Erythrocytes Thiopurine Methyltransferase (TPMT) Activity Immunoassay

USER GUIDE

1. Intended Use

The Patented Biologix TPMT Activity Assay is a competitive micro-well immunoassay for the semi-quantitative determination of TPMT activity in erythrocytes.

2. Introduction

TPMT is a cytoplasmic enzyme that catalyzes the S-methylation of aromatic and heterocyclic sulfhydryl compounds that include several anti-cancer thiopurine drugs such as thioguanine, azathiopurine, and mercaptopurine. TPMT activity exhibits genetic polymorphism in the human population. As a result of these genetic polymorphisms, a significant fraction of the population cannot metabolize these therapeutic drugs. Therefore measurement of TPMT activity levels is important in understanding of the drug metabolism, toxicity, and therapeutic efficacy.

3. Test Principle

The Biologix TPMT Activity assay is a competitive micro-well immunoassay for the semi-quantitative determination of TPMT activity in erythrocytes prepared from lysed red blood cells. TPMP catalyzes the S-methylation of 6-mercaptopurine (6-MP) with the presence of S-adenosylmethionine (SAM), the methyl donor, yielding 6-methylmercaptopurine (6-MMP). 6-MMP is then measured with a micro-well EIA.

TPMT, SAM
6-mercaptopurine (6-MP) -----------------------------> 6-methylmercaptopurine (6-MMP)

Micro-well EIA principal: The Biologix Micro-well EIA is a competitive immunoassay. 6-MMP in the sample and 6-MMP conjugated with the enzyme compete for binding with its specific antibody which is coated on the solid surface of the micro-well. If little or no 6-MMP is present in the sample more enzyme labeled 6-MMP will bind to the antibody on the solid surface. If a large or a significant amount of 6-MMP is present in the sample, less enzyme labeled 6-MMP will bind to the antibody, produce a lower color signal. The absorbance produced is inversely proportional to the amount of 6-MMP in the sample, calibrator or control. Using calibrators of known TPMT activities plotted against their response, the TPMT activity in the unknown samples can be quantified.

TPMT activity definition: One unit of TPMT is defined as a formation of 1 nmol of 6-MMP per ml of packed red blood cells per 60 min incubation at 37 ^{o}C.

4. Reagents

4.1. Sample Preparation Materials

Item Name	Quantity	Preparation Required
6-MP Solution	1 each	No
SAM	1 each	Yes, as described in 'Section 4.3 - Reagent preparation'
Reaction Buffer	1 each	No
Reaction Stop Solution	1 each	No

4.2. Micro-well Assay Materials

Item Name	Quantity	Preparation Required
Antibody Coated Micro-well Plate	1 each	No
ELISA Buffer	1 each	No
Enzyme-Conjugate Concentrate	1 each	Yes, as described in 'Section 4.3 - Reagent preparation'
Calibrators	1 set [*1]	No
Controls	1 set [*2]	No
Wash Concentrate	1 each	Yes, as described in 'Section 4.3 - Reagent preparation'
Substrate	1 each	No
ELISA Stop Solution	1 each	No

[*1]: Calibrator: 6 levels:
Calibrator 1: 0 U
Calibrator 2: 3.75 U
Calibrator 3: 7.5 U
Calibrator 4: 15 U
Calibrator 5: 30 U
Calibrator 6: 60 U

[*2]: Controls: 2 levels:
Low Control: 1 vial, Target conc. 5.6 U
High Control: 1 vial, Target conc. 45 U

4.3. Preparation of Reagents:

4.3.1. Preparation of SAM solution

S-Adenosylmethionine (SAM) was provided as 5 mg powder in a 2-ml micro centrifuge tube. Add 1.8 ml distilled water to dissolve the powder completely before use. The solution is stable at Room Temperature (23 ± 4 °C) for 5 hours.

4.3.2. Preparation of Working Wash Solution

Mix 25 ml of the 20 X Wash Concentrate with 475 ml of deionized or distilled water to make the Working Wash Solution. Store the Working Wash Solution at room temperature. The Working Wash Solution is stable at Room Temperature (23 ± 4 °C) for one month (31 days).

4.3.3. Preparation of the Working Enzyme-Conjugate

The conjugate concentrate has a titer of 1:100. The working Enzyme-Conjugate is prepared by mixing 150 µl of the Enzyme-Conjugate concentrate with 14.85 ml of the ELISA Buffer. The prepared working enzyme-conjugate is stable at 2- 27 °C for 12 hours.

4.4. Expiration:

All reagents expire on the dates shown on their respective labels.

4.5. Warning and Precautions

4.5.1. Proper handling of all reagents is strongly advised. Discard the Substrate if obvious blue color develops.
4.5.2. Keep all containers closed when not in use to avoid contamination and evaporation.
4.5.3. Avoid contact with reagents.
4.5.4. Do not mouth pipet reagents. Handle all reagents as if potentially infectious.
4.5.5. Do not drink or eat near the kit reagents.
4.5.6. Do not use expired reagents.
4.5.7. All wells of the micro-plates are for 'single use only'.

5. Instrument

There is no special instrument required for the assay.

5.1. Equipment for pipetting:

Single and/or multi-channel pipets that can deliver a volume of 10 to 200 µl may be used.

5.2. Plate Washer and Plate Reader.

The recommended procedure for the wash is to use an automatic micro-plate 96 well washer. The washer has to be calibrated per its manufacturer's recommended procedure prior to washing.

Any plate reader that can read 450 nm spectrophotometically can be used. The plate reader has to be calibrated per its manufacturer's recommended procedure prior to be used.

6. Specimen Collection, Handling and Storage

6.1.Specimen Collection:

An ideal specimen is a venous blood collected in EDTA tube. Collected specimens should be shipped to a testing laboratory via '2-8 ^{o}C Next-Day' delivery. Blood specimen can not be frozen. Frozen and/or hemolyzed specimen should be rejected.

6.2. Specimen Handling and Storage:

Upon receipt in the laboratory, process the specimens within 24 hours per the procedure below (7.3. Sample Processing for Micro-well Immunoassay).

Precautions

- Proper handling of all specimens is strongly advised.
- Handle all specimens as if potentially infectious.

6.3. Sample Processing for Micro-well Immunoassay

RBC lysate preparation:

6.3.1. Centrifuge 1 ml of blood in a micro centrifuge tube at 3,000 g for 3 min at RT
6.3.2. Discard the plasma and the buffy coat on top of the red blood cells (RBCs)
6.3.3. Add 750 µl saline (not provided in the kit) and mix the RBC by up-down pipeting using a transfer pipet
6.3.4. Centrifuge the RBC in saline at 3,000 g for 3 min at RT
6.3.5. Discard the supernatant
6.3.6. Repeat Steps: 6.3.3 – 6.3.5 for two more times
6.3.7. Set the last centrifugation at 10,000 g and spin for 10 min
6.3.8. Carefully remove all the supernatant
6.3.9. Label another micro centrifuge tube and fill with 400 ul ice cold distilled water and keep it on ice
6.3.10. Use a provided wide-opening pipette tip to dip into the bottom of the packed red blood cells from Step 6.3.8 above, slowly and accurately draw 100 ul. Slowly dispense the packed RBC into the 400 ul water and rinse the tip by up-down pipeting. Shake the tube for at least twenty times, or vortex the tube for 30 seconds to lyse the RBCs completely. Set the tube on ice for four min.
6.3.11. Centrifuge the RBC lysate at 13,000 g for 10 minutes.
6.3.12. Carefully transfer the top supernatant for testing.

Note: RBC lysate is stable at 2-8 °C within 8 hours of preparation.

Methylation Reaction

1) Add 75 µl Reaction Buffer to a labeled 0.5 – 1.5 ml micro tube
2) Add 10 µl SAM Solution (follow the instruction on label)
3) Add 50 µl lysate from Step 6.3.12 above
4) <u>Warm up the tube at 37 ^{o}C for 10 minutes</u>
5) <u>Warm up the MP Solution to 37 ^{o}C</u>
6) Add 15 ul MP Solution to start the reaction. Make sure the solution is mixed well. Perform a quick spin if necessary to make sure all liquid are at the bottom of the tube.
7) Incubate the tube at 37 ^{o}C for exactly 60 minutes.
8) Add 100 µl Reaction Stop Solution. Mix well.

Note: The reaction mix is stable for 4 hours at room temperature.

7. Micro-Plate Test Procedure

Note: Follow the provided plate map to set up the test
Allow reagents to come to room temperature (23 ± 4 °C) before use.

7.1. Add 100 µl ELISA Buffer to each well.
7.2. Add 10 µl of Calibrators, Controls and unknown specimens to each well per plate map.
7.3. Add 100µl of working Enzyme-Conjugate (step 4.3.3) to all wells and cover the plate.
7.4. Incubate the plate at room temperature (23 ± 4 °C) for 30 minutes.
7.5. Wash-aspirate all wells 5 times with 300 µl Working Wash Solution (step 4.3.2). We strongly recommend to use an automated microtiter plate washer.
7.6. Add 100µl of the Substrate Solution to all wells. Cover the plate and incubate at RT in dark for 30 minutes. Start step 7.7 after 15 min if the color are too dark.
7.7. Add 100 µl ELISA Stop Solution to each well.
7.8. Read the plate at 450 nm within 15 minutes.

8. Calculations and Interpretation of Results

8.1. Data validation:
If the following quality control acceptance criteria are satisfied, the assay is valid:
The OD of the Low Control is greater than the OD of the 15 U Calibrator
The OD of the High Control is lower than the OD of the 15 U Calibrator

8.2. Result Calculation:
A calculation program is provided in the CD within the package. The same program is also available online for download at HTTP://www.TPMTassay.com/download.asp. Type in or transfer your OD readings from Step 7.8 per plate map, the results will be calculated automatically.

8.3. Result Interpretation:
Human TPMT activity levels vary significantly, even in the population that has no known genetic deficiency. According to literatures and current clinical practice, we provided the following SUGGESTED result interpretation guide just for your reference. It is the clinicians' and researchers' responsibility to interpret the results. Therapeutic decisions and research conclusions can NOT be made based on the following reference values.

< 5.5 Unit: Very Low TPMT Activity Level, indicating a possible Homozygous Deficiency genomic type, approximately 0.3% of the human population

5.6-15.5 Unit: Medium TPMT Activity Level, indicating a possible Heterozygous Deficiency genomic type, approximately 11% of the human population

> 15.6 Unit: Normal to High TPMT Activity Level, indicating a possible Wide Type (normal) genomic type, approximately 89% of the human population

9. Possible Sources of Error

9.1. Blood specimen was not shipped in time
9.2. Blood specimen was not shipped properly
9.3. Blood specimen was not processed in time (within 24 hours of receiving)
9.4. Blood specimen was not processed properly
9.5. RBC lysate was not assayed in time (within 8 hours of preparation)
9.6. Non-uniform temperature during sample process and micro-well assay steps

9.7 Uneven washing: For valid result, washing of all wells must be thorough and uniform.
9.8.Uneven pipetting or carry-over may give imprecise results. Pipetting specimens and controls must be done very carefully using one and the same pipettor
9.10 Improper storage of reagents: The color signal and precision may deteriorate significantly if the ELISA plate has been at room temperature for more than 2 hours after coming to RT.

10. Specific Performance Results

10.1 Analytical Sensitivity/Low Detection Limit

The Analytical Sensitivity of the assay is 1.0 U.

10.2 Linearity:

The assay is linear up to 60 U. The lysate for a specimen with more than 60 U (extremely uncommon) may be diluted with Calibrator 1 (0 U) for further testing.

10.3 Intra and Inter assay Precisions

10.3.1 Precision results with calibrator matrix

6-MMP concentrations equivalent to 0, 7.5. 15, 30 U TPMT were spiked in the calibrator matrix. These 4 samples were tested 12 replicates per run for 5 runs.

Intra assay %CV:

Specimen #	SP 1	SP 2	SP 3	SP 4
TPMT (U) Equivalent	0	7.5	15	30
OD	2.505	1.825	1.554	1.297
SD	0.109	0.063	0.047	0.036
N	60	60	60	60
Mean %CV	8.42	7.96	7.10	7.72

Inter assay %CV:

Specimen #	SP 1	SP 2	SP 3	SP 4
TPMT (U) Equivalent	0	7.5	15	30
N	60	60	60	60
Mean %CV	8.14	9.95	9.25	9.32

10.3.2 Precision results with real samples

Sample preparations of a whole blood were tested 12 replicates per run for 5 runs.
Intra assay %CV:

TPMT (U)	19.0
OD	1.485
SD	0.066
N	60
Mean %CV	8.90

Inter assay %CV:

TPMT (U)	19.0
N	60
Mean %CV	9.15

10.4 **Interference**

Sixty-seven (67) compounds, many of them commonly prescribed medicines and abused drugs, were selected for the study. A concentration of 10 µg/ml of each compound was found to have no positive or negative interference with the assay.

Acetaminophen	Ibuprofen
(5)-Acetylmorphine	Imipramine
Acetylsalicylic Acid	Isoxsuprine
(4)-Aminophenyl Sulfone	Ketamine
Amobarbital	Lidocaine
Ampicillin	Loperamide
Atenolol	Meperidine
Atropine	Mephentermine Hemisulfate
Benzoylecgonine	Methadone
Butabarbital	Nalbuphine
Chlordiazepoxide	Nalorphine
Chlorpromazine	Naproxen
Clonazepam	Niacinamide
Clorazepate	Norcocaine
Cocaethylene	Nystatin
Cocaine	Oxycodone
Codeine	Oxymorphone
(-)-Cotinine	Phencyclidine
Diacetylmorphine	Penicillin
Diazepam	Pentobarbital
Diphenhydramine	Phenylalanine
Ecgonine HCl	Phenylephrine
Ecgonine Methyl Ester	(beta)-Phenylethylamine
Fenfluramine	para-Methoxyamphetamine
Fenoprofen	para-Methoxymethamphetamine
Fluoxetine	Procainamide
Gemfibrozil	Procaine
Gentisic Acid	Propranolol
Glipizide	Quinidine
Heroin hydrochloride	Ranitidine
Hydrochlorothiazide	Salbutamol
Hydrocodone	Tolmetin
Hydromorphone	Zomepirac
Hydroxybenzoylecgonine	Δ9-Tetrahydrocannabinol

10.5 **Cross Reactivity**

The following compounds, structurally and/or clinically related to 6-MMP, were tested for their cross-reactivities to the anti-6-MMP antibody at concentrations up to 10,000 ng/ml. The results were as follows:

	Spiked Conc. (ng/ml)	6-MMP Equivalent (ng/ml)	% Cross Reactivity
6-MMP	100	100	100%
6-MP	10,000	<2.5	<0.025%
Thioguanine	10,000	<2.5	<0.025%
Azathiopurine	10,000	<2.5	<0.025%

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Std. Units		1	2	3	4	5	6	7	8	9	10	11	12
0	**A**	Cal. #1	Cal. #1	SP1-1	SP1-2	SP9-1	SP9-2	SP17-1	SP17-2	SP25-1	SP25-2	SP33-1	SP33-2
3.75	**B**	Cal. #2	Cal. #2	SP2-1	SP2-2	SP10-1	SP10-2	SP18-1	SP18-2	SP26-1	SP26-2	SP34-1	SP34-2
7.5	**C**	Cal. #3	Cal. #3	SP3-1	SP3-2	SP11-1	SP11-2	SP19-1	SP19-2	SP27-1	SP27-2	SP35-1	SP35-2
15	**D**	Cal. #4	Cal. #4	SP4-1	SP4-2	SP12-1	SP12-2	SP20-1	SP20-2	SP28-1	SP28-2	SP36-1	SP36-2
30	**E**	Cal. #5	Cal. #5	SP5-1	SP5-2	SP13-1	SP13-2	SP21-1	SP21-2	SP29-1	SP29-2	SP37-1	SP37-2
60	**F**	Cal. #6	Cal. #6	SP6-1	SP6-2	SP14-1	SP14-2	SP22-1	SP22-2	SP30-1	SP30-2	SP38-1	SP38-2
High Control	**G**	High-Control	High-Control	SP7-1	SP7-2	SP15-1	SP15-2	SP23-1	SP23-2	SP31-1	SP31-2	SP39-1	SP39-2
Low Control	**H**	Low Control	Low Control	SP8-1	SP8-2	SP16-1	SP16-2	SP24-1	SP24-2	SP32-1	SP32-2	SP40-1	SP40-2

Note: ***The result calculation program was written according to this specific plate map. If you are going to use the program to calculate your testing results, please strictly follow this plate map.***