January 2004

LiquiChip™ Broad-Range Tyr Kinase Handbook

For detecting and measuring the activity of a wide range of tyrosine kinases

Contents

Kit Contents

LiquiChip Broad-Range Tyr Kinase Kit	
Catalog no.	**922083**
Number of assay points	**100**
LiquiChip Broad-Range Tyr Beads	2 x 0.1 ml substrate-coated LiquiChip bead suspension; 10x stock suspension bead code 29
LiquiChip Phospho-Tyr Antibody	1 x 1 ml
Anti-Mouse Biotin Conjugate	1 x 1 ml
LiquiChip BRK Streptavidin–PE	40 µl of a 50x concentrate
LiquiChip Streptavidin Dilution Buffer	2 x 1 ml
LiquiChip BRK Assay Stop Solution	2 x 1 ml
96-well test plate	1
Handbook	1

Storage

LiquiChip Broad-Range Tyr Beads (stock suspension and dilutions) and LiquiChip BRK Streptavidin-PE should be stored in the dark at 4°C.
Do not freeze!

LiquiChip BRK Assay Stop Solution and LiquiChip Streptavidin Dilution Buffer should be stored at 4°C.

LiquiChip Phospho-Tyr Antibody and Anti-Mouse Biotin Conjugate should be stored at –20°C upon arrival.

After delivery, if stored under these conditions, all reagents can be stored for up to 3 months after delivery.

Note: Once diluted, LiquiChip BRK Streptavidin–PE solution is only stable for up to 1 week at 4°C. If fewer than 96 wells of an assay plate are used, dilute only the required amount of Streptavidin–PE.

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 the LiquiChip Broad-Range Tyr Kinase Kit 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).

Product Use Limitations

The LiquiChip Broad-Range Tyr Kinase Kit is developed, designed, and sold for research purposes only. It is 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.

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 Broad-Range Tyr Kinase Kit:

LiquiChip Phospho-Tyr Antibody

Sensitizer. Risk and safety phrases*: R42/43. S24-26-36/37

LiquiChip BRK 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

* R42/43: May cause sensitization by inhalation and skin contact. R43: May cause sensitization by skin contact. S24: Avoid contact with skin; S26: In case of contact with eyes, rinse immediately with plenty of water and seek medical advice; S36/37: Wear suitable protective clothing and gloves. 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

Phosphorylation of tyrosine residues in proteins plays an important role in controlling most aspects of cellular processes, including growth, movement, cell death, and response to signaling factors. Because they regulate and coordinate entire cellular signaling pathways, tyrosine kinases are an attractive area of research in the drug discovery process. LiquiChip assays measure the interaction of immobilized, bead-bound assay components with reaction partners in solution. The immunoassay-based LiquiChip Broad-Range Tyr Kinase assay enables sensitive analysis of tyrosine kinase activities in a fast, homogeneous, bead-based assay.

Principle

LiquiChip Broad-Range Tyr Beads are coated with a tyrosine-rich protein that is a tyrosine kinase substrate. The substrate beads are incubated with a sample containing one or more active tyrosine kinases and a non-radioactive Mg^{2+}-ATP cocktail, which serves as a phosphate donor. Phosphorylation sites are detected by primary antibodies that specifically recognize phosphorylated tyrosine residues. Primary antibodies are detected using a biotinylated secondary anti-mouse antibody. Secondary antibodies are detected using streptavidin conjugated to highly fluorescent phycoerythrin (Figure 1).

Sensitive and Specific Detection of Tyrosine Kinase Activity

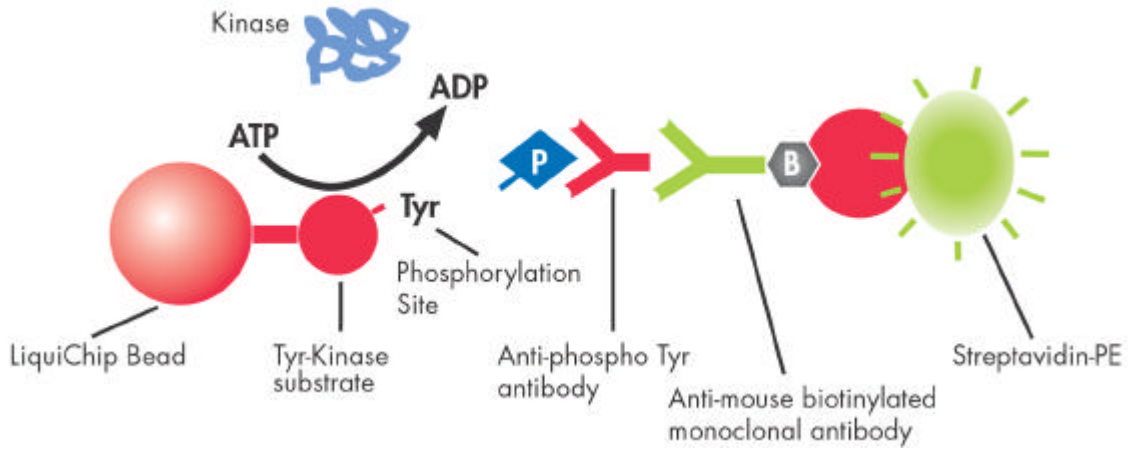

Figure 1 Detection of tyrosine kinase activity using the LiquiChip Broad-Range Tyr Kinase Kit. Phosphorylation sites in the substrate are detected using antibodies that specifically recognize phosphorylated tyrosine residues.

The LiquiChip Broad-Range Tyr Kinase Kit can be used for detection and quantification of a wide range of tyrosine kinase activities, including cytosolic tyrosine kinases (e.g., members of the Src family, Zap70, Csk, and Abl-1) and receptor tyrosine kinases (e.g., EGFR, InsR, and PDGFR alpha). Examples of kinase activities that have been analyzed using the LiquiChip Broad-Range Tyr Kinase Kit are shown in Table 1. It should be pointed out that this is not a comprehensive list of all activities that can be detected.

Table 1. Phosphorylation Activity of Exemplary Purified Kinases Towards the Substrate Immobilized on LiquiChip Broad-Range Tyr Beads

Family	Kinase	Level of phosphorylation
Src Kinases	Src (p60-src, c-Src)	+++
	Fyn	+++
	Lyn	+++
	Lck (Lsk)	+++
	Yes	+++
	Hck (B-cell/myeloid kinase, Bmk)	+++
Other cytosolic kinases	Csk (c-Src kinase)	+++
	Fes/Fps	++
	Zap70 (70 kD Zeta-associated protein)	++
	Abl-1	++
Receptor kinases	EGFR	++
	InsR (insulin receptor)	+++
	PDGFR alpha	++
	FGFR3	+++

Important Notes

Tyrosine kinase assay buffers

As a general buffer for the tyrosine phosphorylation reaction, we recommend using Assay Buffer 1 (see Appendix, page 26, for buffer compositions). The glycerophosphate and orthovanadate components of this buffer serve as phosphatase inhibitors. If cell extracts are assayed, it is recommended that they be prepared using Extraction Buffer, which contains protease inhibitors (see page 17 and Appendix).

When using a buffer different from those listed in the Appendix, make sure that buffer components are compatible with LiquiChip Broad-Range Tyr Beads and the LiquiChip system (see Table 2).

Table 2. Maximum Permissible Concentrations of Assay Buffer Reagents Used with LiquiChip Broad-Range Tyr Beads*

Reagent	Max. conc.	Reagent	Max. conc.
Tween®-20	0.4% (v/v)	EGTA	1.5 mM
Triton® X 100	0.4% (v/v)	Glycerol	5% (v/v)
SDS	0.002% (w/v)	PEG	0.2% (w/v)
EDTA	10 mM	DMSO	2% (v/v)
Ammonium sulfate	0.02% (w/v)	β-Mercaptoethanol	20 mM
Imidazole	50 mM	DTT	5 mM
NaCl	300 mM	$MnCl_2$	10 mM
$MgCl_2$	10 mM	$CaCl_2$	0.2 mM

* Concentrations given are the maximum final concentrations in the kinase reaction.

Controls

Suitable negative controls should always be carried out during each experiment to ensure that the Median Fluorescent Intensity (MFI) obtained is accurate. A microplate well containing all assay components except the test sample serves as a negative control and background value. The sensitivity of the LiquiChip Broad-Range Tyr Kinase Kit is around 0.01 to 0.1 U/ml reaction volume depending on the kinase being measured (see Table 1).

Assays with cell-culture supernatants or lysates

Cell extracts or lysates can be measured directly in a no-wash assay using the LiquiChip Broad-Range Tyr Kinase Kit. A protocol for preparation of cell lysates can be found on page 17 of this handbook. However, if there are any substances (e.g., >30 ng/ml biotin) in your samples that might interfere with assay components, use the wash protocol on page 21. The wash assay must be used if you are using lysates prepared from mouse cells that produce antibodies, because the secondary detection antibody will react with all mouse antibodies present in the sample.

Standards

Assay standards can be generated using a series of dilutions of a purified kinase, or adding a series of dilutions of an inhibitor to a constant amount of kinase. It is recommended that standard curves contain at least five points in addition to a background sample. Standard curve samples should be run in duplicate and span the range of kinase concentrations in the test samples. Further information concerning standard curves and assay analysis can be found in the *LiquiChip Workstation User Manual*.

Stopping the kinase reaction in time-course experiments

The kinase reaction can be efficiently stopped at a distinct time-point by adding the ATPase apyrase (Sigma, cat. no. A 6160). Addition of 10 mU apyrase per well will immediately exhaust all ATP in the reaction, effectively stopping phosphorylation. Without the addition of apyrase, the kinase reaction will proceed during incubation with detection antibodies.

Combining LiquiChip Broad-Range Tyr and Ser/Thr Kinase Kits

Although it is theoretically possible to combine LiquiChip Broad-Range Tyr and LiquiChip Ser/Thr Beads (the bead codes used for each type of bead are different), we do not recommend this, as cross-reactions between antibodies and substrates may lead to inconclusive results. In addition, some Ser/Thr kinases can phosphorylate the substrate immobilized on LiquiChip Tyr Beads, further complicating assay analysis.

LiquiChip Workstation Startup Procedure

1. **Turn on the instrument(s) and the computer. Start the IS instrument software.**

   The warm-up procedure starts immediately and takes around 30 min. During the warm-up procedure you can prepare the system.

2. **Make sure that the sample arm is adjusted to your microplate.**

   See the *LiquiChip Workstation User Manual* for a detailed description of sample-arm adjustment.

3. **Click the "Prime" icon 2–3 times to prime the system.**

   The pressure in the system increases until the operating pressure is reached.

4. **Perform at least one alcohol flush procedure to remove air bubbles. Fill the reservoir with 1.5 to 2.0 ml 70% (v/v) isopropanol or 70% (v/v) ethanol and click the "Alcohol Flush" button.**

5. **Empty the reservoir, fill it with System Fluid, and click the "Wash" button. Repeat the wash procedure 1–2 times.**

6. **For optimal performance, perform a system calibration using the LiquiChip Calibration Bead Kit. Start the calibration by clicking the "CAL1" or "CAL2" button, and follow the instructions given by the software.**

   A calibration must be carried out if the Doublet Discriminator temperature "DD Temp" has changed by more than 3°C since the last calibration. If a calibration is necessary, this is displayed on the "Run Batch" and "Diagnostics" tabs. If the "DD Temp" thermometer is green, system calibration is not required. If it is red, the system should be calibrated. A calibration should be carried out every two weeks, even if the DD Temp has not changed by more than 3°C. If assay validation is required, perform a control of your system using the LiquiChip Control Bead Kit. Start the control procedure by clicking the "CON1" or "CON2" button, and follow the instructions given by the software.

7. **After calibration or control, wash at least 4 times by clicking the "Wash" button. Ensure that the reservoir is filled with System Fluid.**

8. **Now you can start your assay. Import the LiquiChip Tyr template to create a batch and start to measure.**

   Protocols for the creation of templates for assays with and without standards can be found on pages 13 and 14. Alternatively, you can write your own template using the "Create Template" or "Edit Template" icons on the "Acq. Detail" tab in the IS Software.

Shutting Down the LiquiChip Reader

Two maintenance operations can be found under the category "Daily Shutdown" on the "Home" tab.

"Soak" operation

IMPORTANT: The "Soak" operation is used to clean the instrument tubing and should be performed a total of three times at the end of each working day.

The System Fluid contains high concentrations of salt. To prevent salt crystallization in the tubing, the "Soak" operation washes all tubing with distilled water. Wash the reservoir twice with distilled water, fill it with distilled water, and click the "Soak" button. Repeat twice.

"Sanitize" operation

If you have assayed samples containing biohazards or other dangerous substances it is advisable to sanitize your instrument after use. During this operation all tubing that comes into contact with samples and the sampling probe will be washed. To sanitize fill the reservoir with 10% hypochlorite and click the "Sanitize" button.

Software Setup for Tyrosine Kinase Assays with and without Standard Curves

For all tyrosine kinase assays we recommended downloading LiquiChip Tyr Templates, which can be found on the QIAGEN web site at www.qiagen.com/goto/LiquiChipResources . These templates automatically define values for sample volume, sample timeout, bead code, and the number of beads analyzed per bead set. Alternatively, choose one of your own templates from the template list to run batches.

Importing a LiquiChip Tyr Template for Tyrosine Kinase Assays without Standard Curves

1. **Download the LiquiChip Tyr Template from www.qiagen.com/goto/LiquiChipResources .**
2. **Open the Integrated System (IS) Software.**
3. **Click "File" and choose "Import Template".**
4. **Choose the LiquiChip Tyr Template and click "Open".**

   Use this template for creating a batch for tyrosine kinase assays without standards.

Creating a batch for tyrosine kinase assays without standards

5. **Click "New Batch" on the IS Software "Home" tab and choose the LiquiChip Tyr Template by double clicking on it.**

   The imported template will appear at the bottom of the template list. Alternatively, choose an appropriate template from the template list.

6. **Enter the batch name, a short assay description, and your name.**
7. **Enter the number of assay wells by changing "1" next to the "Acquire Patient" button and click "Apply". Enter sample identifications for each plate location.**

   If you wish to start the assay in a specific well, keep the left mouse button depressed and drag and drop the first well to your starting point on the plate. Choose "Save and Load" to start immediately or "Save", if you want to start the batch later. Saved batches can be found under "Open Batch" on the "Home" tab.

8. **Click "Finish" and proceed with your assay in the "Run Batch" tab by loading the 96-well plate and clicking "Start Plate".**

   Information concerning assay analysis can be found in the *LiquiChip Workstation User Manual*.

Creating a Template for Tyrosine Kinase Assays with Standard Curves

For assays that include wells containing standards, the user must create a product and edit the template that is downloaded from the QIAGEN web site.

Importing the template

1. **Download the LiquiChip Tyr Template from www.qiagen.com/goto/LiquiChipResources .**
2. **Open the Integrated System (IS) Software.**
3. **Click "File" and choose "Import Template".**
4. **Choose the LiquiChip Tyr Template and click "Open".**

Creating a product

5. **In the "Acq. Detail" tab, click on "Create New Product".**
6. **Click "New" and select "Standard". Enter product information (product number, name, and manufacturer name).**
7. **Enter "1" in "number of tests" and press "Enter". Enter "Tyr Substr" in the "Test Name" field and your test units (e.g., mU).**
8. **Enter the total number of standard samples excluding the background sample, press "Enter" and enter a description of your standards (e.g., STD 1 to STD 5) in the field "Reagent Name".**
9. **Click "Save" and "Close".**

Editing the template

10. **In the "Acq. Detail" tab, click on "Edit Template", choose the LiquiChip Tyr Template, and click the "Edit" button.**
11. **Enter a template name and choose the template type "Quantitative". Click "Next" and the "Apply Standard/Control" buttons.**
12. **In the "Reagent Product" window select your product at the bottom of the list and click "Open".**
13. **After the product window has closed, the template Wizard reappears. Click on "Bead ID" and select "29". Click on "Curve Fit" and select a curve formula suitable for your assay (e.g., Logistic 4p: y= a+b/(1+(x/c)^d)). Select "Fit of all standards". Click "Next".**

14. **Enter your template commands. With "Acquire Background" and "Acquire Standard" (e.g., STD 1 to STD 5) define the order of your background sample and standards. With "Acquire Test Specimen" you can define the number of your samples. For a repeated selection of the same command, use the "Repeat" function.**

    Each time you have a different number of samples you must redefine your sample number for each new batch.

15. **Click the "Save" and "Close" buttons.**

    Use this template for creating a batch for tyrosine kinase assays using standard curves.

    Further details concerning the generation and editing of templates can be found in the *LiquiChip Workstation User Manual*.

Creating a batch for tyrosine kinase assays with standards

1. **Click "New Batch" on the IS Software "Home" tab and choose your edited tyrosine kinase template by double clicking on it.**

   The template appears at the bottom of the template list.

2. **Add a lot number in the window that appears and click "OK".**

   You can use this lot number to assign an identifier to a particular experiment (date, operator, sample, etc.). Do not enter the lot number printed on the kit box.

3. **Enter your standard concentrations and the expiration date of your assay components.**

   To ensure trouble-free use of LiquiChip assay kits, the expiration date that appears in the "Exp. Date:" field in the "Update Lot Information" screen must be adjusted. After importing or creating a template and lot, this field must be set to a date **3 months after the delivery date** of the kit. Any default date that appears in this field after importing a template must be changed. The expiration date can be set by selecting a date from the calendar that appears in the drop-down menu to the right of the "Exp. Date:" field. Alternatively, the date can be entered directly into the field. The date is entered using the format

   mm/dd/yyyy

   An example is given below.

   Kit delivery date: **18 December 2003** = 12/18/2003

   Enter Exp. Date: **03/18/2004**

4. **Click "Save" and the Batch Setup window will appear.**
   If you wish to use the same template but use different standard concentrations, change the previously defined concentrations using the "New Lot" button in the Batch Setup window.
5. **Enter your batch name, a short assay description, and your name.**
6. **Enter the number of assay wells by changing "1" next to the "Acquire Patient" field and click "Apply". Enter sample identifications for each plate location. Empty wells can be flagged by using "Skip" in the drop-down menu.**
   If you wish to start your assay in a specific well, keep the left mouse button depressed and drag and drop the first well to your starting point on the plate. Choose "Save and Load" to start immediately or "Save", if you want to start the batch later. Saved batches can be found under "Open Batch" on the "Home" tab.
7. **Click "Finish" and proceed with your assay in the "Run Batch" tab by loading the 96-well plate and clicking "Start Plate".**
   Further information on assay analysis can be found in the *LiquiChip Workstation User Manual*.

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 26. When using a buffer different from those listed in the Appendix, make sure that the buffer components are compatible with LiquiChip Broad-Range Tyr Beads (see Table 2, page 9).

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 cell-culture medium without FCS. Repeat.**
3. **Centrifuge cells at 800 x *g* for 5 min and discard the supernatant.**
4. **Resuspend cells in 2 ml Extraction Buffer and incubate for 30 min on ice.**

   If processing fewer than 10^7 cells, reduce the volume of Extraction Buffer accordingly.
5. **Centrifuge lysate at 14,000 x *g* for 20 min at 4°C.**
6. **Pipet supernatant into a fresh tube and use for tyrosine kinase assay.**

   Note: It is possible to measure and compare crude lysates without centrifugation. However, if exact kinase quantification is required, the samples should be diluted 1:4.

Protocol: LiquiChip Broad-Range Tyr Kinase Assay

The following protocol is suitable for assaying active tyrosine kinases in cell extracts or as purified proteins. The volumes given are for 100 assay points/microplate wells. If performing fewer than 100 assay points, reduce the volumes of buffers and reagents accordingly.

Important Notes

- As a general buffer for the tyrosine phosphorylation reaction, we recommend using Assay Buffer 1. If using cleared cell lysates, we recommend using Extraction Buffer. Buffer compositions are provided in the Appendix on page 26.
- When using a buffer other than those listed in the Appendix, make sure that buffer components are compatible with LiquiChip beads (see Table 2, page 9).
- Use the supplied 96-well microplate for this assay. Make sure that the sample arm is adjusted to this microplate.
- If the cell culture medium, kinase sample, or buffer used contains components that may interfere with the kinase assay analysis procedure (e.g., biotin, fetal calf serum, or high concentrations of manganese), use the LiquiChip Broad-Range Tyr Kinase wash protocol on page 21.

Equipment and reagents to be supplied by the user:

- Assay Buffer 1, Extraction Buffer, or other buffer
- Mg^{2+}-ATP cocktail
- Repeating pipet (e.g., Eppendorf Repeater® pipet) with 0.5 ml tip
- Vortex mixer
- Microplate shaker at room temperature (e.g., Heidolph Titramax 100)
- Microplate shaker with temperature control (e.g., Eppendorf Thermomixer Comfort with microplate adapter and lid)
- Aluminum foil

Procedure

1. **Vortex the vials containing LiquiChip Broad-Range Tyr Beads for 30 s at medium speed. Agitate the vials for at least a further 15 min in the dark to completely resuspend the beads.**

   Agitate the beads using an end-over-end shaker or a vortex mixer with a shaker adapter (e.g., Roth, cat. nos. 509.1 and 510.1).

2. **Pipet 900 µl assay buffer into each LiquiChip bead vial to dilute the bead suspension, and vortex at medium speed for 30 s.**

   This yields a final concentration of 125 beads/µl.

3. **Pipet 20 µl assay buffer into each well of the supplied 96-well microplate.**

   If a kinase inhibitor is being added to the reaction, add 10 µl of the inhibitor dilution and 10 µl assay buffer.

4. **Resuspend the diluted bead suspension by vortexing for 1 min at medium speed and pipet 20 µl of the diluted bead suspension into each assay well (= 2500 beads per assay point).**

   Residual amounts of diluted bead suspension can be stored at 4°C in the dark for 3 months.

5. **Pipet 10 µl of your test sample or purified enzyme into each assay well.**

   For the negative control sample (background sample), add 10 µl Assay Buffer.

6. **Start the reaction by pipetting 20 µl of Mg^{2+}-ATP cocktail into each assay well.**

7. **Incubate the plate for 30–60 min at 30°C in the dark on a microplate shaker set to rotate at approximately 600 rpm.**

   To ensure constant, reproducible reaction conditions, we recommend using a thermostat-controlled microplate shaker that can be heated to 30°C. Incubations can be carried out at a lower temperature, but reaction times may need to be extended. The incubation time depends on the activity and concentration of the tested kinase(s). Light can be excluded by wrapping the plate in aluminum foil.

8. **Pipet 10 µl LiquiChip Phospho-Tyr Antibody solution into each assay well. Incubate for 30 min at room temperature (15–25°C) in the dark on a microplate shaker set to rotate at approximately 600 rpm.**

9. **Pipet 10 µl Anti-Mouse Biotin Conjugate into each assay well. Incubate for 30 min at room temperature (15–25°C) in the dark on a microplate shaker set to rotate at approximately 600 rpm.**

10. **Prepare a 1 in 50 dilution of the LiquiChip BRK Streptavidin–PE stock solution using LiquiChip Streptavidin Dilution Buffer (e.g., for 96 assays, pipet 1.96 ml dilution buffer into the tube containing 40 µl LiquiChip BRK Streptavidin–PE). Vortex gently.**

    Once diluted, LiquiChip BRK Streptavidin-PE solution is only stable for up to 1 week at 4°C. If fewer than 96 wells of an assay plate are used, dilute only the required amount of Streptavidin-PE.

11. **Pipet 20 µl of diluted LiquiChip BRK Streptavidin–PE into each assay well and incubate for 30 min at room temperature (15–25°C) in the dark on a microplate shaker set to rotate at approximately 600 rpm.**

12. **Pipet 20 µl of LiquiChip BRK Assay Stop Solution into each assay well and incubate for 5 min at room temperature (15–25°C) in the dark on a microplate shaker set to rotate at approximately 600 rpm.**

13. **Assay the plate on the LiquiChip Reader using the LiquiChip Tyr Template, which can be downloaded from the QIAGEN web site (see page 13).**

Protocol: LiquiChip Broad-Range Tyr Kinase Wash Assay

The following protocol is performed using a 96-well filter plate. The volumes given are for 100 assay points/microplate wells. If performing fewer than 100 assay points, reduce the volumes of buffers and reagents accordingly. A wash assay is recommended if the cell culture medium, kinase sample, or buffer used contains components that may interfere with the kinase assay analysis procedure (e.g., biotin, fetal calf serum, or high concentrations of manganese).

Important notes

- As a general buffer for the tyrosine phosphorylation reaction, we recommend using Assay Buffer 1. Buffer compositions are provided in the Appendix on page 26.
- When using a buffer other than those listed in the Appendix, make sure that buffer components are compatible with LiquiChip beads (see Table 2, page 9).
- Before assaying the microplate, make sure that the sample arm is adjusted to the 96-well filter plate.

Equipment and reagents to be supplied by the user:

- Assay Buffer 1or other buffer
- Mg^{2+}-ATP cocktail
- Repeating pipet (e.g., Eppendorf Repeater® pipet) with 0.5 ml tip
- Vortex mixer
- Microplate shaker at room temperature (e.g., Heidolph Titramax 100)
- Microplate shaker with temperature control (e.g., Eppendorf Thermomixer Comfort with microplate adapter and lid)
- Aluminum foil
- 96-well filter plate (e.g., Pall, cat. no. PN 5042, or Corning, cat. no. 3504)
- QIAvac 96 vacuum manifold (QIAGEN, cat. no. 19504)
- QIAvac Plate Holder (QIAGEN, cat. no.19548)
- Vacuum pump (e.g., KNF Neuberger, model N 840 FT 18)
- Vacuum regulator (QIAGEN cat. no. 19530)

Procedure

1. **Vortex the vials containing LiquiChip Broad-Range Tyr Beads for 30 s at medium speed. Agitate the vials for at least a further 15 min in the dark to completely resuspend the beads.**

   Agitate the beads using an end-over-end shaker or a vortex mixer with a shaker adapter (e.g., Roth, cat. nos. 509.1 and 510.1).

2. **Pipet 100 µl assay buffer into each well of a 96-well filter microplate and incubate for 30 s on a microplate shakerset to rotate at approximately 600 rpm.**

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

3. **Assemble the QIAvac 96 and place the 96-well filter plate on the QIAvac Plate Holder in the manifold.**

4. **Set the vacuum regulator to –600 mbar and apply vacuum to the bottom of the filter plate to remove liquid. As soon as the wells are dry, remove tubing from the vacuum manifold to release vacuum. Blot the bottom of the filter plate on a paper towel.**

5. **Pipet 20 µl of assay buffer into each assay well.**

   If a kinase inhibitor is being added to the assay, add 10 µl of the inhibitor dilution and 10 µl Assay Buffer.

6. **After agitating the beads for 15 min, pipet 900 µl assay buffer into each LiquiChip bead vial to dilute the bead suspension, and vortex at full speed for 90 s.**

   This yields a final concentration of 125 beads/µl.

7. **Pipet 20 µl of the diluted bead suspension into each assay well (= 2500 beads per assay point).**

   Residual amounts of diluted bead suspension can be stored at 4°C in the dark for 3 months.

8. **Pipet 10 µl of your test sample or purified enzyme into each assay well.**

   For the negative control sample (background sample), add 10 µl Assay Buffer.

9. **Start the reaction by pipetting 20 µl of Mg^{2+}-ATP cocktail into each assay well.**

10. **Incubate the plate for 30–60 min at 30°C in the dark on a microplate shaker set to rotate at approximately 600 rpm.**

    To ensure constant, reproducible reaction conditions, we recommend using a thermostat-controlled microplate shaker that can be heated to 30°C. Incubations can be carried out at a lower temperature, but reaction times may need to be extended. The incubation time depends on the activity and concentration of the tested kinase(s). Light can be excluded by wrapping the plate in aluminum foil.

11. **Place the 96-well filter plate on the QIAvac manifold and repeat step 4.**

12. **Pipet 70 µl of assay buffer into each well and incubate for 1 min on a microplate shaker set to rotate at approximately 600 rpm.**

13. **Place the 96-well filter plate on the QIAvac manifold and repeat step 4.**

14. **Pipet 70 µl assay buffer into each well.**

15. **Pipet 10 µl LiquiChip Phospho-Tyr Antibody solution into each assay well. Incubate for 30 min at room temperature (15–25°C) in the dark on a microplate shaker set to rotate at approximately 600 rpm.**

16. **Pipet 10 µl Anti-Mouse Biotin Conjugate into each assay well. Incubate for 30 min at room temperature (15–25°C) in the dark on a microplate shaker set to rotate at approximately 600 rpm.**

17. **Prepare a 1 in 50 dilution of the LiquiChip BRK Streptavidin–PE stock solution using LiquiChip Streptavidin Dilution Buffer (e.g., for 96 assays, pipet 1.96 ml dilution buffer into the tube containing 40 µl LiquiChip BRK Streptavidin–PE). Vortex gently.**

    Once diluted, LiquiChip BRK Streptavidin–PE solution is only stable for up to 1 week at 4°C. If fewer than 96 wells of an assay plate are used, dilute only the required amount of Streptavidin–PE in a separate reaction tube.

18. **Pipet 20 µl of diluted LiquiChip BRK Streptavidin–PE into each assay well and incubate for 30 min at room temperature (15–25°C) in the dark on a microplate shaker set to rotate at approximately 600 rpm.**

19. **Pipet 20 µl of LiquiChip BRK Assay Stop Solution into each assay well and incubate for 5 min at room temperature (15–25°C) in the dark on a microplate shaker set to rotate at approximately 600 rpm.**

20. **Assay the plate on the LiquiChip Reader using the LiquiChip Tyr Template, which can be downloaded from the QIAGEN web site (see page 13).**

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	
a) Fewer than 100 beads per well are analyzed (measured wells are marked red instead of green in the "Run Batch" mode)	Check the number of beads in the bead suspension (see *LiquiChip Applications Handbook*). Analyzing fewer than 30 beads per bead code is not recommended. Increase "Sample Timeout" in the template setup.
b) Sampling probe is not properly adjusted	Adjusting the sampling probe will greatly improve the speed and efficiency of your sample acquisition. To adjust the sampling probe see the *LiquiChip Workstation User Manual*. The sampling probe should be adjusted for each type of 96-well plate or filter plate used.
c) Air has entered the system	Always fill your wells with a volume at least 30 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 80 µl). This additional volume prevents air form entering the system, which can increase sample acquisition time.
d) Sampling probe is clogged	Remove sampling probe, sonicate, and readjust to the correct height (see above). Perform an alcohol flush, a wash step, and a prime procedure to remove air from the system.

	Comments and suggestions
Median florescent intensities are lower than expected	
a) Instrument is not calibrated	Calibrate instrument using LiquiChip Calibration and Control Beads.
b) Doublet discriminator gate is misaligned	Change the "Doublet Discriminator Gate" value from 7500 to 15,000.

Appendix: Buffer Compositions

Assay Buffer 1

20 mM MOPS

25 mM β-glycerophosphate

5 mM EGTA

1 mM DTT

1 mM sodium orthovanadate

Adjust to pH 7.2 using NaOH.

Store buffer at 4°C for up to

1 month.

Mg^{2+}-ATP Cocktail

40 mM $MgCl_2$

0.25 mM ATP

Prepare in assay buffer.

Store in aliquots at –20°C for up to 6 months.

Extraction Buffer (recommended for cell lysates)

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 Extraction Buffer.

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

QIAGEN Companies

Please see the back cover for contact information for your local QIAGEN office.

QIAGEN Distributors

Argentina
Tecnolab S.A.
Tel: (011) 4555 0010
Fax: (011) 4553 3331
E-mail: info@tecnolab.com.ar
Web site: www.tecnolab.com.ar

Austria
VWR International GmbH
Tel: (01) 576 00 0
Fax: (01) 576 00 600
E-mail: info@at.vwr.com
Web site: www.vwr.com

Belgium/Luxemburg
Westburg b.v.
Tel: 0800-1-9815
Fax: (31) 33-4951222
E-mail: info@westburg.nl
Web site: www.westburg.nl

Brazil
Uniscience do Brasil
Tel: 011 3622 2320
Fax: 011 3622 2323
E-mail: info@uniscience.com
Web site: www.uniscience.com

China
Gene Company Limited
Tel: (852)2896-6283
Fax: (852)2515-9371
E-mail:
Hong Kong: info@genehk.com
Beijing: gene@public2.bta.net.cn
Shanghai: gene@public.sta.net.cn
Chengdu: gene@public.cd.sc.cn
Guangzhou:
gzyitao@public.guangzhou.gd.cn

Croatia
INEL Medicinska Tehnika d.o.o.
Tel: (01) 2984-898
Fax: (01) 6520-966
E-mail:
inel-medicinska-tehnika@zg.tel.hr

Cyprus
Scientronics Ltd
Tel: 02-357 22 765416
Fax: 02-357 22 764614
E-mail: a.sarpetsas@biotronics.com.cy

Czech Republic
BIO-CONSULT spol. s.r.o.
Tel/Fax: (420) 2 417 29 792
E-mail: bio-cons@login.cz
Web site: www.bio-consult.cz

Denmark
VWR International A/S
Tel: 43 86 87 88
Fax: 43 86 87 90
E-mail: info@dk.vwr.com
Web site: www.vwr.com

Egypt
Clinilab
Tel: 52 57 212
Fax: 52 57 210
E-mail: Clinilab@link.net

Finland
VWR International Oy
Tel: (09) 804 551
Fax: (09) 8045 5200
E-mail: info@fi.vwr.com
Web site: www.vwr.com

Greece
BioAnalytica S.A.
Tel: (210)-640 03 18
Fax: (210)-646 27 48
E-mail: bioanalyt@hol.gr

Hungary
Kasztel-Med Co. Ltd.
Tel: (01) 385 3887
Fax: (01) 381 0695
E-mail: info@kasztel.hu
Web site: www.kasztel.hu

India
Genetix
Tel: (011)-2542 1714
or (011)-2515 9346
Fax: (011)-2546 7637
E-mail: genetix@nda.vsnl.net.in

Israel
Westburg (Israel) Ltd.
Tel: 08-6650813/4
or 1-800 20 22 20 (toll free)
Fax: 08-6650934
E-mail: info@westburg.co.il
Web site: www.westburg.co.il

Korea
LRS Laboratories, Inc.
Tel: (02) 924-86 97
Fax: (02) 924-86 96
E-mail: webmaster@lrslab.co.kr
Web site: www.lrslab.co.kr

Malaysia
RESEARCH BIOLABS SDN. BHD.
Tel: (603)-8070 3101
Fax: (603)-8070 5101
E-mail: biolabs@tm.net.my
Web site: www.researchbiolabs.com

Mexico
Quimica Valaner S.A. de C.V.
Tel: (55) 55 25 57 25
Fax: (55) 55 25 56 25
E-mail: qvalaner@infosel.net.mx

The Netherlands
Westburg b.v.
Tel: (033)-4950094
Fax: (033)-4951222
E-mail: info@westburg.nl
Web site: www.westburg.nl

New Zealand
Biolab Ltd
Tel: (09) 980 6700
or 0800 933 966
Fax: (09) 980 6788
E-mail:
biosciences@nzl.biolabgroup.com
Web site: www.biolabgroup.com/nzl

Norway
VWR International AS
Tel: 22 90 00 00
Fax: 22 90 00 40
E-mail: info@no.vwr.com
Web site: www.vwr.com

Poland
Syngen Biotech Sp.z.o.o.
Tel: (071) 351 41 06
or 0601 70 60 07
Fax: (071) 351 04 88
E-mail: info@syngen.pl
Web site: www.syngen.com.pl

Portugal
IZASA PORTUGAL, LDA
Tel: (21) 424 7312
Fax: (21) 417 2674

Singapore
Research Biolabs Pte Ltd
Tel: 62731066
Fax: 62734914
E-mail: biolabs@singnet.com.sg

Slovak Republic
BIO-CONSULT Slovakia spol. s.r.o.
Tel/Fax: (02) 5022 1336
E-mail: bio-cons@post.sk
Web site: www.bio-consult.cz

Slovenia
MEDILINE d.o.o.
Tel: (01) 830-80-40
Fax: (01) 830-80-70
or (01) 830-80-63
E-mail: mediline@siol.net

South Africa
Southern Cross Biotechnology
(Pty) Ltd
Tel: (021) 671 5166
Fax: (021) 671 7734
E-mail: info@scb.co.za
Web site: www.scb.co.za

Spain
IZASA, S.A.
Tel: (93) 902.20.30.90
Fax: (93) 902.22.33.66
E-mail: suministros@izasa.es

Sweden
VWR International AB
Tel: (08) 621 34 00
Fax: (08) 760 45 20
E-mail: info@se.vwr.com
Web site: www.vwr.com

Taiwan
TAIGEN Bioscience Corporation
Tel: (02) 2880 2913
Fax: (02) 2880 2916
E-mail: taigen@ms10.hinet.net

Thailand
Theera Trading Co. Ltd.
Tel: (02) 412-5672
Fax: (02) 412-3244
E-mail: theetrad@samart.co.th

Turkey
Medek Medikal Ürünler
ve Saglik Hizmetleri A. S.
Tel: (216) 302 15 80
Fax: (216) 302 15 88
E-mail: makialp@med-ek.com

All other countries
QIAGEN GmbH, Germany

Australia ■ **QIAGEN Pty Ltd** ■ ABN 75 072 382 944 ■ PO Box 25 ■ Clifton Hill ■ Victoria 3068
Orders 03-9489-3666 ■ Fax 03-9489-3888 ■ Technical 1-800-243-066

Canada ■ **QIAGEN Inc.** ■ 2800 Argentia Road ■ Unit 7 ■ Mississauga ■ Ontario ■ L5N 8L2
Orders 800-572-9613 ■ Fax 800-713-5951 ■ Technical 800-DNA-PREP (800-362-7737)

France ■ **QIAGEN S.A.** ■ 3 avenue du Canada ■ LP 809 ■ 91974 COURTABOEUF CEDEX
Orders 01-60-920-920 ■ Fax 01-60-920-925 ■ Technical 01-60-920-930

Germany ■ **QIAGEN GmbH** ■ QIAGEN Strasse 1 ■ 40724 Hilden
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Italy ■ **QIAGEN S.p.A.** ■ Via Grosio, 10/10 ■ 20151 Milano
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Japan ■ **QIAGEN K.K.** ■ Forefront Tower II ■ 13-1, Kachidoki 3 Chome ■ Chuo-ku, Tokyo 104-0054
Telephone 03-5547-0811 ■ Fax 03-5547-0818 ■ Technical 03-5547-0811

Switzerland ■ **QIAGEN AG** ■ Auf dem Wolf 39 ■ 4052 Basel
Orders 061-319-30-30 ■ Fax 061-319-30-33 ■ Technical 061-319-30-31

UK and Ireland ■ **QIAGEN Ltd.** ■ QIAGEN House ■ Fleming Way ■ Crawley ■ West Sussex, RH10 9NQ
Orders 01293-422-911 ■ Fax 01293-422-922 ■ Technical 01293-422-999

USA* ■ **QIAGEN Inc.** ■ 27220 Turnberry Lane ■ Valencia ■ CA 91355
Orders 800-426-8157 ■ Fax 800-718-2056 ■ Technical 800-DNA-PREP (800-362-7737)

* Address effective as of February 1, 2004. Before this date, please mail to QIAGEN Inc., 28159 Avenue Stanford, Valencia, CA 91355.