NaH_2PO_4 \cdot H_2O$ (MW 137.99 g/mol)

Dissolve in 900 ml distilled water and adjust pH to 6.3 using NaOH. Adjust volume to 1 liter. Before use, filter using a 0.45 µm filter.

Coupling biomolecules to LiquiChip Carboxy Beads

Capture molecules without a 6xHis tag or biotin moeity can be immobilized via their free amino groups on LiquiChip Carboxy Beads using N-(3-dimethylaminopropyl)N'-ethylcarbodiimide/N-hydroxysulfosuccinimide (EDC/S-NHS) chemistry. Carboxyl groups on the surface of the beads are activated using EDC and S-NHS to form NHS esters. Amine groups on the biomolecule displace NHS, leading to covalent attachment via peptide bonds. Figure 12 shows the chemical reactions that take place at the surface of the beads.

IMPORTANT: The biomolecule to be immobilized must be free of reagents containing amine groups, e.g., Tris buffers or azide. Any such reagent should be removed by dialysis before immobilization.

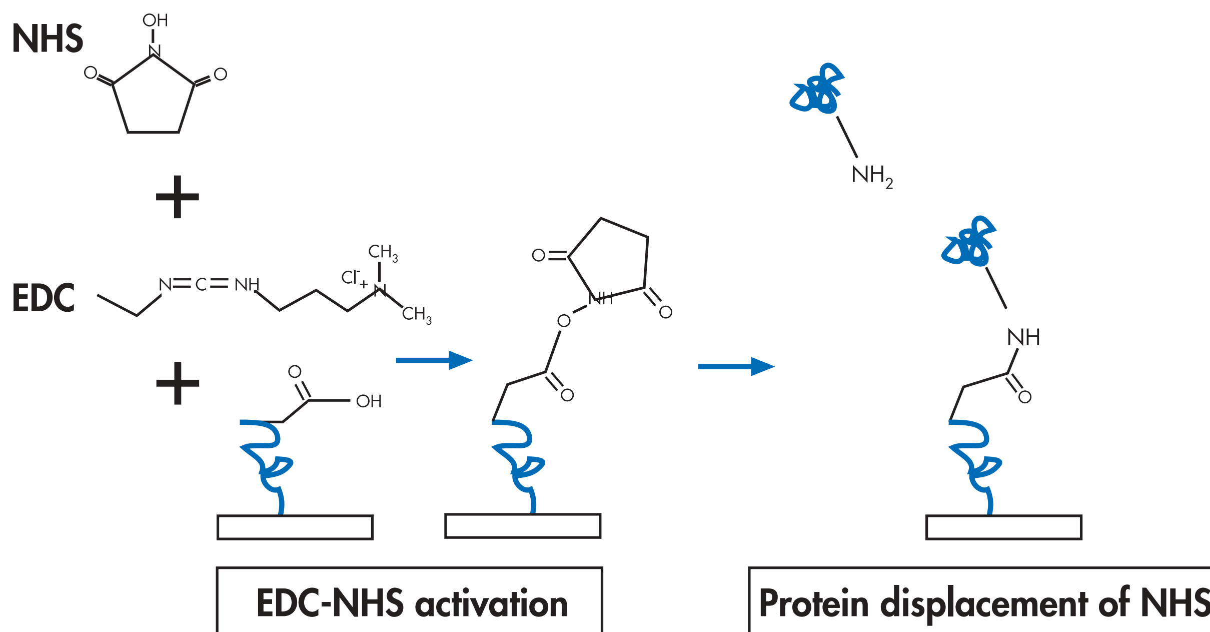

Figure 12 Reaction scheme for EDC-NHS–mediated amine coupling to LiquiChip Carboxy Beads.

Notes

Notes

Ordering Information

Product	Contents	Cat. no.
LiquiChip 200 Workstation	LiquiChip Reader; LiquiChip Microplate Handler and LiquiChip Fluid Module; computer; operating system, TFT-monitor; installation; 3 year warranty on parts and labor; yearly Preventive Maintenance visit	Inquire
LiquiChip 200 Workstation (Analyzer)	LiquiChip Reader, LiquiChip Microplate Handler and Fluid Module, computer, operating system, TFT monitor, installation, 3 year warranty on parts and labor, yearly preventative maintenance visit, LiquiChip Analyzer software	Inquire
Software Pack LiquiChip Policy Manager	Software upgrade LiquiChip Policy Manager (including hardware lock)	9016743
Upgrade LiquiChip Analyzer (Research) +	For xMAP systems operated by Luminex/LiquiChip IS 2.x.: Includes computer, TFT monitor, LiquiChip Analyzer software, upgrade LiquiChip IS 2.3 software, on-site installation and training	9239395
Upgrade LiquiChip Analyzer (Research)	Software upgrade LiquiChip Analyzer Software, on-site installation and training	9239396
LiquiChip Ni-NTA Beads (500)	Ni-NTA Beads code 52; 0.25 ml for 500 homogeneous assay points	922500
LiquiChip Ni-NTA Bead Set A (4 x 500)	Ni-NTA Beads code 50, 52, 54, and 58; 0.25 ml each for 4 x 500 homogeneous assay points	922501
LiquiChip Ni-NTA Bead Set B (4 x 500)	Ni-NTA Beads code 32, 34, 36, and 38; 0.25 ml each for 4 x 500 homogeneous assay points	922503
LiquiChip Ni-NTA Bead Set C (4 x 500)	Ni-NTA Beads code 24, 26, 28, and 30; 0.25 ml each for 4 x 500 homogeneous assay points	922505

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LiquiChip Carboxy Bead Set B (4 x 500)	Carboxy Beads code 31, 33, 35, and 37; 1 ml each for 4 x 500 homogeneous assay points	922404
LiquiChip RCAT Booster Kit (Biotin)	For 96 assay points: RCAT Reaction Mix, RCAT Biotin Antibody, RCAT DNA Polymerase, Buffer	922203
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LiquiChip Filter Plate Adapter	Adapter for LiquiChip Filter Plates	9238368

Product	Contents	Cat. no.
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Penta·His Biotin Conjugate	125 µl Penta·His Biotin Conjugate, 200 µg/ml	34440
Streptavidin-R-PE	250 µg Streptavidin-R-phycoerythrin Conjugate, 1 mg/ml	922721
LiquiChip System Fluid	LiquiChip System Fluid (10x concentrate, 5 liters), for 50 liters working solution	922902
QIAvac 96	Vacuum manifold for processing QIAGEN 96-well plates: includes QIAvac 96 Top Plate, Base, Waste Tray, Plate Holder, Rack of Collection Microtubes (1.2 ml)	19504
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