September 2005

LiquiChip® RCAT™ Handbook

LiquiChip RCAT Cell Signaling Core Kit

LiquiChip Cell Signaling Detection Kits

LiquiChip RCAT Booster Kit (Biotin)

For highly sensitive detection of analytes in bead-based xMAP™ assays

WWW.QIAGEN.COM

Contents

Kit Contents

LiquiChip RCAT Cell Signaling Core Kit For 96 assay points	**Cat. no. 922202**
LiquiChip Buffer 1	2 x 80 ml
LiquiChip Buffer 2	50 ml
LiquiChip Buffer 3	120 ml
LiquiChip RCAT Assay Stop Solution	20 ml
LiquiChip Filter Microplate (1.2 µm)	1
Streptavidin–Phycoerythrin	110 µl of a 1 mg/ml solution
Mixing Vials, 9 ml	3
LiquiChip Solubility Enhancer	500 µl
LiquiChip RCAT Booster Kit (Biotin) For 96 assay points	**Cat. no. 922203**
RCAT Biotin Antibody	5.5 ml
RCAT Reaction Mix	5.25 ml
RCAT DNA Polymerase	20 µl
LiquiChip Buffer 4	40 ml

Storage

The LiquiChip RCAT Cell Signaling Core Kit is shipped with cool packs. Upon arrival, all components should be stored at 2–8°C. Streptavidin–Phycoerythrin should be stored in the dark at 2–8°C. **Do not freeze!**

The LiquiChip RCAT Booster Kit (Biotin) is shipped on dry ice. All components in this box must be stored at –20°C or –70°C upon arrival.

LiquiChip Cell Signaling Detection Kits should be stored at 4°C. LiquiChip beads should be stored in the dark at 4°C. **Do not freeze!**

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).

Quality Control

In accordance with QIAGEN's ISO-certified Total Quality Management System, each lot of LiquiChip Kits is 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.

The following risk and safety phrases apply to the components of the LiquiChip RCAT Cell Signaling Core Kit:

LiquiChip RCAT Assay Stop Solution

Contains formaldehyde. Irritant. Risk and safety phrases*: R43, S24-36/37/39-46

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

* R43: May cause sensitization by skin contact. S24: Avoid contact with skin; S36/37/39: Wear suitable protective clothing, gloves and eye/face protection. S46: If swallowed, seek medical advice immediately and show container or label.

Introduction

The QIAGEN® LiquiChip System is a flexible system for suspension arrays that uses bead-based xMAP technology. 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. 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.

Using this simple assay set up, a typical LiquiChip assay takes place in a procedure in which beads, reagents, and reporter molecules 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.

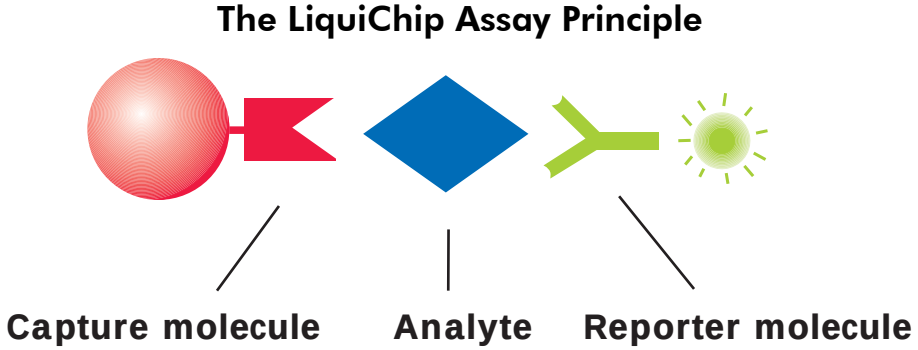


Figure 1 The LiquiChip assay principle. Analytes free in solution interact with bead-bound capture molecules and are detected using reporter molecules specific for the analyte.

LiquiChip RCAT cell signaling assays

The LiquiChip system is based on patented xMAP™ technology, which enables the creation of a "liquid array" for the simultaneous analysis of multiple cell-signaling molecules in a fast bead-based assay.

The modular kit concept of LiquiChip RCAT Cell Signaling Core Kit and LiquiChip Cell Signaling Detection Kits allows researchers to flexibly combine assays according to their individual research needs. It should however be noted that the physical similarity of some analytes (e.g., Total and cleaved PARP) means that they cannot be distinguished by the capture antibodies on LiquiChip Detection Beads. The product sheet supplied with each detection kit will contain information on any such multiplexing restrictions.

The LiquiChip RCAT Cell Signaling Core Kit contains buffers and reagents that provide optimal reaction and detection conditions for LiquiChip cell signaling assays. LiquiChip Detection Kits contain target-specific capture beads and detection antibodies. By using a LiquiChip RCAT Core Kit in conjunction with a set of LiquiChip Detection Kits, a customized "liquid array" can be created.

LiquiChip cell signaling assays are sandwich assays in which analyte-specific antibodies immobilized on LiquiChip beads interact with and bind to cell-signaling molecules in the sample. Bound analytes are then detected using a second specific biotinylated antibody. In conventional xMAP assays, analytes are quantified at this point by addition of Streptavidin R-PE. The LiquiChip RCAT Cell Signaling Core Kit makes use of RCAT, a novel amplification method based on DNA amplification to increase assay sensitivity.

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 2A). The RCAT reaction is initiated by adding a reaction mix containing dNTPs and RCAT DNA Polymerase, which extends the double-stranded annealed sequence (Figure 2B). 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

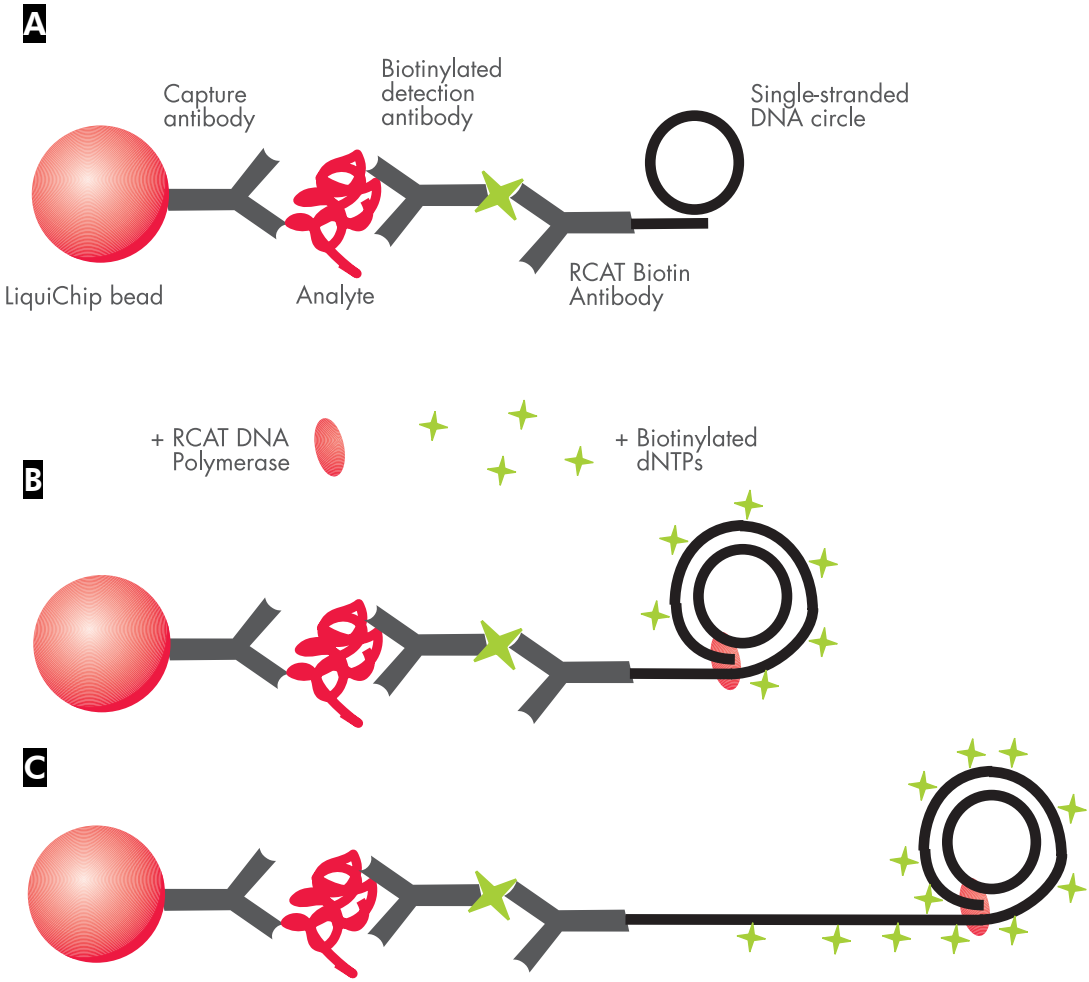


Figure 2 RCAT LiquiChip assay signal amplification technology. **A** The anti-biotin DNA conjugate binds to biotin and the DNA circle anneals to the DNA strand. **B** RCAT DNA Polymerase is added and extends the DNA strand attached to the anti-biotin antibody, using the single-stranded circle as a template. 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.

xMAP Assay Signal Amplification Using RCAT

The LiquiChip RCAT Booster Kit (Biotin) is included in the LiquiChip RCAT Cell Signaling Core Kit, where it is used in conjunction with LiquiChip Cell Signaling Detection Kits to increase assay sensitivity. However, the LiquiChip RCAT Booster Kit (Biotin) can also be used in conjunction with other LiquiChip assays, xMAP assay kits from other suppliers, and with in-house developed assays. The only absolute requirement is that the assay makes use of a biotinylated detection antibody.

To design novel assays, or if existing protocols perform poorly, we recommend basing assays on the wash procedure described on page 19. The recommendations below should also be considered for optimal results. The section "RCAT Signal Amplification of xMAP Assay Signals" on page 23 provides a generic protocol that can be adapted to a wide range of assays.

Assay buffers and microplate

For optimal results we strongly recommend using the assay plate and buffers supplied in the LiquiChip Cell Signaling Core Kit.

Pre-treatment of cell lysates

For some assays using cell lysates, sample pre-treatment using LiquiChip Solubility Enhancer is required. Pre-treatment is especially important when assaying poorly soluble proteins or proteins that form part of a multimeric complex. The pre-treatment procedure is described in detail on page 11.

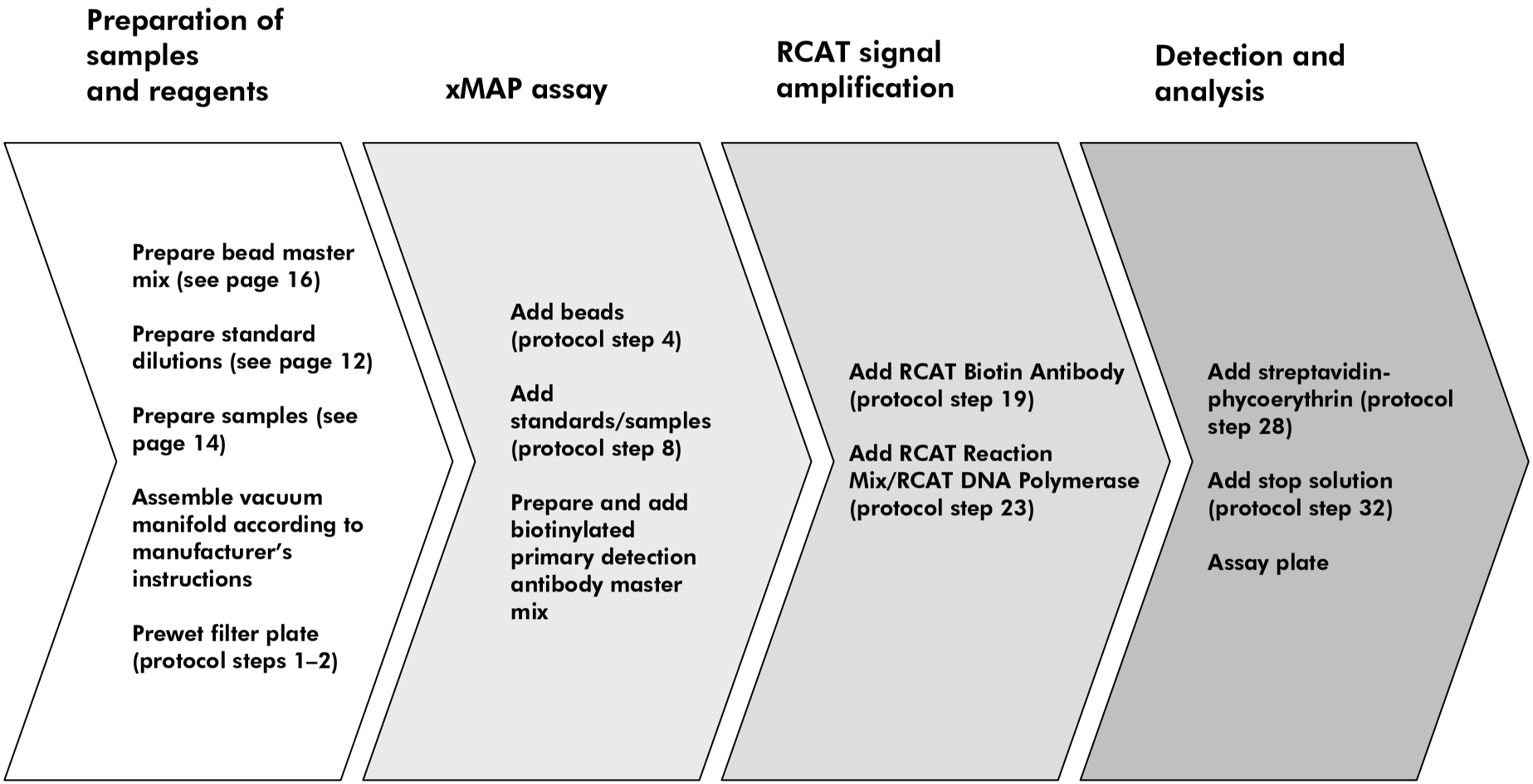
Preparation of serum or plasma samples

If assaying serum or plasma samples, endogenous molecules — such as soluble receptors — can affect assay signals and lead to false-positive or false-negative results. For optimal results, serum, plasma, and the relevant standards should be diluted using buffers included in the LiquiChip Mouse Serum Dilution Kit (cat. no. 922301) and the LiquiChip Human Serum Dilution Kit (cat. no. 922300). See the product sheet included with these kits for more details.

Incubation time of the biotinylated detection antibody

After addition of the biotinylated detection antibody, the signal intensity of many assays can be improved by incubating the reaction overnight at 4°C. However, in some cases overnight incubation may also increase the background signal.

RCAT Signal Amplification Protocol Overview



Standard curves

We recommend that standard curves contain at least 8 points, including a blank. Statistically, at least 8 points are needed to perform the optimal curve-fitting for these assays. Standard-curve samples should be run in duplicate and span the range of analyte concentrations in the test samples. In the assay, 50 µl of standard solution is required for each well.

The additional sensitivity provided by the RCAT system will extend the dynamic range of the assay to lower concentrations. It is recommended that two further dilutions are made, to cover this low-concentration region.

For multiplex assays, prepare a concentrated standard mix and use 1:3 serial dilutions. Standards should be diluted either in LiquiChip Buffer 2, sample buffer, or LiquiChip Serum Standard Diluent (see page 11).

Blanks are prepared by adding 50 µl of LiquiChip Buffer 2, assay buffer, or LiquiChip Serum Standard Diluent to the relevant well(s). Additional negative controls for lysate samples can be prepared by substituting identically processed lysis buffer for the cell lysate sample(s).

Suggested standard concentrations and analyte suppliers are provided in the product sheet supplied with each LiquiChip Cell Signaling Detection Kit.

Protocol: Preparation of Cell Lysates

This protocol can be used for processing approximately 10^7 mammalian (e.g., Jurkat, HeLa, or A 431) cells. Cells should be carefully washed before lysis to remove traces of growth medium components (e.g., >30 ng/ml biotin) that may interfere with the assay.

Buffer compositions are provided in the Appendix on page 30. Alternatively, cells can be lysed using the Qproteome™ Mammalian Protein Prep Kit (QIAGEN cat. no. 37901).

Equipment and reagents to be supplied by the user

- Cell–culture medium or PBS buffer
- Cell lysis buffer
- Centrifuge at 4°C

Procedure

1. **Culture cells according to your protocol.**
2. **Wash cells by centrifuging at 800 x *g* for 5 min and resuspending in 15 ml PBS buffer or cell-culture medium without fetal calf serum. Repeat.**
3. **Centrifuge cells at 800 x *g* for 5 min and discard the supernatant.**
4. **Resuspend cells in 2 ml cell lysis buffer and incubate for 30 min on ice.**

   If processing fewer than 10^7 cells, reduce the volume of cell lysis buffer accordingly.
5. **Centrifuge lysate at 14,000 x *g* for 20 min at 4°C.**
6. **Pipet supernatant into a fresh tube.**
7. **If using cell lysates and your sample does not require pre-treatment, adjust the total protein concentration of the cell lysate to 1 mg/ml.**

   Prepared lysates can be stored at –80°C for up to one week.

Protocol: Pre-Treatment of Cell Lysates

Depending on the target protein, it may be necessary to perform a sample pre-treatment procedure when using cell lysate samples (see page 11). When using LiquiChip Cell Signaling Detection Kits, the product sheet supplied with the kit will indicate whether this procedure is necessary for your particular analyte. If performing a multiplex assay, pretreatment must be performed if it is required for any of the analytes being assayed. This protocol generates sufficient pre-treated lysate for 10 assay wells. If you plan to use more wells, adjust the volumes accordingly.

Buffer compositions are provided in the Appendix on page 30.

Equipment and reagents to be supplied by the user

- Cell lysis buffer
- LiquiChip Buffer 1 and LiquiChip Solubility Enhancer (supplied in the LiquiChip RCAT Cell Signaling Core Kit, cat. no. 922202)

Procedure

1a. If using cell lysates and your sample does not require pre-treatment, adjust the total protein concentration of the cell lysate to 1 mg/ml, and pipet 495 µl LiquiChip Buffer 1 or wash buffer and 5 µl cell lysate into a clean microcentrifuge tube and vortex briefly and store on ice.

Lysates can be stored at –80°C for up to one week.

1b. If using cell lysates and your sample requires pre-treatment, pipet 2.5 µl LiquiChip Buffer 1, 2.5 µl LiquiChip Solubility Enhancer, and 5 µl cell lysate into a clean microcentrifuge tube. Briefly vortex and incubate for 30 min on ice.

It is important that the order of pipetting given in the protocol step is strictly followed.

2b. After incubation, pipet 490 µl LiquiChip Buffer 1 into the tube containing the cell lysate.

Treated lysates can be stored at –80°C for up to one week.

Protocol: Preparing a LiquiChip Bead Master Mix

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.

Procedure

1. **Centrifuge each tube of beads that you plan to use in your assay at low speed (100 x *g*) for 30 s, and vortex gently for 5 s to resuspend the beads.**
2. **Prepare a LiquiChip bead master-mix by pipetting 100 µl of each bead suspension that you plan to use into a 9 ml mixing vial (supplied with the LiquiChip RCAT Cell Signaling Kit), and making up the total volume to 5500 µl using LiquiChip Buffer 1 (see Table 1).**

Table 1. Pipetting Scheme for Producing a LiquiChip Bead Master Mix

Degree of multiplexing	LiquiChip Buffer 1 (µl)	Each bead type (µl)	Total volume (µl)
Singleplex	5400	100	5500
Duplex	5300	100	5500
Triplex	5200	100	5500
4-plex	5100	100	5500
5-plex	5000	100	5500
6-plex	4900	100	5500
7-plex	4800	100	5500
8-plex	4700	100	5500
9-plex	4600	100	5500
10-plex	4500	100	5500
n-plex	5500 – 100*n*	100	5500

3. **Vortex the bead master-mix and store in the dark at 4°C until use.**

   The bead master-mix is stable for up to 4 weeks if stored in the dark at 4°C. Light should be excluded by wrapping the tube in aluminum foil.

Protocol: Preparing a LiquiChip Antibody Master Mix

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.

Procedure

1. **Gently vortex each tube of antibody that you plan to use in your assay for 5 s, and centrifuge at low speed (100 x *g*) for 30 s.**
2. **Prepare an antibody master-mix by pipetting 100 µl of each antibody that you plan to use into a 9 ml mixing vial (supplied with the LiquiChip RCAT Cell Signaling Kit), and making up the total volume to 5500 µl using LiquiChip Buffer 2 (see Table 2).**

Table 2. Pipetting Scheme for Producing an Antibody Master Mix

Degree of multiplexing	LiquiChip Buffer 2 (µl)	Each antibody type (µl)	Total volume (µl)
Singleplex	5400	100	5500
Duplex	5300	100	5500
Triplex	5200	100	5500
4-plex	5100	100	5500
5-plex	5000	100	5500
6-plex	4900	100	5500
7-plex	4800	100	5500
8-plex	4700	100	5500
9-plex	4600	100	5500
10-plex	4500	100	5500
n-plex	5500 – 100*n*	100	5500

3. **Vortex the antibody master mix and store on ice until use.**
   The antibody master mix is stable for up to 4 weeks if stored at 4°C.

Protocol: Washing LiquiChip Beads During Assay Procedures

The protocols in this handbook contain several wash steps. At each wash step follow the protocol below. Ensure that the correct buffer is used for the wash step, and that it performed the recommended number of times.

Materials and reagents to be supplied by the user

- Repeating pipet (e.g., Eppendorf® Repeater® pipet)
- Orbital shaker
- Vacuum manifold for 96-well plate (e.g., QIAvac 96, QIAGEN cat. no. 19504)

Procedure

1. **Place the 96-well filter plate on the vacuum manifold and apply vacuum to the bottom of the filter plate to remove liquid. As soon as the wells are dry (approximately 5 s), release vacuum. Blot the bottom of the filter plate on a paper towel.**
2. **Wash beads by adding 200 µl buffer to each well. Cover plate and resuspend the LiquiChip beads by shaking on a microplate shaker for 30 s in the dark. Place the plate on the vacuum manifold and remove the wash buffer by applying vacuum.**

Protocol: LiquiChip RCAT Cell Signaling Assays

This protocol is suitable for signal amplification in LiquiChip xMAP assays that use LiquiChip Cell Signaling Detection Kits.

Materials and reagents to be supplied by the user

- Vortex mixer
- Repeating pipet (e.g., Eppendorf® Repeater® pipet)
- LiquiChip Cell Signaling Detection Kit(s)
- 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)
- Analyte standard solutions (see detection kit product sheet for recommended suppliers and concentrations)

Important points before starting

- Depending on the analyte, it may be necessary to perform a sample pre-treatment procedure when using cell lysate samples (see page 11). When using LiquiChip Cell Signaling Detection Kits, the product sheet supplied with the kit will indicate whether this procedure is necessary for your particular analyte. If performing a multiplex assay, pretreatment must be performed if it is required for any of the analytes being assayed.
- 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.
- After each incubation, allow orbital shaker to come to a complete stop before removing filter plate.
- Always wear gloves when handling LiquiChip 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 the filter plate lid as a support during pipetting steps.

Things to do before starting

- Before use, LiquiChip Buffers 1, 2, and 3 should be brought to room temperature (15–25°C) by placing them at room temperature overnight. These buffers can be stored at room temperature for up to one week with no loss in performance.
- Before use LiquiChip Buffer 2 must be supplemented with LiquiChip Solubility Enhancer at a concentration of 1 in 200. For 96 assay points, pipet 200 µl LiquiChip Solubility Enhancer into a 40 ml aliquot of LiquiChip Buffer 2, and mix by gently vortexing.
- Prepare serial dilutions of the required standard or standard mixture (see detection kit product sheet for recommended suppliers and concentrations).
- Assemble the vacuum manifold according to the manufacturer's instructions.
- If using cell lysates and your sample does not require pre-treatment (see page 11), adjust the total protein concentration of the cell lysate to 1 mg/ml, and pipet 495 µl LiquiChip Buffer 1 and 5 µl cell lysate into a clean microcentrifuge tube and vortex briefly and store on ice.

Procedure

1. **Pipet 200 µl LiquiChip Buffer 1 into each assay well and incubate the filter microplate for 10 min on the bench.**

   This step pre-wets the filter in each assay well. Wells that will not be used should either be filled with buffer or sealed with a tape sheet before liquid is removed by vacuum.

2. **Place the 96-well filter plate on the vacuum manifold and apply vacuum to the bottom of the filter plate to remove liquid. As soon as the wells are dry (approximately 5 s), release vacuum. Blot the bottom of the filter plate on a paper towel.**

   If the plate is not blotted, drops of liquid on the underside of the plate may lead to reagents in the wells being drawn through the membrane by capillary action. To avoid damage to the filter plate during vacuum processing, ensure that the applied vacuum does not exceed –100 mbar.

3. **Pipet 150 µl LiquiChip Buffer 1 into each assay well.**

4. **Vortex the LiquiChip Bead master-mix at high speed for 15 s. Add 50 µl of bead master-mix suspension to each well.**
5. **Cover the filter plate and shake for 30 s in the dark at room temperature (15–25°C) on a microplate shaker set to rotate at 600 rpm.**
6. **Place the 96-well filter plate on the vacuum manifold and apply vacuum to the bottom of the filter plate to remove liquid. As soon as the wells are dry (approximately 5 s), release vacuum. Blot the bottom of the filter plate on a paper towel.**
7. **Carefully pipet 50 µl of LiquiChip Buffer 2 into each well.**

   Ensure that LiquiChip Buffer 2 has been supplemented with LiquiChip Solubility Enhancer (see "Things to do before starting").
8. **Carefully pipet 50 µl of standard, sample, or control into the relevant well of the microplate, according to the batch setup.**

   Use a new pipet tip for each well. For blank wells use LiquiChip Buffer 2 and for negative controls for cell lysate samples use cell lysis buffer.
9. **Incubate samples in the dark for 2 h at room temperature (15–25°C) on a microplate shaker set to rotate at 600 rpm.**
10. **During the incubation prepare the detection antibody master mix (see page 17).**
11. **Wash beads twice using LiquiChip Buffer 1.**
12. **Pipet 50 µl LiquiChip Buffer 2 into each well.**

    Ensure that LiquiChip Buffer 2 has been supplemented with LiquiChip Solubility Enhancer (see "Things to do before starting").
13. **Add 50 µl of antibody master mix to each well.**
14. **Cover plate and incubate for 2 h in the dark at room temperature (15–25°C) on a microplate shaker set to rotate at 600 rpm.**

    For some analytes, signal-to-noise ratio may be increased by overnight incubation at 4°C (see detection kit product sheet).

    During incubation remove RCAT Biotin Antibody and RCAT Reaction Mix from storage and bring to room temperature shortly before use.
15. **Wash beads twice using LiquiChip Buffer 3.**
16. **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.**
17. **Wash beads twice using LiquiChip Buffer 4.**
18. **Pipet 10 µl RCAT DNA Polymerase directly into the tube containing RCAT Reaction Mix and vortex gently.**

19. **Pipet 50 µl of the RCAT Reaction Mix/DNA Polymerase prepared in step 18 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.**
20. **Incubate the filter plate for 1 h in the dark at 37°C without shaking.**
21. **Wash beads twice using LiquiChip Buffer 3.**
22. **Dilute Streptavidin–Phycoerythrin by adding 100 µl Streptavidin–Phycoerythrin (1 mg/ml) to 5400 µl LiquiChip Buffer 3 in the mixing vial provided.**

    Diluted Streptavidin-Phycoerythrin is not stable, and should be stored at 2–8°C in the dark. If performing fewer than 96 assays, prepare only as much diluted Streptavidin-Phycoerythrin as required.
23. **Carefully pipet 50 µl diluted Streptavidin-Phycoerythrin 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.**
24. **Incubate the filter plate for 30 min in the dark at 37°C without shaking.**
25. **During the incubation, prepare the LiquiChip Reader.**

    Bead codes for the respective detection kits can be found on the product sheet supplied with the kit and on bead vial tube labels.
26. **Wash beads once using LiquiChip Buffer 3.**
27. **Add 150 µl LiquiChip RCAT Assay Stop Solution to each well.**

    Avoid air bubbles during pipetting.
28. **Cover plate and incubate for 5 min in the dark at room temperature (15–25°C) on a microplate shaker set to rotate at 900 rpm.**
29. **Assay the plate on the LiquiChip Reader using your assay template.**

    Ensure that the sampling probe is adjusted for a filter microplate. Before assaying the plate remove any bubbles from wells using a syringe needle. To avoid sample carryover, use a clean needle for each well.

    Choose the following settings in the IS software template:

Sample Vol (µl):	100
Sample Timeout (sec):	100
Bead Count	100 per region

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). 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 (protocol steps 16–33) 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 30.

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.
- After each incubation, allow orbital shaker to come to a complete stop 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 the filter plate lid as a support during pipetting steps.

Things to do before starting

- 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.

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), your usual assay buffer, 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 RCAT Reaction Mix and vortex gently.**
6. **Pipet 50 µl of the RCAT Reaction Mix/DNA Polymerase prepared in step 22 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), your usual assay buffer, 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. **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: Buffer Compositions

Cell lysis buffer

20 mM Tris·Cl, pH 7.4

100 mM NaCl

1% (v/v) NP-40

1 mM DTT

5 mM β-glycerophosphate

1 mM sodium orthovanadate

1 mM EDTA

5 mM Pefabloc® SC (Roche, cat. no. 1429876)

1 U/ml Benzonase® (Novagen, cat. no. 70746-3)

5 mM NaF

1 µg/ml Aprotinin*

1 µg/ml Pepstatin A*

1 µg/ml Leupeptin*

Add inhibitors immediately before use and do not store buffer.

* Aprotinin, Pepstatin A, and Leupeptin are available in a convenient EDTA-free mini tablet (Boehringer, cat. no. 1 836 153).

PBS-T Buffer

10 mM Na_2HPO_4

1.7 mM KH_2PO_4

154 mM NaCl

0.05% ProClin 300

0.05% Tween® 20

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

Summary of Assay Steps

Prewet filter plate

↓

Add 150 µl LiquiChip Buffer 1 + 50 µl bead master mix, apply Vacuum

↓

Add 50 µl LiquiChip Buffer 2+ 50 µl sample — Incubate 2 h @ RT

↓

Prepare primary antibody master mix

↓

Wash beads twice using LiquiChip Buffer 1

↓

Add 50 µl LiquiChip Buffer 2 + 50 µl antibody mix — Incubate 2 h @ RT

↓

Wash beads twice using LiquiChip Buffer 3

↓

Add 50 µl RCAT Biotin Antibody — Incubate 30 min @ RT

↓

Wash beads twice using LiquiChip Buffer 4

↓

Add 50 µl RCAT Reaction Mix/DNA Polymerase — Incubate 1 h @ 37°C

↓

Wash beads twice using LiquiChip Buffer 3

↓

Add 50 µl Streptavidin–Phycoerythrin — Incubate 30 min @ 37°C

↓

Wash beads once using LiquiChip Buffer 3

↓

Add 150 µl LiquiChip RCAT Assay Stop Solution — Incubate 5 min @ RT

↓

Assay plate

All incubations should be carried out in the dark.

Ordering Information

Product	Contents	Cat. no.
LiquiChip RCAT Cell Signaling Core Kit	For 96 assay points: Buffers, Reagents, RCAT DNA Polymerase, Streptavidin–Phycoerythrin, Filter Plate, Mixing Vials	922202
LiquiChip Total PARP Detection Kit	For 96 assay points: LiquiChip Human Total PARP Beads (bead code 18), Human Total PARP Antibody	923030
LiquiChip Cleaved PARP Detection Kit	For 96 assay points: LiquiChip Human Cleaved PARP Beads (bead code 28), Human Cleaved PARP Antibody	923031
LiquiChip Active Caspase 3 Detection Kit	For 96 assay points: LiquiChip Human Caspase 3 Beads (bead code 27), Human Caspase 3 Antibody	923032
LiquiChip Active Caspase 8 Detection Kit	For 96 assay points: LiquiChip Human Caspase 8 Beads (bead code 25), Human Caspase 8 Antibody	923033
LiquiChip Total IKBa Detection Kit	For 96 assay points: LiquiChip Human Total IKBa Beads (bead code 20), Human Total IKBa Antibody	923034
LiquiChip Phospho-IKBa Detection Kit	For 96 assay points: LiquiChip Human Phospho-IKBa Beads (bead code 29), Human Phospho-IKBa Antibody	923035
LiquiChip Cytochrome c Detection Kit	For 96 assay points: LiquiChip Human Cytochrome c Beads (bead code 31), Human Cytochrome c Antibody	923036
LiquiChip Total NFkB Detection Kit	For 96 assay points: LiquiChip Human NFkB Beads (bead code 33), Human NFkB Antibody	923037
LiquiChip Total GSK3 Detection Kit	For 96 assay points: LiquiChip Human GSK3Beads (bead code 35), Human GSK3 Antibody	923038

Product	Contents	Cat. no.
LiquiChip RCAT Booster Kit (Biotin)	For 96 assay points: RCAT Reaction Mix, RCAT Biotin Antibody, RCAT DNA Polymerase, Buffer	922203
Related products		
LiquiChip Human Serum Dilution Kit	LiquiChip Human Serum Standard Diluent (2 x.6 ml), LiquiChip Human Serum Dilution Buffer (2 x 10 ml)	922300
LiquiChip Mouse Serum Dilution Kit	LiquiChip Mouse Serum Standard Diluent (2 x 6 ml), LiquiChip Mouse Serum Dilution Buffer (2 x 10 ml)	922301
LiquiChip Filter Microplates (10)	96-well filter microplates plus lids, 10 per case	922920
LiquiChip Filter Plate Adapter	Adapter for LiquiChip Filter Plates	9238368
LiquiChip Control Beads	Classification Control Beads (CON1, 5 ml); Reporter Control Beads (CON2, 5 ml)	922912
LiquiChip Calibration Beads	Classification Calibration Beads (CAL1, 5 ml); Reporter Calibration Beads (CAL2, 5 ml)	922911
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
Vacuum Regulator	For use with QIAvac manifolds	19530
Software Pack LiquiChip Policy Manager	Software upgrade LiquiChip Policy Manager (including hardware lock)	9016743

Product	Contents	Cat. no.
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
Qproteome Mammalian Protein Prep Kit	For approximately 100 protein preparations from cultured mammalian cells: Buffer, Reagents, Protease Inhibitor Solution, Benzonase	37901

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