Volumetric Absorptive Micro Sampler (VAMS or Mitra) in Clinical Diagnostic:

-Therapeutic Drug Monitoring and Diagnostic Biomarkers

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Exagen Diagnostics Inc.

exagen DIAGNOSTICS

Acknowledgements

Neoteryx LLC (Provides All Mitra)

Dr. Stuart Kushon

Bobby Virasingh

Clinical Trial Patients Recruiting

Site 1: Dr. Smitha Reddy

Site 2: Dr. Puja Chitkara

Thermo Fisher Scientific

Dr. Anastasia Kalli

Dr. Raimund Fiedler (ThermoFisher)

Advanced Analytical Technology:

- LC-MS/MS and Immunological based Assay

10 ml Venous Whole Blood

Dried Blood Spot (DBS), 30-50 µl

The hematocrit (HCT) of blood affects its viscosity and so gives different-sized DBS spots.

VAMS (Mitra™)

HCT range: 20-65%

10.6 ± 0.4 µl

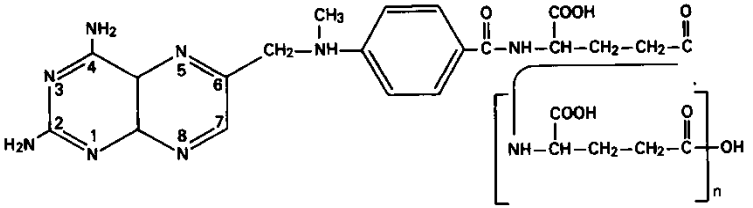
- Spooner et al., Bioanalysis (2015), 7(6), 635-659

Comparison Study: DBS vs Mitra

- *Therapeutic Drug Monitoring: Methotrexate*
  - *LC-MS/MS method development for Methotrexate polyglutamates (MTXPG1-5);*
  - *Analytical Validation;*
  - *Clinical Validation;*
  - *clinical trial Study.*
- *Diagnostic Biomarker: Rheumatoid Factor (RF)*
  - *Immunological based assay for Anti-CCP IgG, Anti-Mrf IgM, Anti-Arf Ig A and Anti-MCV antibodies;*
  - *Analytical and Clinical Validation;*
  - *clinical trial Study (undergoing….)*

LC-MS/MS method for measuring MTXPGn in DBS and Mitra

Methotrexate (MTX) is a prodrug activated to MTX polyglutamates (MTXPGn)
$MTXPG_1$, $MTXPG_2$, $MTXPG_3$, $MTXPG_4$, $MTXPG_5$



DBS Sample Preparation:

- *Add 30 µl whole blood (WB) on Whatman 903 DBS card;*
- *Dry it for at least 3 hours at RT;*
- *Punch DBS by using 6 mm puncher head into 96 well plate; One spot is equivalent to 14 µl whole blood;*
- *Add 70 µl water, vortex plate on speed 5 for 30 min;*
- *Remove 10 µl eluent for hemoglobin quantification;*
- *In 40 µl eluent, add 40 µl (50 nM) internal standard (IS): MTX-d3 and $MTXPG_3$-d3*
- *Add 10 µl 70% perchloric acid for protein precipitation;*
- *Centrifuge at 14,000 rpm for 5 min.*
- *Inject 20 µl supernatant to LC-MS/MS*

Mitra Sample Preparation

Dip Mitra tip into WB for 5 sec. to absorb 10 µl WB;

- *Dry it for at least 3 hours at RT;*
- *Put Mitra tip into a Eppendorf;*
- *Add 70 µl water, vortex plate on speed 1 for 30 min;*
- *Remove 10 µl eluent for hemoglobin quantification;*
- *In 40 µl eluent, add 40 µl (50 nM) internal standard (IS): MTX-d3 and $MTXPG_3$-d3*
- *Add 10 µl 70% perchloric acid for protein precipitation;*
- *Centrifuge at 14,000 rpm for 5 min.*
- *Inject 20 µl supernatant to LC-MS/MS*

LC-MS/MS method for measuring MTXPGn in DBS and Mitra

LC Conditions

Column (Thermo column) :

Pentafluorophenyl (PFP):

2.1 x 50 mm 2.6 µm particle

Mobile Phase:

A: 0.1%formic acid, 0.01% Triethylamine in water,

B: 0.1%formic acid, 0.01% Triethylamine in ACN

Time (min)	%B	Flow Rate (ml/min)
0.0	5	0.5
0.5	5	0.5
2.0	60	0.5
3.0	100	0.5
4.0	100	0.5
5.0	5	0.5
6.0	5	0.5

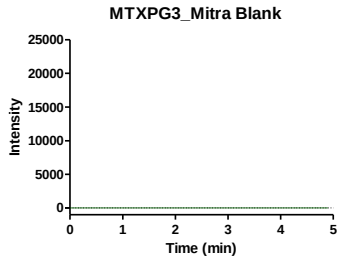
Mass Spectrometer Conditions

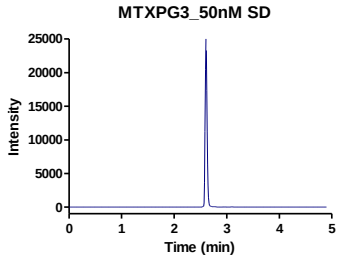
TSQ-Quantiva triple quadruple MS (ThermoScientific)

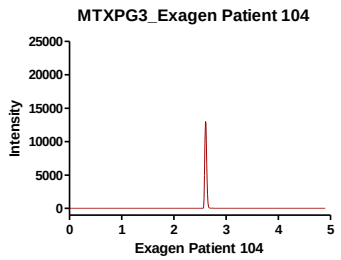
Parameters	Value
Spray Voltage (positive ion)	3700 (V)
Sheath Gas	45 (Arb)
Aux Gas	5 (Arb)
Sweep Gas	1 (Arb)
Ion Transfer Tube Temp	325 (°C)
Vaporizer Temp	317 (°C)

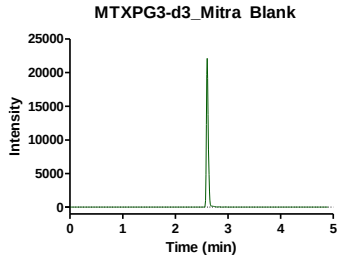
Compound	Precursor (m/z)	Product (m/z)
MTX	455.18	308.03
MTX-d3	458.22	311.12
MTXPG2	584.21	308.04
MTXPG3	713.23	308.09
MTXPG3-d3	716.36	311.13
MTXPG4	842.3	308.22
MTXPG5	971.33	308.12

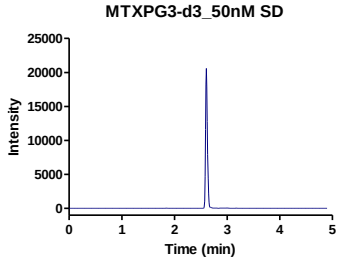
LC-MS/MS method for measuring MTXPGn in DBS and Mitra

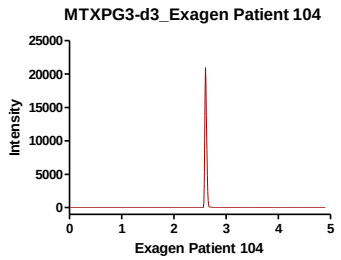


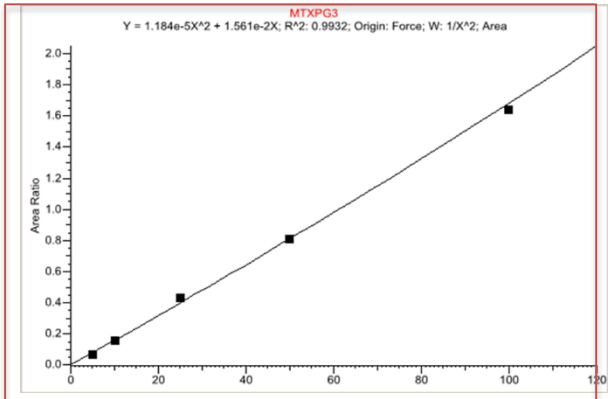












Quadratic SD curve With 1/x² weight

- *No interfering peaks were observed in Mitra and DBS samples prepared from blank blood;*
- *No carryover;*
- *No Matrix effect for both Mitra and DBS extract (post column infusion experiment);*
- *Calibration range: 5-100 nM for DBS and Mitra, LOD: 1 nM and LOQ: 5 nM;*
- *Precision were within 15% for both DBS and Mitra, 20% at LOQ.*

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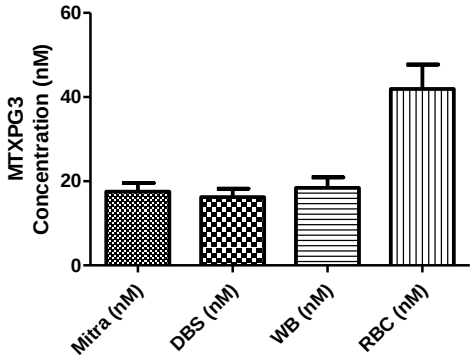
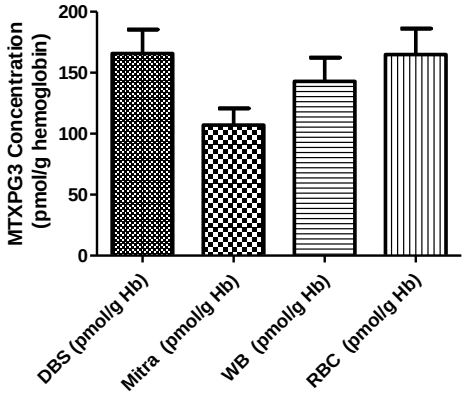
Clinical Validation by using Venous Whole Blood

28 Exagen patients which received MTX treatment

- *Measured MTXPG$_{1-5}$ in Whole Blood, DBS, Mitra and RBC;*
- *Measured hemoglobin concentration in each sample;*
- *Measured hematocrit (HCT) value for each patient;*

Hemoglobin Concnetration				
Filename	DBS (g/L)	Mitra (g/L)	WB (g/L)	RBC (g/L)
16CE010099	95	154	134	228
16CE010100	105	189	156	252
16CE010101	80	154	102	224
16CE010102	115	189	147	216
16CE010103	85	168	125	268
16CE010104	85	147	124	244
16CE010105	90	147	115	292
16CE010106	100	182	127	270
16CE010107	95	182	134	280
16CE010109	100	175	134	210
16CE010110	85	154	129	272
16CE010111	85	161	132	270
16CE010113	110	175	132	274
16CE010114	145	182	137	270
16CE010115	115	147	129	226

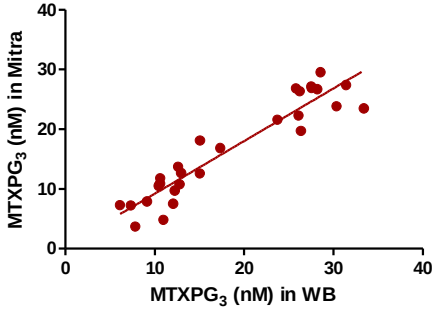


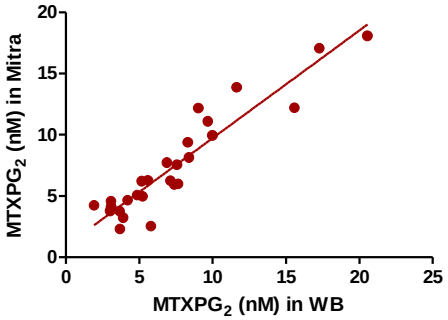


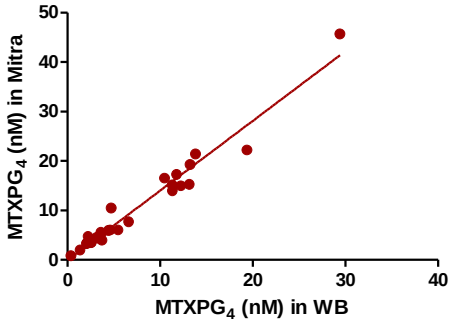
Elution recovery of hemoglobin from DBS and Mitra are different.

Results from Venous Blood Prepared Mitra and DBS (n=28)

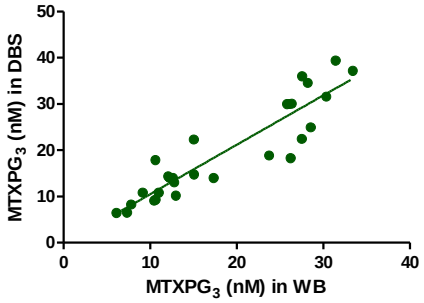
Correlation of Mitra with Whole Blood

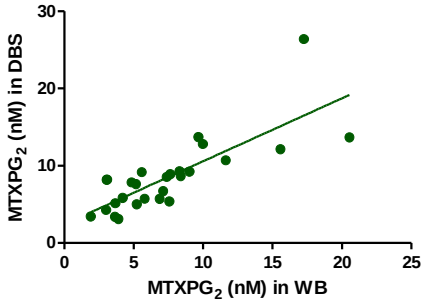


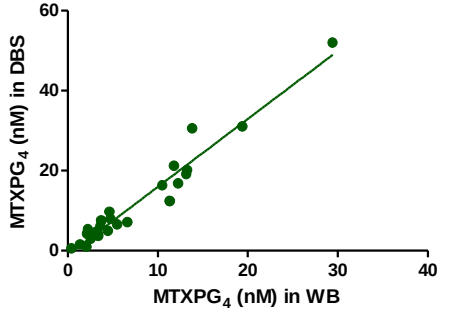




Correlation of DBS with Whole Blood







	Mitra (nM)	DBS (nM)
r^2	0.8839	0.8368

	Mitra (nM)	DBS (nM)
Slope	0.9344 ± 0.06641	1.184 ± 0.1026

	Mitra (nM)	DBS (nM)
r^2	0.8755	0.6166

	Mitra (nM)	DBS (nM)
Slope	0.9364 ± 0.07062	1.049 ± 0.1655

	Mitra (nM)	DBS (nM)
r^2	0.9596	0.9399

	Mitra (nM)	DBS (nM)
Slope	1.450 ± 0.05839	1.778 ± 0.08822

exagen DIAGNOSTICS

clinical trial: 44 Patients were Recruited in Two Clinical Sites

Clinical Study Case# 1

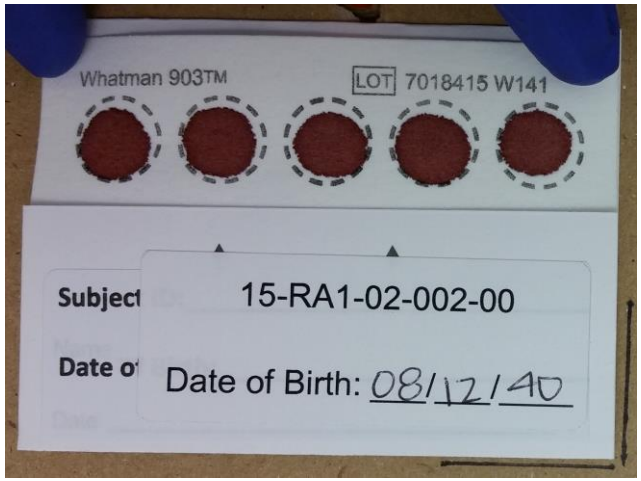
$MTXPG_3$ (nM)

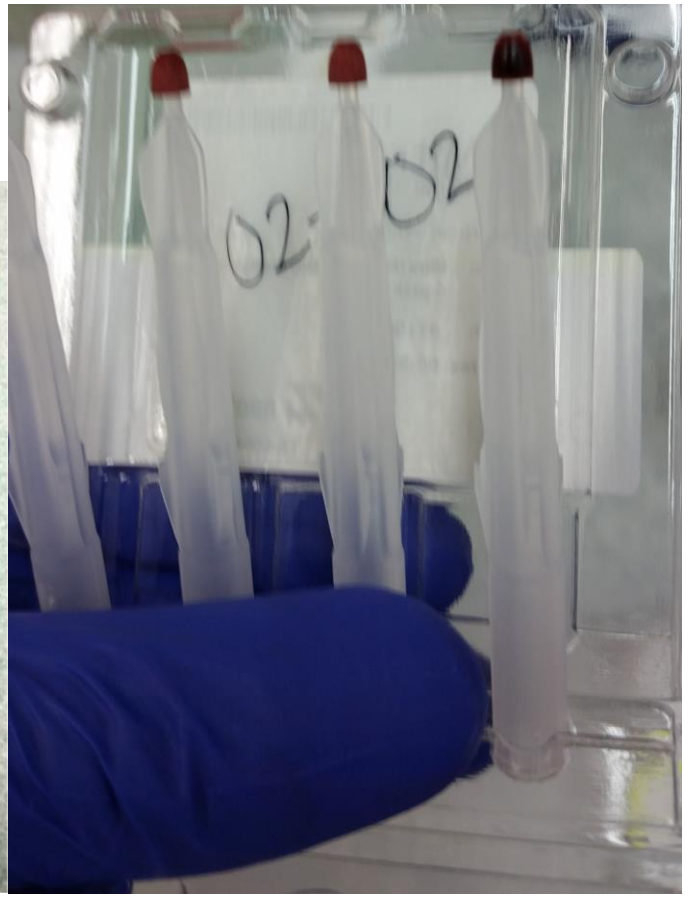
Capillary Blood:

DBS: 14.6 nM;

Mitra: 13.9 nM;

Venous Whole Blood: 13.9 nM





clinical trial: 44 Patients were Recruited in Two Sites

Clinical Study Case# 2

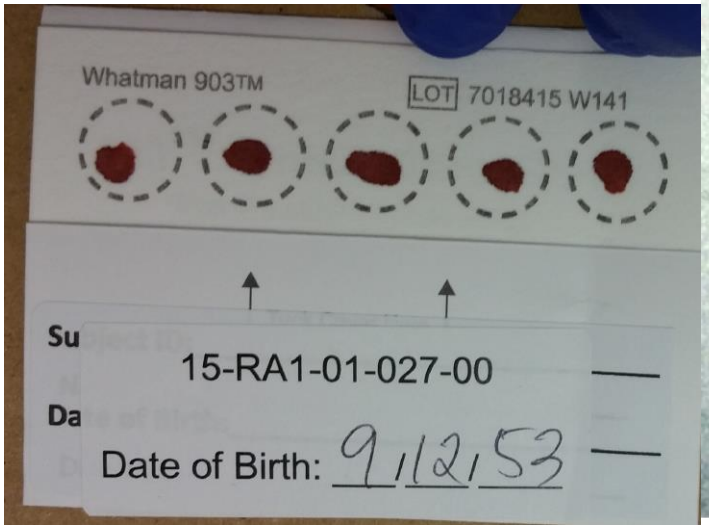
$MTXPG_3$ (nM)

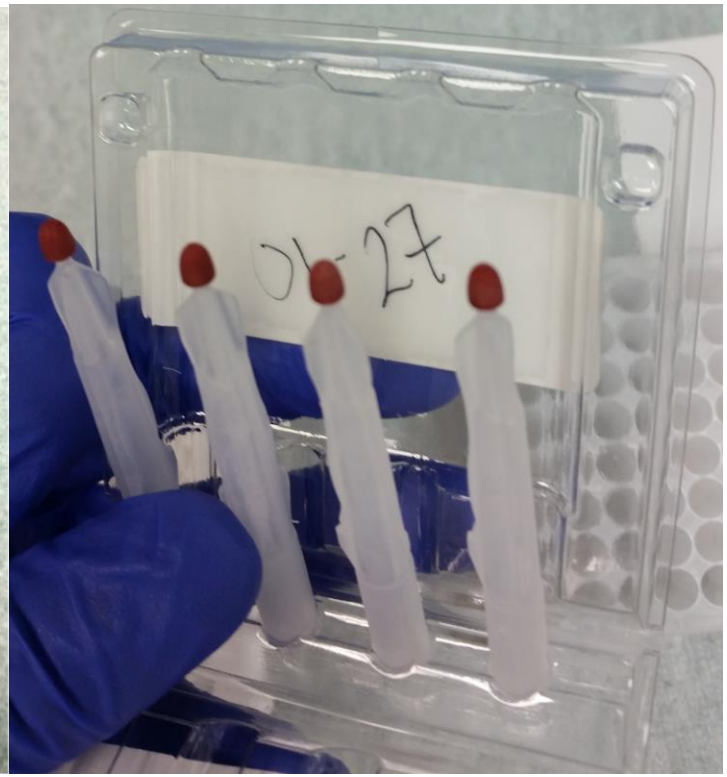
Capillary Blood:

DBS: 19.4 nM;

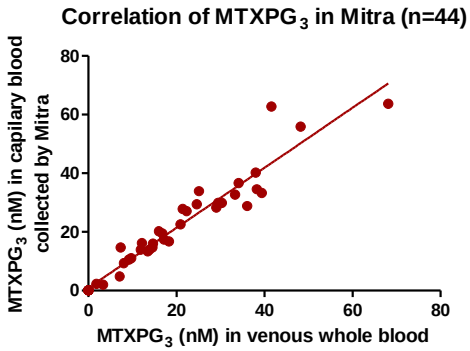
Mitra: 28.2 nM;

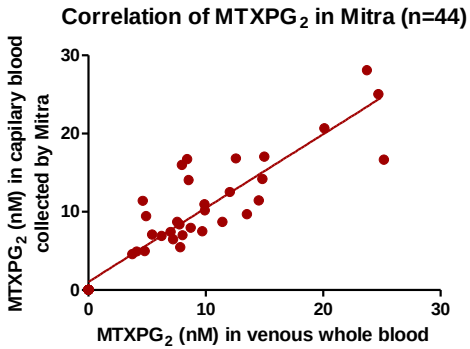
Venous Whole Blood: 29.0 nM

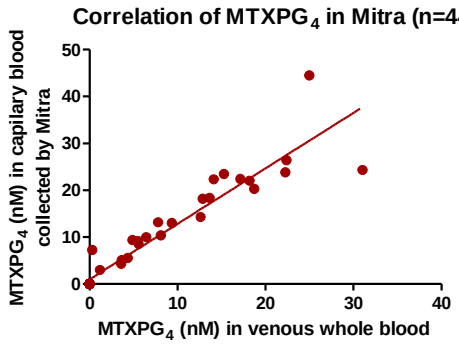


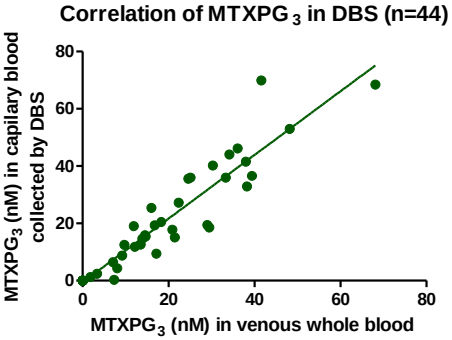


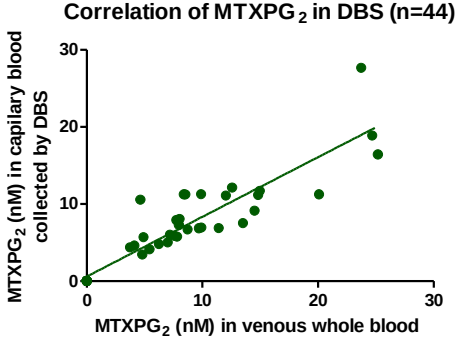
Results Comparison: Venous Blood and Capillary Blood (Mitra and DBS)

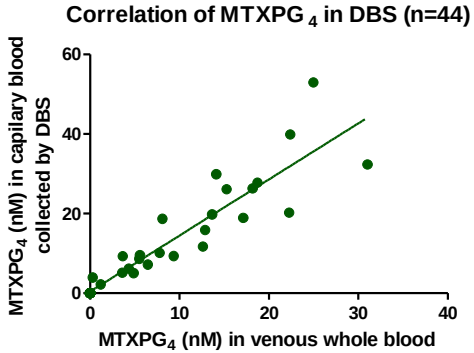












	Mitra (nM)	DBS (nM)
r²	0.9318	0.8844

	Mitra (nM)	DBS (nM)
Slope	1.063 ± 0.04439	1.194 ± 0.06663

	Mitra (nM)	DBS (nM)
r²	0.8333	0.8349

	Mitra (nM)	DBS (nM)
Slope	1.038 ± 0.07165	0.8343 ± 0.05724

	Mitra (nM)	DBS (nM)
r²	0.8914	0.8652

	Mitra (nM)	DBS (nM)
Slope	1.272 ± 0.06848	1.561 ± 0.09624

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Conclusions:

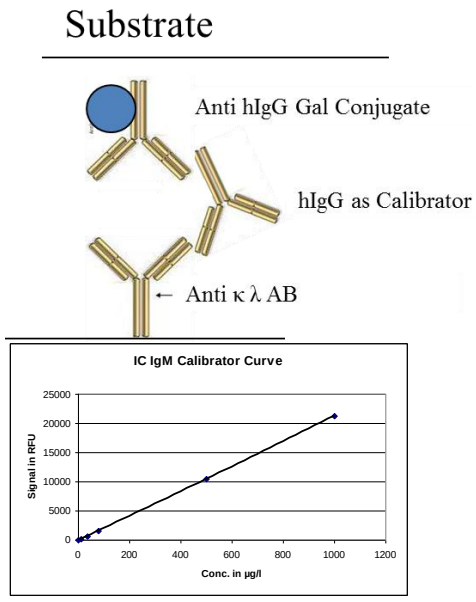
Using Mitra to collect capillary blood could represent venous whole blood for monitoring MTX treatment in patients.

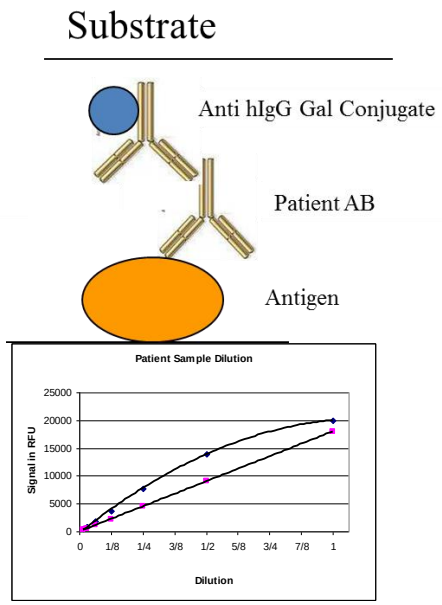
Comparison Study: DBS vs Mitra

- *Therapeutic Drug Monitoring: Methotrexate*
  - *LC-MS/MS method development for Methotrexate polyglutamates (MTXPG1-5);*
  - *Analytical and Clinical Validation;*
  - *clinical trial Study*
- *Diagnostic Biomarker: Rheumatoid factor (RF)*
  - *Immunological based assay for Anti-CCP IgG, Anti-Mrf IgM, Anti-Arf Ig A and Anti-MCV antibodies*
  - *Analytical and Clinical Validation;*
  - *clinical trial Study (undergoing….)*

Rheumatoid Arthritis (RA) Diagnostic Panel

- *Four serological markers measured in our clinical laboratory: anti-IgM RF, anti-IgA RF, anti-CCP and anti-MCV*
- *An indirect solid phase enzyme immunoassay (ELISA) was used for the quantitative measurement of Anti IgG class autoantibodies against mutated citrullinated vimentin (MCV);*
- *The method for determining anti-IgM RF, anti-IgA RF, anti-CCP is a quantitative Fluoroenzyme immunoassay on the Phadia 250 instrument (EliA system);*
- *All EliA test of one method (IgG, Ig M, Ig A) can be processed with same conjugate and calibration curve; Antigen: CCP, Mrf, Arf, 70, ro, la et. Al. 25 different markers*





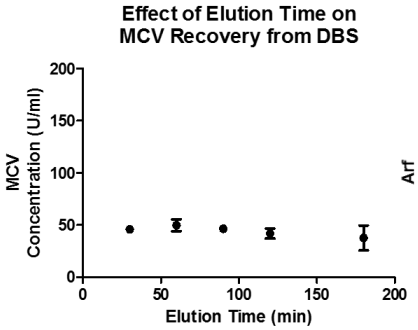
Sample Preparation for Immunological Based Assay

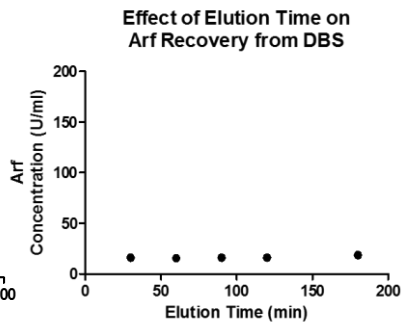
DBS Sample Preparation:

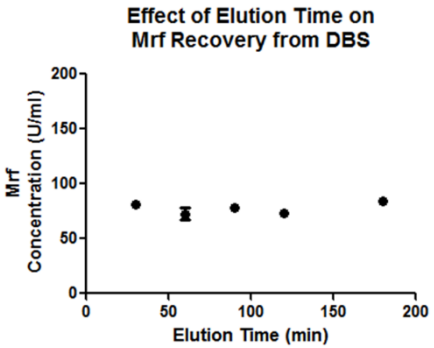
- *Add 30 µl venous whole blood (WB) on Whatman 903 card;*
- *Dry it for at least 3 hours at RT;*
- *Punch DBS by using 6 mm puncher head into 96 well plate; One spot is equivalent to 14 µl whole blood;*
- *Add 1.0 ml EliA Diluent which are PBS buffer containing BSA, detergent and sodium azide (0.095 %). Shake on a test tube rocker for 30 min;*
- *Put tube on Phadia instrument and measure anti-IgM RF, anti-IgA RF, anti-CCP.*

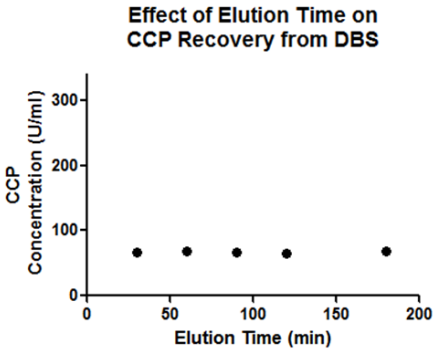
Mitra Sample Preparation:

- *Dip Mitra tip into venous WB for 5 sec. to absorb 10 µl WB;*
- *Dry it for at least 3 hours at RT;*
- *Put Mitra tip into a test tube;*
- *Add 1.0 ml EliA Diluent which are PBS buffer containing BSA, detergent and sodium azide (0.095 %). Shake on a test tube rocker for 30 min;*
- *Remove Mitra tip;*
- *Put tube on Phadia instrument and measure anti-IgM RF, anti-IgA RF, anti-CCP.*



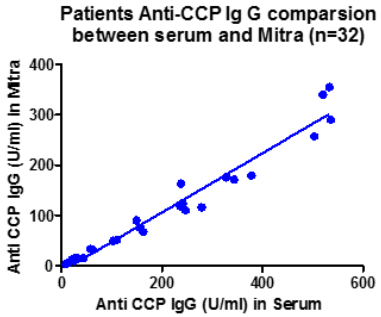






Anti-CCP IgG in DBS and Mitra

Select Anti-CCP positive patients, prepare DBS and Mitra by using venous WB

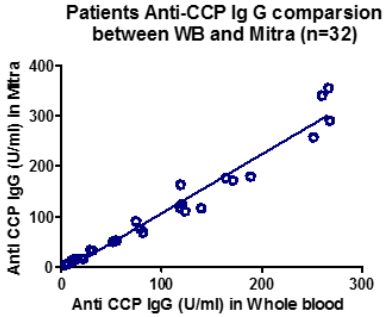


Deming regression line slope

Slope	0.5952 ± 0.02351

The correlation coefficient of linear regression:

r²	0.9553

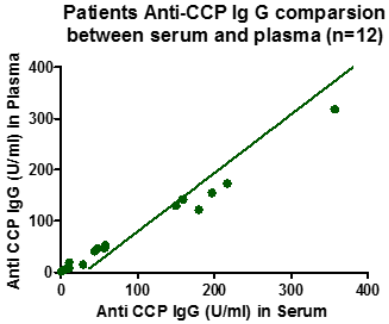


Deming regression line slope

Slope	1.209 ± 0.04774

The correlation coefficient of linear regression:

r²	0.9553

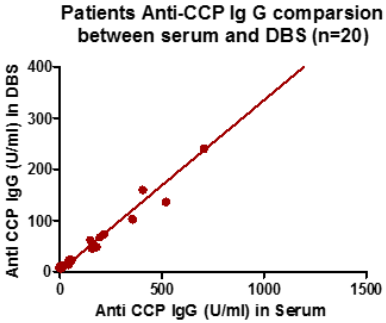


The correlation coefficient of linear regression:

Slope	1.157 ± 0.04120

Deming regression line slope

r²	0.9777

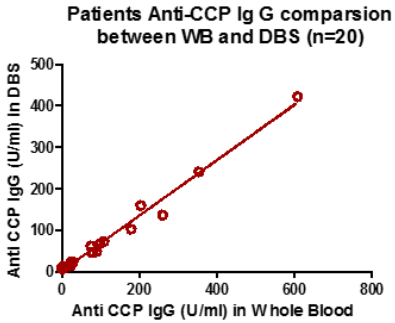


The correlation coefficient of linear regression:

Slope	0.3340 ± 0.009926

Deming regression line slope

r²	0.9844



The correlation coefficient of linear regression:

Slope	0.6284 to 0.7121

Deming regression line slope

r²	0.9844

Original Method: Measure anti-CCP in patient serum.

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Anti-CCP IgG in DBS and Mitra

Select Anti-CCP negative patients, prepare DBS and Mitra by using venous WB

Patient ID	CCP Serum	CCP DBS
70916	1.5	8.3
70917	2.2	12
70881	1	7.4
70883	1.5	8.7
70826	1.6	12
70874	1.2	7.2
70914	1.1	7.5
70960	0.9	48
70909	1.6	9.9
70915	1.1	9.3
70923	2	12
70919	1.2	12
70829	1.5	18
70922	0.9	12
70875	0.9	7.9
70832	0.7	9
70913	1	7.8
70836	0.4	9.2
70820	1.8	9
70818	0.5	7

Patient ID	CCP serum	CCP Mitra
88630	0.8	1.3
88633	1.0	1.7
88802	1.4	2.2
88827	1.6	3.0
88819	1.3	2.0
88823	0.9	1.7
88845	1.4	3.4
88876	1.1	2.5
88872	2.4	2.3
88376	3.1	3.1
88414	1.0	2.7
88389	1.4	1.8
88384	1.5	1.9
88085	1.2	2.5
88350	1.6	2.1
88306	0.9	1.3
88308	1.0	1.0

**Non specific absorption cause high background in DBS*

Continue to finish our clinical trial….

exagen DIAGNOSTICS