Chapter 11

High-Throughput Screening of the Cyclic AMP-Dependent Protein Kinase (PKA) Using the Caliper Microfluidic Platform

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Abstract

Inhibitors of kinase activities can be mechanistically diverse, genomically selective, and pathway sensitive. This potential has made these biological targets the focus of a number of drug discovery and development programs in the pharmaceutical industry. To this end, the high-throughput screening of kinase targets against diverse chemical libraries or focused compound collections is at the forefront of the drug discovery process. Thus, the platform technology used to screen such libraries must be flexible and produce reliable and comparable data. The Caliper HTS microfluidic platform provides a direct determination of a peptidic substrate and phosphorylated product through the electrophoretic separation of the two species. The resulting data are reliable and comparable among screens and cover a broad range of biological targets, provided there is a definable peptide substrate that permits separation. Here we present a method for the high-throughput screening of the cyclic AMP-dependent protein kinase (PKA) as an example of the simplicity of this microfluidic platform.

Key words: Microfluidics, Kinase, PKA, High-throughput screening, HTS, Caliper.

1. Introduction

Kinases are one of the leading drug targets of the pharmaceutical industry *(1–3)*. This is in part due to the early success of Gleevec, an inhibitor of the kinase activity of the Bcr–Abl fusion protein involved in chronic myeloid leukemia (CML) *(4)*. To this end, there has been significant technological advancements in the method and platform development of kinase assays in the high-throughput screening arena. Many assays rely on

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secondary activities to detect phosphorylation by kinases using either phospho-specific antibodies (AlphaScreen, PerkinElmer) *(5)*, metal chelates (IMAP, Molecular Devices) *(6)*, protease sensitivity (Z′-LYTE, Invitrogen), or ADP detection reagents (Kinase-Glo, Promega) *(7)*. The Caliper microfluidic platform has the advantage of directly measuring the status of both a phosphorylated product and a substrate peptide with an accurate determination of the ratio of the two species *(8)*. The technology uses capillary electrophoresis to separate the reaction products into two distinct peaks using a combination of voltage and pressure. The assay relies on the design of a peptide that serves as a substrate for the kinase and is electrophoretically neutral so as to produce clear separation between the negatively charged products relative to the substrate. Samples can be processed in ~20 s and the microfluidic chips have 12 channels allowing parallel processing.

Kinase reactions are assembled in the presence of an inhibitor on a 384-well plate and terminated with EDTA at a kinetically determined end point. Terminated reactions are processed on the Caliper LabChip 3000. A single plate can be read in ~20 min. The output of the assay is a ratio of the product height and the peak sum of product and substrate referred to as PSR (product/sum ratio). Because of this, small variations in the fluorescent output will have very little impact on the PSR value, insuring the reproducibility of the data and the reliability of the assay. Typically the kinetic end point of the assay is validated in the range of 0.3–0.5 PSR and insured to be in the linear phase of the reaction. From an operational standpoint, most kinase reactions are terminated after 3 hr.

Here we present an example of the platform detailing the high-throughput screening of the cyclic AMP-dependent protein kinase (PKA). The percent inhibition cutoff for determining active compounds is 13.3% (*see* **Section 3.7**, Step 4 for a discussion of assay threshold). The Z′ (*11*) for the assay is 0.85. Prior to HTS the PKA assay was developed with a validated peptide substrate and the biochemical parameters for the assay determined including ATP K_m, rate, specific activity, and control inhibitor validation. The operating buffer is standardized for serine/threonine kinases and the concentration of ATP used is twice the K_m. With this description as a template, other kinases may be substituted after development and validation of the assay. The microfluidic platform additionally offers biological diversity with the ability to assay several peptide-based target classes including phosphatase, protease, and histone acetylase and deacetylase activities.

2. Materials

2.1. Proteins and Peptides

1. Cyclic AMP-dependent protein kinase catalytic subunit (PKA) was purchased from Sigma, catalog number C-8482. PKA is a human, recombinant, His-tagged protein expressed in *Escherichia coli*.
2. The peptide used for detection of PKA phosphorylation is labeled at the amino-terminal with carboxyfluorescein (FAM). The amino acid sequence is LRRASLG (Kemptide) *(9)* and was synthesized by CPC Scientific. The peptide is dissolved in 40% DMSO to yield a 1 mM solution.

2.2. Kinase Reaction

1. Master buffer (2×): 200 mM HEPES, pH 7.5, 0.2% BSA (w/v), 0.02% Triton X-100 (v/v).
2. Enzyme buffer (2.5×): 1× master buffer, 2.5 mM DTT, 25 mM $MgCl_2$, 25 μM sodium orthovanadate, 25 μM β-glycerophosphate, 25 μM ATP, 0.08 nM PKA.
3. Substrate buffer (2.5×): 1× master buffer, 2.5 μM FAM-LRRASLG.
4. Termination buffer (1.55×): 1× master buffer, 31 mM ethylenediamine tetraacetic acid (EDTA).
5. Compound (5×) in 1× master buffer.

2.3. Enzyme Validation

1. Enzyme buffer (2×): 1× master buffer, 2 mM DTT, 20 mM $MgCl_2$, 20 μM sodium orthovanadate, 20 μM β-glycerophosphate, 20 μM ATP, 2% DMSO.
2. Substrate buffer (2×): 1× master buffer, 2 μM FAM-LRRASLG.
3. Termination buffer (1.4×): 1× master buffer, 28.8 mM EDTA.

2.4. Compound Preparation

1. Compound buffer (5×): 1× master buffer, 50 μM compound, 5% DMSO (from compound).

2.5. Staurosporine Assay Plate Preparation

1. Staurosporine (Calbiochem) (3 mM) dissolved in 100% DMSO.
2. Inhibitor dilution buffer: 1× master buffer, 5% DMSO.

2.6. Microfluidic Analysis

1. Separation buffer: 1× master buffer, 0.36% DMSO, 20 mM EDTA, 0.1% coating reagent (supplied by Caliper).
2. Row marker dye: 1× master buffer, 20 mM EDTA, 0.75 μM FAM-LRRASLG.

3. Methods

The following procedure assumes access to equipment needed to accomplish a high-throughput screen. In addition to a Caliper LabChip 3000, liquid-handling instrumentation needed to create compound-containing assay plates and distribute enzymatic reaction components (BioMEK; Beckman Coulter, multidrop; Thermo Labsystems) is required. Prior to the high-throughput screening of PKA, the kinase activity was subjected to a rigorous assay development process in an effort to determine the biochemical parameters, performance, and validation of the assay. The first step of the assay development process is optimizing a peptide substrate that is both an efficient and a biologically relevant substrate for the kinase and whose product and substrate populations can be separated into two distinct peaks. Microfluidic separation of the peptide is optimized through the manipulation of voltage and pressure on the Caliper instrument. The ATP K_m had been determined to be 4.5 $\pm$ 0.7 μM. Inhibition of the PKA activity is measured at twice the K_m or 10 μM.

Before initiating the PKA screen, the enzymatic activity is first validated to insure a quantity of PKA that results in a product/sum ratio (PSR) of 0.3–0.5 after a 3 hr incubation period. This is required as the activity of PKA may vary from vendor to vendor and lot to lot. Validation is accomplished with an enzyme titration at a fixed ATP concentration and a kinetically determined end point. Statistical validation of the assay is then determined using experimentally designed assay plates that result in known values of inhibition derived from an IC_{50} titration of staurosporine.

With the assay validated, the high-throughput screen is begun by first preparing assay plates that contain 5 μl of a 5× concentration of the compound (50 μM) in the aqueous buffer. Assay plates are created through the addition of aqueous buffer to daughter plates containing 1 mM compound in neat DMSO. Daughter plates are created directly either from 10-mM master plates or from intermediate 3-mM mother plates. Prior to screening, we typically quantitate the compounds by high-performance liquid chromatography (HPLC) coupled with chemiluminescent nitrogen detector (CLND) in the aqueous buffer relevant for follow-up and IC_{50} determination *(10)*. We have determined previously that the aqueous concentrations of the compounds vary dramatically compared to their DMSO stocks *(10)*. Each of the assay plates is designed to contain a statistically significant number of positive controls: zeros without inhibitor and 100% inhibitor controls that contain 20 mM EDTA. With the assay plates ready, a 2.5× enzyme buffer is added to the plate using a

multidrop device and the reaction initiated by the addition of the 2.5× buffer that contains the fluorescently labeled peptide substrate. The addition of the enzyme buffer first allows potential prebinding of the inhibitor prior to the initiation of the reaction. After a 3 hr incubation period the reaction is terminated with 20 mM EDTA. Terminated reactions are stable for more than 24 hr, so there are no time constraints on plate reading. The plates are read on the Caliper instrument and the data collected for analysis.

3.1. Enzyme Validation

1. To insure that the enzyme is performing to the desired activity within the 3-hr time period, PKA is titrated to the fluorescently labeled substrate peptide. The assays are designed such that two solutions are consecutively added: one that contains the kinase and a majority of the reaction components including ATP and Mg^{++} and one that contains the peptide. All assays are performed on 384-well plates, which have 16 rows and 24 columns. For convenience the enzyme dilutions are completed directly on the assay plate. A 125-µl-capacity multichannel pipette is ideal for this task.
2. Dilute PKA 1:10 in 1× master buffer.
3. For an eight-point serial dilution, add 99 µl of 2× enzyme buffer to Row M1 on a 384-well plate, then 50 µl of 2× enzyme buffer to wells M2–M8.
4. Add 1 µl of diluted PKA to well M1 for a 1:1000 dilution of PKA. Lot #092K0330 had an initial concentration of 2.3 µM. The starting concentration of the dilution is therefore 0.0023 µM.
5. Dilute serially adding 50 µl of M1 to the 50 µl of M2, mix and repeat from M3 to M8.
6. Add 10 µl of 2× substrate buffer to rows A and E, wells 1–24.
7. Initiate the reaction by adding 10 µl of the diluted PKA eight wells at a time to wells A1–8, A9–16, and A17–24 for triplicate determination. To row E, add 10 µl of 1× master buffer as a comparative control for peak mobility (substrate only). Seal plates and incubate for 3 hr at room temperature. The final concentrations of PKA are 1.3, 0.65, 0.325, 0.163, 0.081, 0.041, 0.020, and 0.010 nM.
8. Stop reactions with 50 µl of termination buffer.
9. Analyze plate on the Caliper instrument.
10. Plot data as PSR on the *y*-axis and the concentration of PKA on the *x*-axis, fitting the data to a hyperbola. Determine the concentration of PKA that results in a PSR of 0.3 or 30% substrate-to-product conversion. This is the amount of enzyme to be used in the assay for HTS.

3.2. Staurosporine IC_{50} Determination

1. The IC_{50} value of staurosporine will be determined for PKA to use for validation of the assay below. Here reagents outlined for the kinase reaction (2.2) will be used.
2. Prepare a 50 μM stock of staurosporine from 3 mM in the inhibitor dilution buffer.
3. Serial dilute 50 μM staurosporine 15 times in the inhibitor dilution buffer. There will be 16 staurosporine assay points total. Add 5 μl of each concentration to wells A1–A16 and E1–E16 (most to least concentrated) for duplicate IC_{50} determination. These are 5× concentrations of inhibitor. In rows A17–24 and E17–24, add 5 μl of inhibitor dilution buffer.
4. To wells A1–A24 and E1–E24, add 10 μl of 2.5× enzyme buffer followed by 10 μl of 2.5× substrate buffer. Cover plate, incubate at room temperature, and after 3 hr, stop the reaction by adding 45 μl of termination buffer.
5. Analyze the samples in the Caliper instrument and extract the PSR values relative to the staurosporine concentrations.
6. Based on the average of the zeros (wells A17–24 and E17–24) derive the percent inhibition from the staurosporine-containing wells (*see* **Note 5**). Plot the percent inhibition on the *y*-axis and the staurosporine concentration on the *x*-axis. If using Excel, fit the data to a dose–response, single-site model to derive the IC_{50}. We previously determined the staurosporine IC_{50} for PKA to be 4 nM. Also, determine from the fit the amount of staurosporine required for 20, 50, and 70% inhibition for the assay validation below.

3.3. Assay Validation

1. Create three assay plates according to **Fig. 11.1**. The plates are designed to have statistical quantities of staurosporine that result in 0, 20, 50, and 70% inhibition at a 5× concentration. The amount of staurosporine used in the validation is based on the IC_{50} determination of the inhibitor. Five microliters of each inhibitor concentration is added to the designated wells on the assay plate. This process is best suited for robotic liquid handling-instrumentation.
2. Add 10 μl of 2.5× enzyme buffer followed by 10 μl of 2.5× substrate buffer using a multidrop or similar liquid-handling instrument. Seal plates, incubate at room temperature, and after 3 hr terminate with 45 μl of termination buffer.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A	0	100			20	20			50	50			70	70										
B	0	100			20	20			50	50			70	70										
C	0	0			20	20			50	50			70	70										
D	0	0			20	20			50	50			70	70										
E	0	100			20	20			50	50			70	70										
F	0	100			20	20			50	50			70	70										
G	0	0			20	20			50	50			70	70										
H	0	0			20	20			50	50			70	70										
I					20	20			50	50			70	70									0	100
J					20	20			50	50			70	70									0	100
K					20	20			50	50			70	70									0	0
L					20	20			50	50			70	70									0	0
M					20	20			50	50			70	70									0	100
N					20	20			50	50			70	70									0	100
O					20	20			50	50			70	70									0	0
P					20	20			50	50			70	70									0	0

Fig. 11.1. HTS plate layout format with controls and validation pattern.

3. Read plates on the Caliper instrument and extract the percent inhibition values from the plates using the 0 and 100% inhibition controls designated on the Well Analyzer software.
4. Perform statistical analysis on the plate data both considering well variation and plate-to-plate variation in the average and standard deviation of the values to insure that assay performance meets quality standards. Additional experiments may be performed on consecutive days to achieve day-to-day variation of the assay as well. For validation, it is more important that the inhibition values are consistent among the wells rather than being exactly the values predicted from the staurosporine IC_{50} curve.

3.4. Assay Plate Preparation

1. Assay plates containing compounds and controls at 5× concentration according to the plate map in **Fig. 11.1** are created using automated liquid-handling instrumentation (i.e., Biomek FX, Beckman Coulter).
2. Daughter plates containing 1 mM compound in neat DMSO are diluted to 50 μM with assay buffer.
3. DMSO (0%) and 20 mM EDTA (100%) controls are added to the wells indicated in **Fig. 11.1**.
4. Five microliters of diluted compound are transferred to the assay plate, which is now ready for the addition of the kinase reaction reagents.

3.5. HTS Kinase Reaction

1. To an assay plate containing 5 μl of compound, add 10 μl of 2.5× enzyme buffer followed by 10 μl of 2.5× substrate buffer using a multidrop (Thermo Labsystems). Cover plates and incubate for 3 hr at room temperature. Terminate reactions with 45 μl of termination buffer. Measure phosphorylation of peptide on the Caliper instrument.

3.6. Caliper Operation

1. Prepare a microfluidic chip by first rinsing the chip with deionized water and suctioning off the excess fluid with a vacuum line fitted with a micropipette tip. One must insure not to directly suction the microfluidic channels
2. Fill the waste wells with 0.42 ml of separation buffer and each of the upstream wells with 65 μl of separation buffer.
3. Load chip into the cassette and secure the lid.
4. Flow separation buffer through the instrument lines and fill each of the two dye troughs with 1.5 ml of row marker dye.
5. Place cassette with microfluidic chip into the instrument, lock into place, and with the software, lower the sippers into the troughs containing the separation buffer.
6. Follow the directions of the Caliper software to align the optics and insure correct flow into all 12 channels, test the voltage of the chip, validate the baseline, and determine the simultaneous sample output of the chip.
7. Load the assay plates into robotic stacks and design a job using the instrument software with the upstream voltage settings set at –2700 V and the downstream at –800 V. The pressure setting is –1.5 psi, dye and sample sip time set at 0.2 sec, initial delay, post dye, and post sample sip times set at 20 sec, and the final delay sip time set at 120 sec.

3.7. Data Analysis

1. The data from each plate are first visually inspected as a color-coded plate map (formulated within the Caliper software) to identify any anomalous patterns associated with liquid handling or the Caliper readout before transferring into the corporate database (**Fig. 11.2**).
2. To eliminate fluorescent anomalies, an Excel-based template is used to automatically remove any data point whose fluorescent signal of both product and substrate is above or below 6 standard deviations of the average signal within each microfluidic channel.

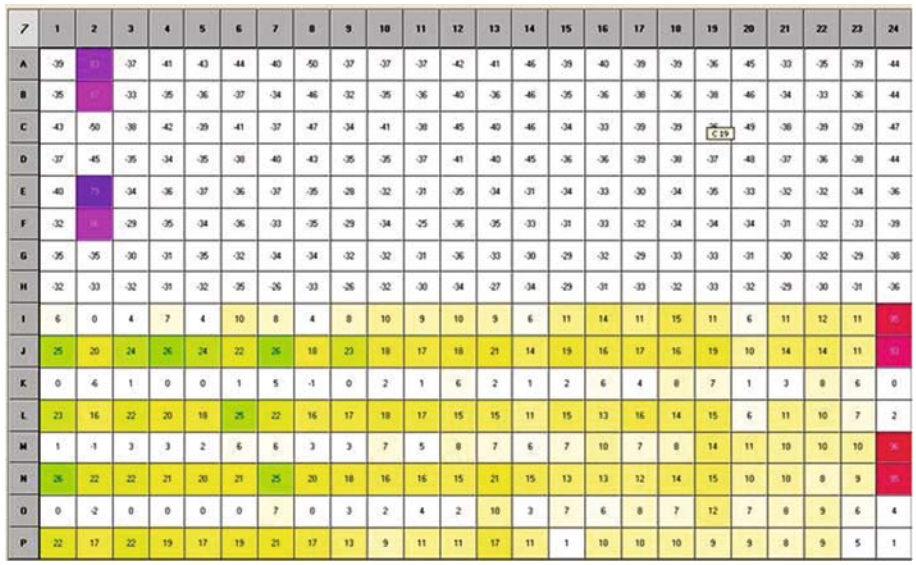

Fig. 11.2. HTS screening data. Anomalous patterns caused by liquid-handling error in rows I through P would be noted by the *yellow* and *green* color.

3. Control outliers are eliminated and a statistical equilibration is performed on the 12 channels to adjust for variability in individual channels. The normalized 0 and 100% controls are then used to calculate percent inhibition values for each compound tested.
4. The assay threshold is determined by the interquartile range or IQR to identify distribution outliers in lieu of the sample standard deviation. The IQR is calculated, where Q(X) represents the quantile below which *x*% of the data is observed

$$IQR = Q(.75) - Q(.25)$$

The IQR represents the width of exactly 50% of the data. When applied to the assay data, the bulk of the distribution representing the *inactives* will fall inside the interval:

$$[Q(.25) - 1.5 \cdot IQR, Q(.75) + 1.5 \cdot IQR]$$

All points outside the interval are potential *actives*. For a normally distributed population this method will bound 99.3% of the data; approximately the same percentage bound by an interval based on 3σ. An interval of 3.95·IQR will bound 99.9999999% of the data, which is equivalent to an interval based on 6σ.

5. A Z′ value is calculated for each plate *(11)*. The compounds from any plate whose Z′ is below 0.5 are rescreened.

3.8. HTS Results

Statistics for the PKA screen are shown in **Table 11.1**. The total number of points is the number of wells screened, while the total number of samples is the number of compound batches screened. Based on the determination of 3σ and 6σ, there are three data populations:

1. Compounds without effect are those whose range is ±3σ.
2. A grey area is determined to be between 3σ and 6σ (<13.3%).
3. Potent hits are those whose values are >6σ >1̃3.3%.

Table 11.1
HTS statistics

Total number of points	261,432	
Total number of samples	115,158	
Number of samples (3σ−/3σ+)	111,497	96.8%
Number of samples (3σ+/6σ+)	1,375	1.2%
Number of samples (>6σ+)	224	0.19%
6σ cutoff	13.34%	
Boolean samples	508	

Boolean samples noted in **Table 11.1** are those samples where replicate measurements fall in both the active and inactive populations. **Figure 11.3** is a Gaussian distribution of the sample results around the center of the assay or those compounds without effect. **Figure 11.4** is the distribution of samples >3σ with a standard deviation determined among replicate points. **Figure 11.5** is a plot of the minimum and maximum values for two replicate points that conform to a linear progression.

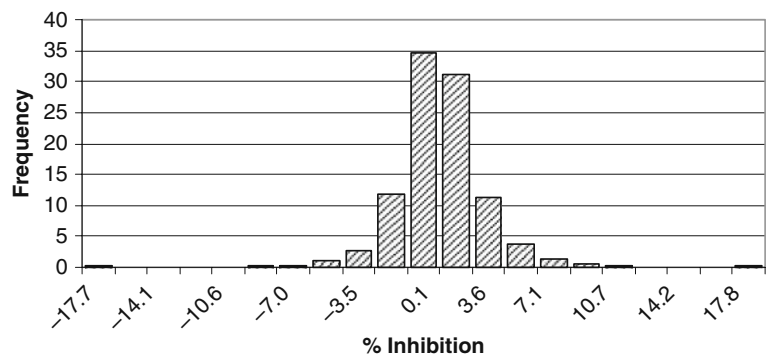

Fig. 11.3. Gaussian distribution of the data around the center of the assay (no effect). The percent inhibition of the assay points was binned within intervals of 2.96% and plotted as a histogram with the frequency of the range on the *y*-axis.

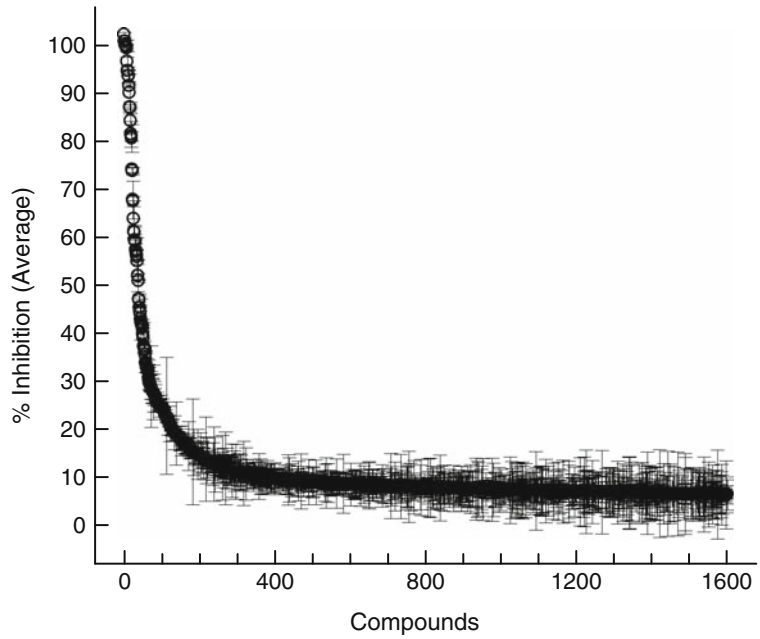

Fig. 11.4. Distribution and grey area of the outlier samples. The average and the standard deviation of duplicate assay points were plotted with rank order potency up to 100% inhibition of PKA activity. The 6σ cutoff for the screen was 13.3% inhibition.

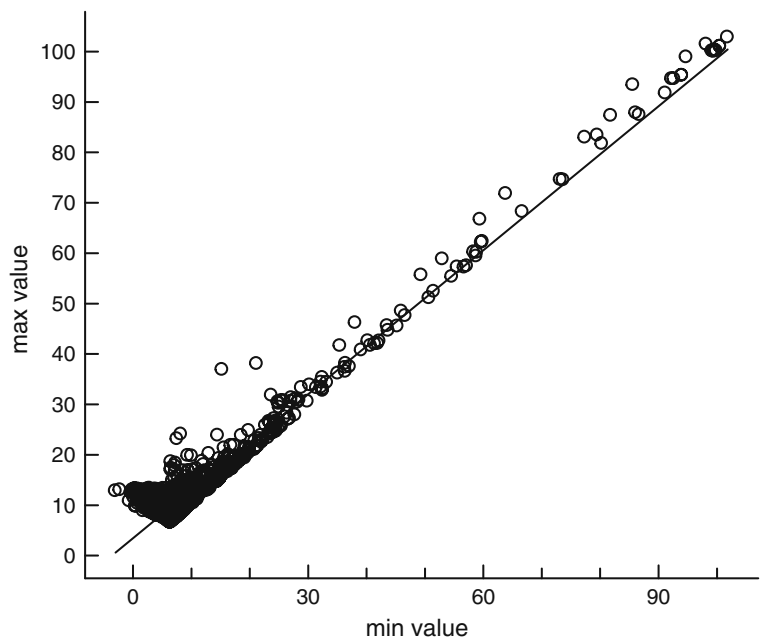

Fig. 11.5. Plot of minimum and maximum outliers of the sample duplicates. Scatter diagram with linear R^2 highlights the precision of the duplicate outliers.

4. Notes

1. All solutions should be prepared in filtered water that has a resistance of 18.2 Ω-cm and a total organic content of less than five parts per billion.
2. The peptide is solubilized in 40% DMSO and water (v/v) to a concentration of 1 mM.
3. The fluorescently labeled peptide is quantitated by measuring the absorbance of the fluorescein fluorophore at A_{492} using an extinction coefficient of 79,000 $cm^{-1}M^{-1}$. The concentrated peptide is diluted in 50 mM sodium carbonate, pH 9 prior to measuring the absorbance. Following quantitation, the peptide is examined on the Caliper to determine the degree of purity. The accepted level of purity, where the contaminant does not interfere with product peak assignment, is >95%.
4. The Caliper instrument can be run continuously for 5 days to shorten machine preparation and prolong the life of the microfluidic chip. All buffer solutions are stable throughout this time period. Only the chip needs to be refreshed daily with separation buffer.
5. Percent inhibition calculation

$$\left(1 - \left(\left(\mathrm{PSR}_{\mathrm{compound}} - \mathrm{PSR}_{100}\right)\left(\mathrm{PSR}_{0} - \mathrm{PSR}_{100}\right)\right)\right) \times 100.$$

6. Z′ calculation:

$$1 - \left(\left(3 \times \left(\mathrm{stdev}_{0} + \mathrm{stdev}_{100}\right)\right)/\left(\text{absolute value of } \left(\mathrm{average}_{0} - \mathrm{average}_{100}\right)\right)\right).$$

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