PTK

Protein Tyrosine Kinase Assay

PamGene

Protein Tyrosine Kinase Assay on PamStation®12
Version 3.0

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1 INTRODUCTION

1.1 Intended use

The protein tyrosine kinase assay on PamChip® arrays on the PamStation®12 instrument is used to determine tyrosine kinase activity of recombinant kinases or kinases in lysates from tissues and cells and is intended for research purposes only.

1.2 Description of the assay

The protein tyrosine kinase assay performed on PamChip® arrays on the PamStation®12 is a flow-through microarray assay to determine the activity of tyrosine kinases. The phosphorylation activity is determined using peptides immobilized on the PamChip® arrays (Figure 1). The phosphorylation activity is detected with a fluorescently labelled antibody and recorded by a CCD-camera in the PamStation®12. The PamStation®12 is specifically intended for the processing of one up to three PamChip®4 microarrays. Both the instrument and the PamChip® microarrays are products of PamGene International B.V. A schematic diagram of the assay is shown in Figure 1. During the assay, the assay mix is pumped up and down through the porous 3D arrays. The progress of the reaction in time is indicated in (pump) cycles. Figure 1 indicates the cycle numbers found for the images.

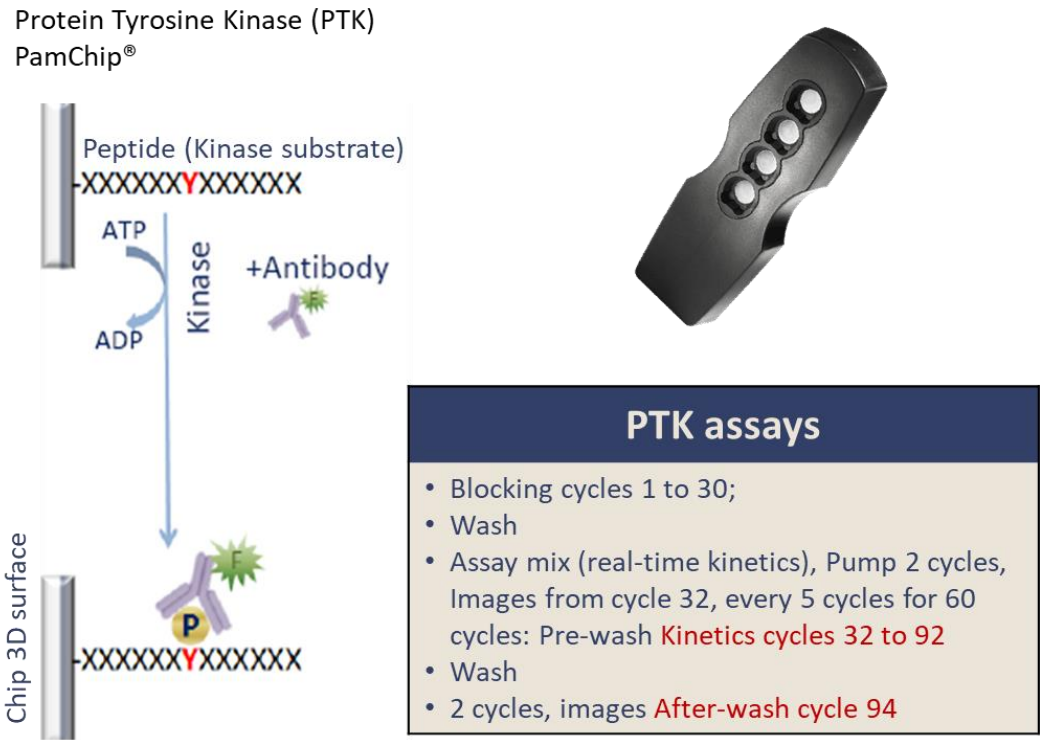
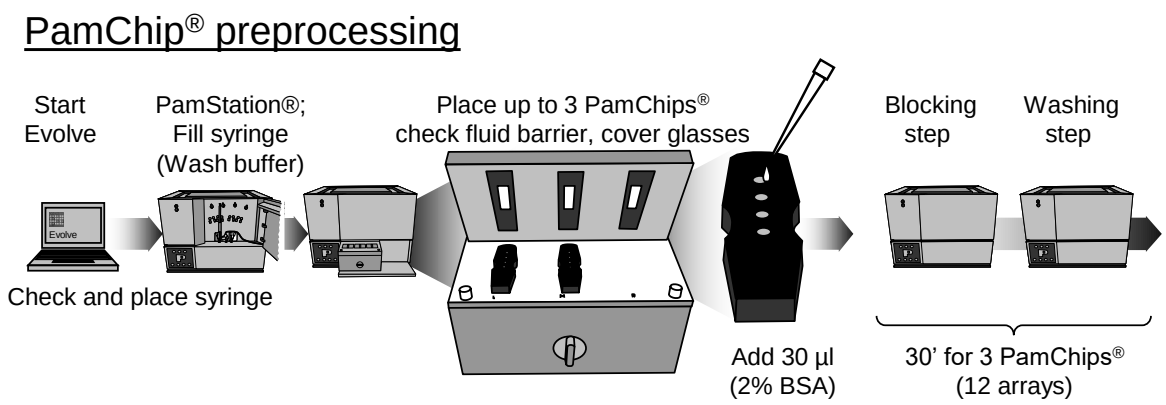


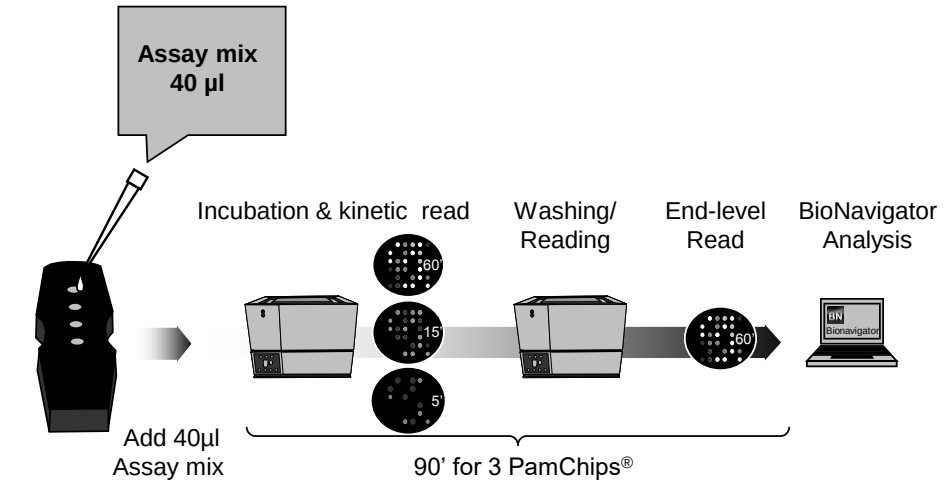
Figure 1 : The PTK assay. Schematic diagram of protein tyrosine kinase assay (left); PamChip®4 microarray (right, top); Cycle numbering using the standard EVOLVE PTK assay protocols (right, bottom)

Notes:

- Please refer to the PamStation®12 Operator Manual for details related to instrument procedures.
- Please refer to the BioNavigator Manual for details related to image quantification and data analysis.

1.3 Quick guide





2 KINASE ACTIVITY PROFILING PROTOCOL

2.1 Sample preparation

Prepare lysates according to PamGene Protocol 1160 *Preparation of lysates of Cell Lines or Purified Cells* or PamGene Protocol 1150 *Preparation of lysates of Tissue Sections*. For additional protocols, request us at support@pamgene.com.

2.2 Materials

Material/Equipment	Supplier	Catalog No.	Storage
PamStation®12 System	PamGene	31500	N/A
Protein Tyrosine Kinase PamChip®	PamGene	32508	+4°C
PTK reagent kit	PamGene	32112	-20°C
M-PER™ Mammalian Extraction Buffer	Thermo Fischer Scientific	78503 78501 or 78505	RT
Halt™ Phosphatase Inhibitor Cocktail (100x)	Thermo Fischer Scientific	78428	+4°C
Halt™ Protease Inhibitor Cocktail, EDTA free (100x)	Thermo Fischer Scientific	78437	+4°C
Water (ultrapure grade or equivalent)	Various suppliers		RT

Antibody solution (PY20-FITC):

- Upon first use, and recommended before each use: Spin the stock solution for 5 minutes at maximum speed (16.000 x g) in a pre-cooled centrifuge at +4°C to spin down fluorescent aggregates[1]
- Carefully pipette from the supernatant layer
- Store the supernatant at -20°C protected from light

10x PTK additive:

- In case of precipitation, incubate 10x PTK additive for 5 minutes at +37°C and mix well[2]
- When dissolved, store the 10x PTK additive at room temperature. When precipitate is observed again, incubate at +37°C as described above

[1] Fluorescent aggregates may occur upon shipment or storage of the antibody solution.
[2] Precipitation might occur upon freezing of the 10x PTK additive.

2.2.1 Preparation of experiment

- For assays with **kinase Inhibitors** and assays with **recombinant kinases,** See Section 0 Tips and Tricks
- Prepare the experimental setup, considering the desired analysis of data, following guidelines provided[3]
- Prepare a pipetting scheme for the preparation of the Assay "Basic mix". Use one of the pipetting schemes (assay calculation sheets) provided[3]
- For each new run, a fresh Assay "Total mix" should be prepared, just prior to loading the samples onto the array. This step is to make sure that the Kinase and ATP are combined at the latest time prior to adding to arrays
- As shown in the excel sheet, multiply the volumes shown in the table with the number of arrays and add at least 10% (e.g. 4 arrays, multiply volumes with 4.4) to account for pipetting losses
- As shown in the excel sheet, prepare a pipetting scheme for the dilutions of the lysate samples with 10x Inhibitor in M-PER solution and M-PER
- Prepare the information needed for the annotation of the samples

2.2.2 Preparation of solutions

The following solutions must be prepared fresh and can be used for one day. Keep all solutions on ice

- **1x PK wash buffer:**
  - Dilute 300 µl of 10x PK buffer with ultrapure water to a final volume of 3.0 ml
  - Use the remaining 100 µl 10x PK buffer for the preparation of the Assay "Basic mix"
- **ATP solution (4 mmol/l or 1 mmol/l)**
  - Dilute 4 µl ATP stock solution (100 mmol/l) with 96 µl ultrapure water to a final volume of 100 µl

  or
  - Dilute 1 µl ATP stock solution (100 mmol/l) with 99 µl ultrapure water to a final volume of 100 µl (e.g. when using kinase inhibitors in the assay)
- **DTT solution (1 mol/l):**
  - Check label of tube containing DTT and dissolve the solid DTT in the indicated amount of ultrapure water

[3] https://www.pamgene.com/en/KinaseAssays.htm (Requires a PamCloud Login)

- **M-PER with protease and phosphatase inhibitors**
  - 10x Inhibitors in M-PER solution: Add 2 µl Protease Inhibitor Cocktail (100x) and 2 µl Phosphatase Inhibitor Cocktail (100x) to 16 µl of M-PER

2.2.3 Assay Protocol

Pre-processing:

- Leave the closed pouch with PamChip® arrays 10 minutes at room temperature before opening
- Check the waste bottle, and empty if needed
- Start PC and PamStation®12 system
- Start Evolve software. Press “Initialize” button
- Open the Evolve Runner application
- Open supplied Evolve protocol[4] (e.g. 1200PTKlysvXX.PS12Protocol)
- Fill a syringe with 1x PK wash buffer, check that the needle is not clogged, that it dispenses properly and does not leak. Place the syringe in position 2 (see Evolve protocol)
- Start protocol by pressing the “Run” button
- Select the one, two or three PamChip® arrays that will be tested. Use chips from the same production batch (e.g. 631**275**102). Check on the box that you have the right PamChips (PTK)
- Enter the barcode and select the appropriate article number (see PamChip® pouch)
- Open the PamChip Annotator and annotate your samples. This can also be done during or after the run
- Press “OK”
- Press the “Load” button and the incubator will appear
- Check fluid barriers, and replace when a well has turned white
- Check the cover glasses and clean with a (moist) dust free cloth. Dry, and replace in instrument with indication “TOP” on top

Blocking:

- Place 1-3 Protein Tyrosine Kinase PamChip®s in the incubator on the place indicated by the barcodes
- Apply 30 µl of 2% BSA to the middle of each array. Do not touch the surface of the arrays with pipette tip!
- Press the “Load” button

Note: During blocking and washing procedure, Assay “Total mix” and samples can be prepared.

[4] https://www.pamgene.com/en/KinaseAssays.htm (Requires a PamCloud Login)

Prepare the "Lysate dilution mix":

- After thawing on ice, the lysates could optionally be centrifuged for 5 minutes at >10.000 x g at +4°C to remove precipitates
- Calculate the lysate volume required per array (based on desired total protein per array; usually 5 µg per array). Excel worksheets are provided to prepare the "Lysate dilution mix"[5]. Add M-PER (without inhibitor) to make a total volume of 9 µl. Add 1 µl of the 10x inhibitor in M-PER solution to this tube
- Store "Lysate dilution mix" on ice
- To account for pipetting, extra volumes are factored in, as shown in Assay calculation sheets[5]

Prepare the Assay "Basic mix":

- Spin the stock solution for 5 minutes at maximum speed (16.000 x g) in a pre-cooled centrifuge at +4°C to spin down fluorescent aggregates. Carefully pipette from the supernatant layer
- Prepare the Assay "Basic mix" according to the Table, without the "Lysate dilution mix". Excel worksheets are provided to prepare the "Basic mix"[5] (with at least 10% extra to account for pipetting losses, See Section 2.2.1). Add components in the order indicated in the Table and keep on ice.

Mix gently, do not vortex

Solution	Volume (µl) for 1 array
Water	20.6 (Make to 30 µl)
10x PK buffer	4
100x BSA solution	0.4
1 M DTT solution	0.4
10x PTK additive	4
Detection antibody	0.6
4 mM ATP[6]	4
Sample ("Lysate dilution mix")	10
Total Volume	**40**

[5] https://www.pamgene.com/en/KinaseAssays.htm (Requires a PamCloud Login)

[6] For assays with kinase Inhibitors and assays with recombinant kinases, See Section 3 Tips and Tricks

Prepare the Assay "Total mix":

- To the "Lysate dilution mix", in labelled tubes on ice, add the Assay "Basic mix", according to the assay set up, as shown in the Excel assay calculation sheets[4]. Keep on ice
- "Total mix" should be prepared, just prior to loading the samples onto the array

Continue the assay run after Blocking Step

- Press the "Load" button to unload the incubator
- Apply 40 µl complete Assay "Total mix" per array
- Press the "Load" button and the kinetic run is started
- After completion of the run, press the "Load" button and remove the PamChips®.

End of day procedure

- See Section 4 and the Operator Manual for details.
- Discard the 1x PK wash buffer in the syringe
- Clean syringe according to protocol
- Rinse the barrel (first without the needle) and plunger with ultrapure water. Attach the needle to the barrel, fill with ultrapure water, place the plunger and rinse the needle. Repeat this 3 times. Dry needle, syringe and barrel thoroughly before storing, preferably by using compressed air
- Empty and rinse waste bottle
- Replace the fluid barrier when one of the wells has absorbed fluid and turned white
- Clean cover glasses if necessary

Safety & Precautions

- For equipment: see Operator Manual
- For materials: see supplier's information

Cleaning tools and equipment

- For equipment: see Operator Manual

3 TIPS AND TRICKS:

3.1 Assay

- The material the arrays are made of is very brittle. Do not touch the arrays with the pipette tip to prevent breaking
- Samples should be applied directly after the blocking and wash steps
- Complete drying of arrays after blocking affects assay performance negatively. When a long time passes between washing and application of the assay mixture, the arrays may dry completely (white appearance). When it is expected that the time between the end of the washing step and application of assay mix will be more than 15 min., the PamChip®4 disposable(s) may be removed from the instrument and stored in the original pouch to prevent further drying

3.2 Lysates

- Lysates can be very sensitive to freeze-thaw cycles. Preferably use a never thawed aliquot for every experiment
- Routinely 1-10 µg protein per array is used in PTK assay
- When more than 10 µg protein per array is used, the membrane can become clogged during the assay
- As a control for occurrence of non-specific binding of the antibodies to peptides, an assay without ATP or the addition of a generic PTK inhibitor like staurosporin (10 µM final concentration) can be performed

3.3 Kinase Inhibitors

- The assay set up/ calculation files can be found from the link provided in Section 2.2.1 or on request (Email: support@pamgene.com)
- When working with (ATP competitive) kinase inhibitors, the assay is performed with 100 µM ATP. ATP is not added to the Assay “Basic mix” but later to the “Total mix”
- Most kinase inhibitors can be dissolved at a concentration of 10mM in DMSO (recommended). Check the suppliers information for solubility in DMSO and after dissolving, check for precipitates. These stock solutions can be stored according to the supplier’s instructions
- Shortly before the assay, dilute the kinase inhibitor in DMSO to yield a solution of 50x the final assay concentration. Prepare an Assay “Total mix” where 0.8 µl is reserved for DMSO or Inhibitor in DMSO. Add sample just before application onto the arrays

- Preferably, the DMSO assay concentration should not exceed 2%
- Some inhibitors bind slowly to the kinase. To assure proper binding, add the inhibitor before adding ATP

3.4 Recombinant kinases

- Recombinant kinases can be used in the assay. They can be diluted with 1x PK buffer. The M-PER can be replaced by ultrapure water
- For a recombinant kinase the optimal kinase input must be determined by testing a concentration series of the kinase. Since the sensitivity of the PamChip® assay is similar to a radioactive assay, the input suggested by the supplier is a good starting point. When such information is not available, 2, 20 and 200 ng per array can be tested to determine the optimal concentration. As control, an incubation without ATP must be performed to show ATP dependency of the reaction
- When preparing a concentration series of a kinase, one assay master mix containing the highest concentration kinase is made and one without kinase. The latter is used to dilute the kinase containing assay master mixture. This method avoids errors due to variation in pipetting small volumes
- Since recombinant kinases may be sensitive to freezing–thawing cycles, the recombinant kinases must be treated according to the instruction of the manufacturer. In many cases, aliquoting of the recombinant kinases is suggested to avoid freezing–thawing cycles
- Sample assay calculation sheets provided[7] can be adapted to your assay set-up

[7] https://www.pamgene.com/en/KinaseAssays.htm (Requires a PamCloud Login)

4 DETAILED INFORMATION FOR RUNNING AN EXPERIMENT

- Make sure the instrument and the PC are connected properly as described in the Operator Manual of the PamStation®12
- Once in a while check the available free disk space on the computer (~400 Mb per run)

Waste bottle

- Before starting an assay, check that the waste bottle is empty and that it is attached in the proper way to the instrument (screwed to the red cap). The waste bottle must be positioned straight in the dedicated holder at the right-hand side of the instrument. Make sure that the tubes to the waste bottle are not blocked by the door
- At the end of the run(s) empty the waste bottle by unscrewing the glass part of the bottle. Dispose of the contents in the appropriate way. Check that the inlets in the lid of the bottle are clean
- There is no sensor to warn of a full waste bottle. If the bottle is full, aspiration errors might occur and the aspiration pump can be damaged

Syringes

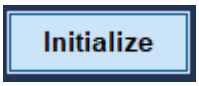
- Preparation of the syringes: A syringe comprises of three parts; a needle, a glass barrel and a stainless-steel plunger. Assembly is done by attaching the needle with its luer lock to the barrel. When the needle cannot be fixed with the luer lock and can be turned around and around, replace the needle
- When assembled as described above, fill the syringe with 3 ml 1x PK wash buffer. Place the plunger in the barrel. Remove any air bubbles in the syringe. Check that the needle of the syringe is not clogged. To test the needle performance, dispense with a short powerful burst; the dispense jet must be straight and continuous for 2 – 3 cm. Check that the needle does not leak
- If not OK, replace the needle, and test again as described above. If OK, place it inside the instrument in the 2nd position (according to the Evolve protocol)
- Needles must be handled with care. It is very important to clean the needles as soon as possible after use to prevent clogging by crystallization of salts. Cleaning can be done by rinsing the barrel (first without the needle) and plunger with ultrapure water. Thereafter, attach the needle to the barrel, place the plunger and rinse the needle. Repeat this process 3 times. To prevent corrosion and increase the lifespan of the needles, the inside of the needle must be dried thoroughly, preferably by using compressed air

- If a needle is clogged, an ultrasound bath can be used to facilitate cleaning. Replace the syringes every 6 months
- For further preparation and cleaning of the syringe see Operator Manual

PamStation®12

The PamChip® assays come with standard protocol(s) for running PTK or STK kinase assays with cell lysates or recombinant kinases on PamStation®12. The PamStation®12 Operator Manual can be consulted for designing custom protocols. For running existing protocols, the Protocol Runner can be used. For creating or adapting existing protocols the Protocol Editor can be used (see Operator Manual).

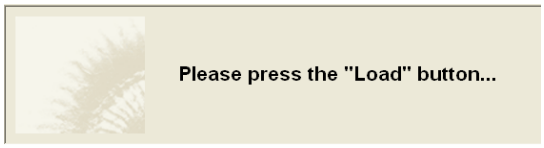
- After opening the Evolve software the “Initialize” button appears. Press the “Initialize” button to connect the PC to the PamStation®12



- Open the Evolve Runner application
- Open the supplied Evolve protocol[8] (e.g. 1200PTKlysv04.PS12Protocol)
- A large green “Run” button will pop-up. Click on the green “Run” button to start the protocol

- At the end of the protocol, this button will change into a red “Stop” button. **While this red button can be used to stop a protocol at any time, it should only be used in case of emergency (see Operator Manual for further instructions).**

- The protocol will always start with a “Load” step. During the “Load” step the instrument will ask the operator to press the “Load” button.



- During the “Load” step in the Evolve protocol:
  Check the fluid barriers
  Clean the cover glasses

Fluid barriers

- Fluid barriers are filled with a liquid absorbing material to prevent liquid from entering the PamStation in case of a leaking or broken array. The material turns white when it becomes wet. Check that the fluid barriers are in place. A fluid barrier must be

[8] File Name: 1200PTKlysv04.PS12Protocol; https://www.pamgene.com/en/evolve-protocols.htm

replaced when one of the wells has turned white. For more information see Operator Manual

Cover glasses

- Check before every experiment that the cover glasses are clean, by taking them out of the wagon and inspecting for irregularities
- When the wagon is in the unload position, pull the cover glass gently from the wagon
- Clean it with water and a piece of dust free optical quality cloth. Replace the cover glass with the TOP side up. This side contains the word “TOP”

5 SUPPORT

PamGene aims to provide total support to enable customers to fully realize the benefits of the PamGene technology and to maximize use of the PamStation®12 system.

The software components and data handling are essential interfaces to the system and are supported by a dedicated software support team through email, telephone and onsite visits, if required.

Please contact support on:

☎ +31 (0)73 615 89 00
✉ support@pamgene.com

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6 RIGHTS AND RESTRICTIONS

6.1 Intellectual property rights

The use of PamChip® microarrays is licensed, among others, under patent and patent applications issued to PamGene International B.V.

Any patents, patent applications and other proprietary rights named in this manual are included for reference purposes only. No licenses to use the processes or products covered by such patents, patent applications or proprietary rights are granted by or implied by PamGene International B.V. It is the responsibility of the user to determine whether a license is required and obtained.

6.2 Trademarks

PamStation®12 and PamChip® are trademarks of PamGene International B.V., registered in one or more countries.

6.3 Disclaimer

PamGene International B.V. reserves the rights to change its products and services at any time to incorporate technological developments. This manual is subject to change without notice.

Although this manual has been carefully prepared with every precaution to ensure accuracy, **PamGene International B.V.** can assume no liability for any errors or omissions, or for any direct or indirect damages resulting from application of this information.

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