European Commission

STANDARD OPERATING PROCEDURE

for assessing specificity of DIO1 interaction using Alkaline phosphatase (ALP) testing as secondary readout, version 1.0

EURL ECVAM validation study of a battery of mechanistic methods relevant for the detection of chemicals that can disrupt the thyroid hormone system

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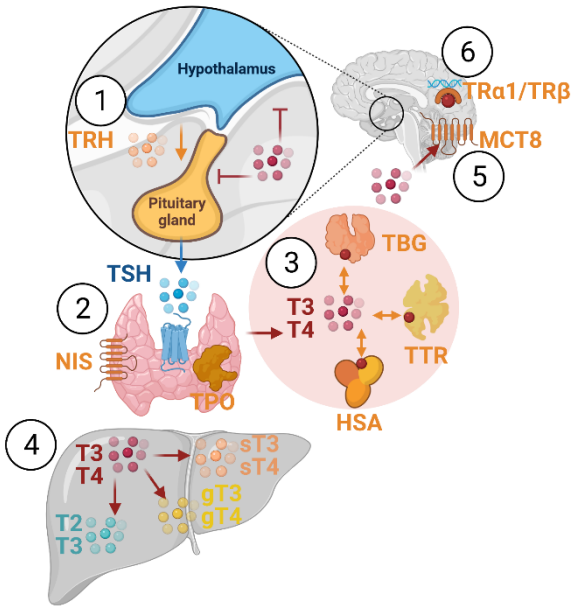

2023

Joint Research Centre

This Standard Operating Procedure (SOP) has been prepared within the context of a collaboration agreement with the Joint Research Centre (JRC) Directorate for Health, Consumers and Reference Materials (Chemicals Safety and Alternative Methods Unit F3 / EURL ECVAM), for the validation of mechanistic methods to identify potential modulators of thyroid hormone signalling. It aims to provide evidence-based scientific support to the European policymaking process. The contents of this publication do not necessarily reflect the position or opinion of the European Commission. Neither the European Commission nor any person acting on behalf of the Commission is responsible for the use that might be made of this publication. For information on the methodology and quality underlying the data used in this publication for which the source is neither Eurostat nor other Commission services, users should contact the referenced source. The designations employed and the presentation of material on the maps do not imply the expression of any opinion whatsoever on the part of the European Union concerning the legal status of any country, territory, city or area or of its authorities, or concerning the delimitation of its frontiers or boundaries.

This SOP is part of a series of 2 SOPs used to measure the " "Deiodinase 1 activity based on Sandell-Kolthoff reaction":

1. SOP "Colorimetric assessment of deiodinases activity based on Sandell-Kolthoff reaction with human microsomes: DIO1-SK assay" version 2.0 (used in Part2 of the validation study)
2. **SOP "Assessing specificity of DIO1 interaction using Alkaline phosphatase (ALP) testing as secondary readout" version 1.0 (used in Part 2 of the validation study)**

The method was developed by Kostja Renko when working at Charité Universitatsmedizin Berlin (DE), and subsequently implemented by the EU-NETVAL test facility BASF SE within the validation study.

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Standard Operation Procedure (SOP)

Colorimetric method for assessing deiodinases activity based on Sandell-Kolthoff reaction with human microsomes: DIO1-SK assay

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Assessing specificity of DIO1 interaction using Alkaline phosphatase (ALP) testing as secondary readout

AUTHOR

BASF SE

1. INTRODUCTION

1.1. BACKGROUND AND OBJECTIVE

The alkaline phosphatase (ALP) is a homodimeric enzyme, containing two Zn and one Mg ion on each catalytic site, which are crucial for catalytic function. ALP catalyses the dephosphorylation of compounds and has important functions in bone mineralization and intestinal absorption. Important human isoforms are the tissue-nonspecific alkaline phosphatase (TNAP), the placental alkaline phosphatase, Germ cell alkaline phosphatase, and the germ cell alkaline phosphatase (GCALP). TNAP is expressed in the developing nervous system, skeletal tissues, kidney, and the liver (Millán 2006).

Alkaline phosphatase activity is usually monitored by the formation of yellow *para*-nitrophenol from *para*-nitrophenylphosphate (PNPP) as a function of alkaline phosphatase-mediated dephosphorylation activity with an absorption maximum of 405 nm.

TNAP activity is also present in human liver microsomes and is used in this method as a secondary enzyme activity testing for the DIO1-SK assay. Inhibition in both the DIO1-SK assay as well as on ALP activity might indicate towards unspecific inhibition, for example due to unspecific protein binding or modification.

1.2. REFERENCES

Dahl, R., E. A. Sergienko, Y. Su, Y. S. Mostofi, L. Yang, A. M. Simao, S. Narisawa, B. Brown, A. Mangravita-Novo and M. Vicchiarelli (2009). "Discovery and validation of a series of aryl sulfonamides as selective inhibitors of tissue-nonspecific alkaline phosphatase (TNAP)." Journal of medicinal chemistry **52**(21): 6919-6925.

Millán, J. L. (2006). "Alkaline phosphatases." Purinergic signalling **2**(2): 335-341.

2. MATERIAL

Table 1: Used apparatus

Apparatus	Requirements[1] Suggested type[2]
Analytical balance	capable of accurately weighing up to 30 g with 0.1 mg readability[1]

Pipets capable of delivering 1 to 10 µL	
Pipets capable of delivering 10 to 100 µL	
Pipets capable of delivering 100 to 1000 µL	
Multichannel pipette capable of delivering 10 to 100 µL	
Multichannel dispenser capable of delivering 50 to at least 1000 µL	
Repeater pipette	
Pipets for higher volumes	serological pipettes, e.g. 10, 25, 50 mL[2]
Incubator	capable of keeping temperatures of 37°C, 5 % CO_2 and ≥90 % humidity[1]
pH meter with electrode and calibration buffers	capable of reading +/- 0.1 pH units[1]
Photometer for absorbance measurement	The photometer used must be able to heat up to 37°C[1] e.g., Sunrise™ Absorbance Reader, INSTSUN-3, Tecan Trading AG[2]

Table 2: Used chemicals and reagents in ALP activity testing.

Chemicals / reagents	Requirements[1] Suggested type[2]
6-Propyl-2-thiouracil (6PTU) CAS: 51-52-5 MW: 170.23 g/mol	e.g. 6-Propyl-2-thiouracil, VETRANAL™, analytical standard, Supelco[2]
Dimethyl sulfoxide (DMSO) CAS: 67-68-5 MW: 78.13 g/mol	e.g. dimethyl sulfoxide (Reag. Ph. Eur.) for analysis, ACS, PanReac AppliChem[2o]
Human liver microsomes	e.g. Human Microsomes, 50 Donors, HMMCPL, Gibco[2] or Microsomes from Liver, Pooled, from human, Sigma-Aldrich[2]
Diethanolamine (DEA) CAS: 111-42-2 MW: 105.14 g/mol	e.g. Diethanolamine, reagent grade, ≥98.0%, Sigma-Aldrich[2]
Magnesium chloride ($MgCl_2$) CAS: 7791-18-6 MW: 203.30 g/mol	e.g. Magnesium chloride hexahydrate, ACS reagent, 99.0-102.0%, Sigma-Aldrich[2]
Para-Nitrophenyl phosphate (PNPP) CAS: 333338-18-4 MW: 371.14 g/mol	e.g. Phosphatase substrate, 5 mg tablets, 4-Nitrophenyl phosphate disodium salt hexahydrate, Sigma-Aldrich[2]
10% (w/w) Hydrogen chloride (HCl) CAS: 7647-01-0	e.g. Hydrochloric acid 10%, EMPROVE® EXPERT Ph Eur,JP,NF, Sigma-Aldrich[2]

MW: 36.46 g/mol	
TNAP inhibitor CAS: 496014-13-2 MW: 344.38 g/mol	e.g. TNAP Inhibitor - CAS 496014-13-2 – Calbiochem, Sigma-Aldrich[2]

Table 3: Material that is used in ALP activity testing.

Material:	Requirements[1] Suggested type[2]
Volumetric flask	certified with defined volume[1]
Assay plates (96 well format)	e.g. tissue culture plates, 96 well plate, flat bottom, polystyrene, 0.34 cm^2, sterile, 108/cs, TPP[2]
Microcentrifuge tubes 1.5 mL	e.g. Eppendorf® Safe-Lock microcentrifuge tubes, volume 1.5 mL, natural, Eppendorf AG[2]

Table 4: Software that is used in ALP activity testing.

Software	Requirements[1] Suggested type[2]
Statistics software	Able to perform regression analysis that reflect assay characteristics and able to calculate inhibitory concentrations[1] e.g. GraphPad Prism 8, GraphPad[2]

3. TEST SYSTEM

The minimum requirements for human liver microsomes are described in Table 5. The human microsomes should be tested for all known human liver microsomal contaminations in compliance with GIVIMP (OECD, 2018).
The used microsome concentration per well for the ALP reactions is the same than the determined microsome batch-specific concentration for DIO1 testing. Microsome batch-specific DIO activity testing is further specified in SOP: DIO1-SK assay.
The microsomes should be stored at ≤ -80°C until required for use.

Table 5: Minimum requirements for the used microsome batch.

Species	human
Tissue	liver
Sex	mixed gender
Pool	≥25 donors
Age	various
Demonstrated absence of the following contaminations	Hepatitis B Hepatitis C Human Immunodeficiency Virus (HIV)

4. CONTROLS

The reference item “tissue-nonspecific (TN) alkaline phosphatase (AP) inhibitor” (2,5-Dimethoxy-N-(quinolin-3-yl)benzenesulfonamide, CAS no.: 496014-13-2) is a described specific inhibitor of TNAP (Dahl, Sergienko et al. 2009) and was used as the reference item for ALP testing. The DIO1 and TPO inhibitor 6-Propyl-2-thiouracil (6PTU) was used as negative control in ALP testing since 6PTU does not inhibit ALP activity.

Table 6: Overview of the used controls in ALP activity testing.

Controls:	
Reference item (RI)	Quantitatively controls ALP inhibition in the assay and is used for normalization to maximum inhibition in the assay. In addition to control replicates on each assay plate of the highest concentration, a concentration-response curve of the reference item is performed on each assay day. Used in ALP activity testing: TNAP inhibitor at a maximum assay concentration of $3.16*10^{-5}$. Concentration-response curves of TNAP inhibitor ranged from $3.16*10^{-5}$ to 10^{-9} M.
Negative control (NC)	A substance that leads to no inhibition of ALP activity. Used in ALP activity testing: 6-Propyl-2-thiouracil at a maximum assay concentration of 10^{-3} M.
Solvent control (SC)	Blank control. The solvent control ensures that the response does not originate from the applied solvent and shows no ALP inhibition. Solvent controls are used to normalize the inhibition observed with the test items to the maximum possible ALP activity. Final solvent concentration in the assay: Dimethyl sulfoxide (DMSO) at an assay concentration of 1%.

Table 7: Information on the reference item TNAP

Name:	2,5-Dimethoxy-N-(quinolin-3-yl)benzenesulfonamide
CAS No.:	496014-13-2
Molecular weight [g/mol]:	344.38
Storage conditions:	2-8°C
Solvent	DMSO
Stock solution [mol/L]:	10^{-1}
Storage conditions of stock solution	2-8°C

Table 8: Information on the negative control 6PTU

Name:	6-Propyl-2-thiouracil
CAS No.:	51-52-5

Molecular weight [g/mol]:	170.23
Storage conditions:	RT
Solvent	DMSO
Stock solution [mol/L]:	10^{-1}

5. METHOD

5.1. PRE-ASSAY

5.1.1. ALP assay buffer preparation

1. Thaw Diethanolamine (DEA) at 37°C (melting point of DEA: 28°C): big volumes need to be thawed overnight; once thawed, small aliquots of DEA can be prepared for future ALP assay buffer preparation
2. Prepare a 200 mM $MgCl_2$ stock solution (1000x stock) by weighing in 40.66 mg $MgCl_2$ and dissolving in 1 ml diH_2O; vortex to aid dissolution
3. Prepare 500 ml ALP assay buffer (20 mM DEA / 200 µM $MgCl_2$) in diH_2O: weigh in 1.05 g of thawed DEA and dissolve by addition of 500 ml diH_2O; add 500 µl of 200 mM $MgCl_2$ (1000x stock) to the DEA solution
4. Adjust pH to 9.8 by dropwise addition of 10% (w/w) HCl
5. Store the ALP assay buffer at 4°C

5.1.2. Solubility testing of a test item in ALP assay buffer

In the context of ALP testing, the solubility of test items needs to be assessed in their stock solutions, in prepared dilutions and under assay conditions. The solubility of the test item in solvent (DMSO preferred) as a stock solution as well as the dilution in water was already assessed in the DIO1-SK assay (see SOP: DIO1-SK assay) and the defined conditions will be used for the preparation of the stock solution in the ALP activity testing.

1. Prepare the stock solution of the test item in DMSO; use the concentration that was determined in DIO1-SK testing
2. Gently mix at room temperature. Vortex the tube if necessary
3. Prepare a 10% (v/v) dilution of the test item stock solution in diH_2O as determined in DIO1-SK testing
4. Add 100 µl of the prepared dilution, 400 µl diH_2O and 500 µl ALP assay buffer to a 24-well plate to test solubility under assay conditions; make sure the added solutions are mixed, either by shaking or pipetting
5. Visually check by using a microscope if the test item solution is dissolved under assay conditions
6. If the test item hasn't dissolved, use water bath sonification for up to 5 mins or warm the solution to 37°C for up to 60 mins; repeat step 5 to check if the test item is dissolved
7. If the test item is not soluble under assay conditions, dilute the test item stock solution in DMSO (e.g., by reducing the concentration by a factor of 10), prepare the resulting 10% (v/v) dilutions of the test item stock solutions in diH_2O as well the solutions under assay conditions as described in step 4

8. Visually check by using a microscope if the test item solution is dissolved under assay conditions
9. Repeat step 7 until the test item is fully dissolved

5.1.3. Preparation of the reference item TNAP inhibitor stock solution

The TNAP inhibitor is used as the reference item on the first plate on each assay day with a full concentration-response curve. Additionally, the highest concentration of the reference item is included on all plates in at least 6 replicates and is used to determine the background reaction occurring at full ALP inhibition.
The stock solution of the TNAP inhibitor can be prepared prior to the assay run and can be stored at 4°C. The dilutions in DMSO for the concentration-response testing are prepared fresh on each day of assay performance.

Stock solution

1. Weigh in a respective amount of reference item in a brown glass vial and dissolve in DMSO resulting in a concentration of 100 mM (ALP-RI-S0*); if the amount of the reference item in a supplied vial is ≤5 mg, add the amount of DMSO to the vial directly resulting in a concentration of 100 mM (ALP-RI-S0)
2. Store ALP-RI-SO at 4°C

*The stock solution is labelled with “ALP” here to prevent mix-ups of long-termed stored solutions

5.2. ASSAY RUN

5.2.1. Preparation of dilutions

5.2.1.1. Preparation of stock solutions and dilutions of the test items

Use the test item concentration that was fully dissolved in the stock solution and the resulting 10% (v/v) dilution diH_2O as well as solution under assay conditions to prepare the stock solution of the test item:

1. Weigh the appropriate amount of test item into a glass vessel and dissolve in DMSO to prepare the stock solution for test item 1 (TI1-S0)
2. For the preparation of the test item dilutions, the test item stock solution is subsequently diluted seven times with diH_2O and the appropriate solvent in a 1:10 ratio to obtain eight test item dilutions with a solvent concentration of 10 % (v/v) as shown in Table 9.

Table 9: Preparation of the test item dilutions using test item stock solutions

Name of the test item dilution	diH_2O [µL]	DMSO [µL]	Test item	Dilution factor
TI1-C1	450	-	50 µL TI1-S0	1:10
TI1-C2	405*	45*	50 µL of TI1-C1	1:10
TI1-C3	405*	45*	50 µL of TI1-C2	1:10
TI1-C4	405*	45*	50 µL of TI1-C3	1:10
TI1-C5	405*	45*	50 µL of TI1-C4	1:10
TI1-C6	405*	45*	50 µL of TI1-C5	1:10
TI1-C7	405*	45*	50 µL of TI1-C6	1:10
TI1-C8	405*	45*	50 µL of TI1-C7	1:10

*A 10 % (v/v) solvent / diH2O solution can be prepared and 450 µL of the dilution can be added instead

5.2.1.2. Preparation of a stock solution and dilution of the reference item

The stock solution of the reference item TNAP inhibitor was prepared prior to the assay and was stored at 4°C.

1. On the day of analysis, prepare the reference item dilutions from the 100 mM reference item stock solution (ALP-RI-S0) according to Table 10; RI-D0 is used as a predilution and will not be used for testing in the assay
2. Label the subsequent reference item dilutions derived from the reference item stock solution adequately (e.g. RI-D1, RI-D2,…., RI-D8)

Table 10: Preparation of the dilutions for the reference item TNAP inhibitor.

Name of the reference item dilution	Reference item dilution concentration [M]	diH_2O [µL]	DMSO [µL]	Reference item [µL]	Final concentration of reference item in the assay [M]
RI-D0	$3.16*10^{-3}$	450	34.2	15.8 µL of ALP-RI-S0	-
RI-D1	$3.16*10^{-4}$	405*	45*	50 µL of RI-D0	$3.16*10^{-5}$
RI-D2	$3.16*10^{-5}$	405*	45*	50 µL of RI-D1	$3.16*10^{-6}$
RI-D3	10^{-5}	405*	45*	50 µL of RI-D2	10^{-6}
RI-D4	$3.16*10^{-6}$	405*	45*	50 µL of RI-D3	$3.16*10^{-7}$
RI-D5	10^{-6}	405*	45*	50 µL of RI-D4	10^{-7}
RI-D6	$3.16*10^{-7}$	405*	45*	50 µL of RI-D5	$3.16*10^{-8}$
RI-D7	10^{-7}	405*	45*	50 µL of RI-D6	10^{-8}
RI-D8	10^{-8}	405*	45*	50 µL of RI-D7	10^{-9}

*You can also prepare a 10 % DMSO / diH_2O solution and add 450 µL of the dilution

5.2.1.3. Preparation of a stock solution and dilution of the negative control

Prepare a 10^{-1} M stock solution for the negative control 6PTU fresh on the day of analysis.

1. Based on the amount of stock solution needed, weigh an appropriate amount of 6PTU into a suitable vessel.
2. Add the appropriate amount of DMSO and vortex the vessel

Example:
To prepare 1 mL of a 10^{-1} M stock solution of 6-Propyl-2-thiouracil in DMSO with a molecular weight of 170.23 g/mol, 17 mg of the substance was weighed into a vessel and solved in 1 mL of DMSO.

$$m = c * V * M = 0.1 \frac{mol}{l} * 0.001\ l * 170.23 \frac{g}{mol} = 0.017\ g = 17\ mg$$

3. Prepare the negative control dilution from the 10^{-1} M negative control stock solution according to Table 11.

Table 11: Preparation of the negative control 6-propyl-2-thiouracil (6PTU) dilution.

Negative control dilution [M]	diH_2O [µL]	DMSO [µL]	Negative control [µL]	Final concentration of negative control in the assay [M]

10^{-2}	450	-	50 µL of 10^{-1} M negative control stock solution	10^{-3}

5.2.1.4. Preparation of the PNPP substrate solution

The substrate solution must be prepared fresh on each day. Keep substrate in the dark to prevent loss of activity in the assay.

1. Dissolve 5 mg PNPP in 449.8 µl diH_2O to a final concentration of 30 mM to prepare a 100x PNPP stock solution
2. Dilute the 100x PNPP stock solution 1:100 (v/v) in ALP assay buffer to prepare a 1x PNPP solution with a final concentration of 0.3 mM of PNPP; 6 ml of 1x PNPP solution is needed for one 96-well plate of ALP testing

5.2.2. Microsome incubation with test items

1. Prepare the reference item stock solution as well as dilutions as described in 5.2.1.2, the negative control stock solution as well as dilution as described in 5.2.1.3 and the test item stock solutions as well as dilutions as described in 5.2.1.1.
2. Preheat the absorbance reader to 37°C
1. On the first plate of an assay day, add 10 µL of the reference item dilutions to a 96-well plate to perform a full concentration-response testing. For the solvent control, add 10 µl of a 10 % (v/v) DMSO in diH_2O solution. For the negative control, add 10 µl of the prepared dilution of the negative control. Add 10 µl of the test item dilutions to the 96-well plate.
   A proposed plate layout for the first run of an assay day is shown in Table 12: plate layout for the first plate of an assay day of the ALP activity testing; a proposed plate layout for additional runs on the same assay day is shown in Table 13.
3. Add 40 µL of a defined protein dilution (resulting in the calculated amount of enzyme per sample well calculated in SOP: DIO1-SK assay that is specific for the used microsome batch) to the wells
4. Add 50 µl 1x PNPP solution to each well using a multichannel pipette
1. As soon as possible after the application of 1x PNPP solution, determine the optical density (OD) in a plate reader with the following settings:
   - Absorption parameters: 415 nm (±2 nm)
   - Initial shaking: weak for 5 s
   - Target temperature: 37°C, valid temperature range 35 – 39°C
   - Measurement of the OD every minute for 60 min (other time periods are possible, make sure the detection is in linear range), also measuring the initial OD

Table 12: plate layout for the first plate of an assay day of the ALP activity testing

	1	2	3	4	5	6	7	8	9	10	11	12
A	SC			RI-C1			NC			RI-C1		
B	RI-C1			RI-C2			RI-C3			RI-C4		
C	RI-C5			RI-C6			RI-C7			RI-C8		
D	TI1-C1			TI1-C2			TI1-C3			TI1-C4		

E	TI1-C5	TI1-C6	TI1-C7	TI1-C8
F	TI2-C1	TI2-C2	TI2-C3	TI2-C4
G	TI2-C5	TI2-C6	TI2-C7	TI2-C8
H	SC	RI-C1	SC	NC

SC **solvent control** 1% DMSO — **RI** **reference item** TNAP inhibitor $3.16*10^{-5}$ M — **NC** **negative control** 6PTU 10^{-3} M — **TI** **test item**

Table 13: plate layout for additional plates of an assay day of the ALP activity testing

	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
A	SC			RI-C1			NC			RI-C1		
B	TI3-C1			TI3-C2			TI3-C3			TI3-C4		
C	TI3-C5			TI3-C6			TI3-C7			TI3-C8		
D	TI4-C1			TI4-C2			TI4-C3			TI4-C4		
E	TI4-C5			TI4-C6			TI4-C7			TI4-C8		
F	TI5-C1			TI5-C2			TI5-C3			TI5-C4		
G	TI5-C5			TI5-C6			TI5-C7			TI5-C8		
H	SC			RI-C1			SC			NC		

SC **solvent control** 1% DMSO — **RI** **reference item** TNAP inhibitor $3.16*10^{-5}$ M — **NC** **negative control** 6PTU 10^{-3} M — **TI** **test item**

5.2.3. Evaluation of the data

1. Determine the ΔOD_{21min} values via subtraction of the 60-minute values of all samples from the initial measured values:

   $$\Delta OD_{60min} = OD_{60min,415nm} - OD_{0min,415nm}$$

2. Determine the ΔOD-BG values, by subtracting the mean of ΔOD_{60min} values of the inhibited $3.16*10^{-5}$ M TNAP inhibitor reference item controls from the ΔOD_{60min} values of all samples:

   $$\Delta OD\text{-}BG = \Delta OD_{60min} - \overline{\Delta OD_{60min,RI}}$$

Where “RI” represents the reference item TNAP inhibitor

3. Normalize the values of the test item to the respective solvent control values via division of the test item(s) ΔOD-BG values by the mean of the ΔOD-BG values of the respective solvent control, generating Alkaline phosphatase (ALP) activity values. State ALP activity values in %. Keep in mind that test items with differing solvents need to be normalized to their respective solvent controls:

$$ALP\ activity = \frac{\Delta OD\text{-}BG_{TI}}{\Delta OD\text{-}BG_{SC}} * 100$$

where “TI” represents the test item at used concentrations and “SC” the solvent control

4. Plot the ALP activity values of the different test item concentration samples in a statistics software with ALP activity values on y-axis (linear) and test item concentrations on x-axis (logarithmic)

5. Use a curve-fit algorithm to visualize a concentration-response relationship (e.g. “[Inhibitor] vs. response -- Variable slope (four parameters)” in GraphPad Prism 8):

$$Y = Bottom + (Top - Bottom) / (1 + 10^{(LogIC50 - x) * HillSlope)})$$

where “Top” represents the maximal response, “Bottom” represents the lowest response, and “HillSlope” describes the steepness of the curve.

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