INTENDED USE
This test kit is intended for use in the quantitative determination of human calprotectin (neutrophil cytoplasmic protein S100A8/A9) levels in stool samples. It is for research use only. The test is useful for detecting inflammatory bowel disease (IBD) such as ulcerative colitis and Crohn’s disease.

INTRODUCTION
Quantitative determination of fecal calprotectin is an indication of the severity of bowel inflammation. Also, higher levels of calprotectin in the stool are associated with an increased risk of relapse in patients with inflammatory bowel disease (IBD).1 Low stool calprotectin levels correlate well with a low risk for intestinal allograft rejection. This assay uses specific monoclonal antibodies to ensure only calprotectin is detected.

ASSAY PRINCIPLE
This ELISA is designed, developed and produced for the quantitative measurement of human calprotectin in stool samples. The assay utilizes the two-site “sandwich” technique with two selected antibodies that bind to different epitopes of human calprotectin. Assay standards, controls and patient samples are added directly to wells of a microtiter plate that is coated with antibody to calprotectin. After a short incubation period, the plate is washed and horseradish peroxidase (HRP)-conjugated human calprotectin specific monoclonal antibody is added to each well. After the second incubation period, a “sandwich” of solid-phase antibody – human calprotectin – HRP-conjugated monoclonal antibody is formed. The unbound monoclonal antibodies and buffer matrix are removed in the subsequent washing step. For the detection of this immunocomplex, the well is then incubated with a substrate solution in a timed reaction and then measured in a spectrophotometric microplate reader. The enzymatic activity of the immunocomplex is measured as absorbance. A standard curve is generated by plotting the absorbance versus the respective human calprotectin concentration for each standard on a point-to-point or 4-parameter curve fitting. The concentration of fecal human calprotectin in test samples is determined directly from this standard curve.

REAGENTS: Preparation and Storage
This test kit must be stored at 2 – 8°C upon receipt. For the expiration date of the kit refer to the label on the kit box. All components are stable until this expiration date.

Prior to use all reagents to come to room temperature. Reagents from different kit lot numbers should not be combined or interchanged.

1. Calprotectin Antibody Coated Microplate (Cat. No. 30439)
   One microplate with twelve by eight strips (96 wells total) coated with calprotectin antibody. The plate is framed and sealed in a foil zipper bag with a desiccant. This reagent should be stored at 2 – 8°C and is stable until the expiration date on the kit box.

2. Calprotectin Tracer Antibody (Cat. No. 30440)
   One vial containing 0.6 mL HRP-labeled anti-human calprotectin antibody in a stabilized protein matrix. This reagent must be diluted with Tracer Antibody Diluent (Cat. 30458) before use. This reagent should be stored at 2 – 8°C and is stable until the expiration date on the kit box.

3. ELISA Wash Concentrate (Cat. No. 10010)
   One bottle contains 30 mL of 30-fold concentrate. Before use the contents must be diluted with 870 mL of demineralized water and mixed well. Upon dilution, this yields a working wash solution containing a surfactant in phosphate-buffered saline with a non-azide, non-mercury preservative. The diluted wash solution may be stored at room temperature and is stable until the expiration date on the kit box.

4. ELISA HRP Substrate (Cat. No. 10020)
   One bottle contains 12 mL of tetramethylbenzidine (TMB) with hydrogen peroxide. This reagent should be stored at 2 – 8°C and is stable until the expiration date on the kit box.

5. ELISA Stop Solution (Cat. No. 30559)
   One bottle contains 12 mL of 2N Hydrochloric Acid (HCl). This reagent should be stored at 2 – 8°C and is stable until the expiration date on the kit box.

6. Calprotectin Standards (Cat. No. 30571 – 30577)
   Seven vials containing human calprotectin in a lyophilized bovine serum-based matrix with a non-azide, non-mercury preservative. Refer to vials for exact concentration for each standard. These reagents should be stored at 2 – 8°C and are stable until the expiration date on the kit box.

7. Calprotectin Controls (Cat. No. 30578 – 30580)
   Three vials containing human calprotectin in a lyophilized bovine serum-based matrix with a non-azide, non-mercury preservative. Refer to vials for exact concentration range for each control. Both controls should be stored at 2 – 8°C and are stable until the expiration date on the kit box.

8. Tracer Antibody Diluent (Cat. No. 30458)
   One vial containing 12 mL ready-to-use buffer. It should be used only for calprotectin antibody dilution according to the assay procedures. This reagent should be stored at 2 – 8°C and is stable until the expiration date on the kit box.

9. Assay Buffer (Cat. No. 30485)
   One bottle containing 12 mL ready-to-use buffer. It should be used according to the assay procedures. This reagent should be stored at 2 – 8°C and is stable until the expiration date on the kit box.
10. Extraction Buffer Concentrate (Cat. No. 30473)
One bottle containing 120 mL of 5-fold concentrate. Before use the
contents must be diluted with 480 mL of demineralized
water and mixed well. Upon dilution, this yields a ready-to-use
Extraction Buffer for fecal sample extraction and dilution. The
diluted Extraction Buffer may be stored at room temperature
and is stable until the expiration date on the kit box.

SAFETY PRECAUTIONS
The reagents must be used in a professional laboratory. The source
material for reagents containing bovine serum was derived in the
contiguous 48 United States. It was obtained only from healthy donor
animals maintained under veterinary supervision and found free of
contagious diseases. Wear gloves while performing this assay and
handle these reagents as if they are potentially infectious. Avoid
contact with reagents containing TMB, hydrogen peroxide, or
hydrochloric acid. TMB may cause irritation to skin and mucous
membranes and cause an allergic skin reaction. TMB is a suspected
carcinogen. Hydrochloric acid may cause severe irritation on contact
with skin. Provide good ventilation in process area to prevent
formation of vapor. Do not breathe mist, vapors, spray. Do not get in
eyes, on skin, or on clothing. Do not ingest or inhale fumes. On
contact, flush with copious amounts of water for at least 15 minutes.
Use Good Laboratory Practices.

MATERIALS REQUIRED BUT NOT PROVIDED
1. Fecal sample collection tube (Epitope Catalog No: 30356)
2. Precision single channel pipettes capable of delivering 50
µL, 100 µL, 500 µL, etc.
3. Disposable pipette tips suitable for above volume
dispensing.
4. Disposable plastic 100 mL and 1000 mL bottle with caps.
5. Aluminum foil.
6. Deionized or distilled water.
7. Plastic microtiter well cover or polyethylene film.
8. ELISA multichannel wash bottle or automatic (semi-
automatic) washing system.
9. Spectrophotometric microplate reader capable of reading
absorbance at 450 nm and 650 or 630

SPECIMEN COLLECTION
1. Only one fecal sample is required. Fresh fecal sample must be
collected by using Epitope Diagnostics Fecal Sample Collection
Tube (Cat. No. 30356). This tube is specially designed for easy
collection of a substantially small amount of fecal sample into the
tube pre-filled with sample extraction buffer. The collected fecal
sample may be transported at ambient temperature, stored at 2-8 °C
and tested within 3 days. Fecal sample may be stored below -20 °C
for a longer storage period. Avoid more than three freeze-thaw
cycles for each specimen.

The validation data of this test were generated by using Fecal
Sample Collection Tube (Cat. No. 30356). To order this tube,
please order Fecal Calprotectin/NGAL Sample Collection kit
(Cat. No. KT-843). Each kit contains 48 tubes filled with
extraction buffer. A different calprotectin test result may be
obtained by using a different type of fecal sample collection
tube.

2. It is an alternative to collect fecal sample with a commercial stool
sample collection device. The collected sample can be stored at 2-8°C
for up to 6 days. The collected sample should be diluted in two
steps with 1:40 and 1:9 before measurement. Following is a detailed
sample extraction process.
(a) Label and tare an empty polypropylene tube together with an
inoculation loop.
(b) Weigh 50 – 100 mg of stool using the inoculation loop by placing it
into the pre-tarred tube.

(c) Record the net amount of sample and break the inoculation loop;
leave the lower part of the loop in the tube.
(d) Add Extraction Buffer (39 parts of the stool volume, 1 g stool = 1
ml) into the tube.
(e) Vortex to dissolve stool sample. Let the sample set at room
temperature vertically for 30 min for sedimentation or centrifuge the
sample at 3000 x g for 5 minutes.
(f) Transfer 0.15 mL clear supernatant (no particles) to a clean tube
with 1.2 ml Extraction Buffer. Mix the sample by gently vortexing.
This extracted sample is ready to be measured for fecal Calprotectin.

ASSAY PROCEDURE
1. Reagent Preparation
(1) Prior to use allow all reagents to come to room
temperature. Reagents from different kit lot numbers
should not be combined or interchanged.
(2) ELISA Wash Concentrate (Cat. 10010) must be diluted to
working solution prior to use. Please see REAGENTS
section for details.
(3) Reconstitute all assay standard level 1 to level 7 (Cat.
30571-30577) and controls (Cat. 30578-30580) by adding
0.5 mL of demineralized water to each vial. Allow the
standards and controls to sit undisturbed for 5 minutes,
and then mix well by inversions or gentle vortexing. One
must make sure that all solid is dissolved completely prior
to use. These reconstituted standards and controls may be
stored at 2 – 8°C for up to 3 days or at –10°C or below for
long-term storage. Do not exceed 3 freeze-thaw cycles.

(h) Test Configuration

<table>
<thead>
<tr>
<th>ROW</th>
<th>STRIP 1</th>
<th>STRIP 2</th>
<th>STRIP 3</th>
<th>STRIP 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>STD 1</td>
<td>STD 5</td>
<td>C 2</td>
<td>SAMPLE 5</td>
</tr>
<tr>
<td>B</td>
<td>STD 1</td>
<td>STD 5</td>
<td>C 2</td>
<td>SAMPLE 6</td>
</tr>
<tr>
<td>C</td>
<td>STD 2</td>
<td>STD 6</td>
<td>C 3</td>
<td>SAMPLE 7</td>
</tr>
<tr>
<td>D</td>
<td>STD 2</td>
<td>STD 6</td>
<td>C 3</td>
<td>SAMPLE 8</td>
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<tr>
<td>E</td>
<td>STD 3</td>
<td>STD 7</td>
<td>SAMPLE 1</td>
<td>SAMPLE 9</td>
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<td>F</td>
<td>STD 3</td>
<td>STD 7</td>
<td>SAMPLE 2</td>
<td>SAMPLE 10</td>
</tr>
<tr>
<td>G</td>
<td>STD 4</td>
<td>C 1</td>
<td>SAMPLE 3</td>
<td>SAMPLE 11</td>
</tr>
<tr>
<td>H</td>
<td>STD 4</td>
<td>C 1</td>
<td>SAMPLE 4</td>
<td>Etc.</td>
</tr>
</tbody>
</table>

(5) Place a sufficient number of calprotectin-coated microwell
strips (Cat. 30439) in a holder to run human calprotectin
standards, controls and unknown samples in duplicate.
3. The standard curve is generated by the corrected absorbance of all standard levels on the ordinate against the standard concentration on the abscissa using point-to-point or log-log paper. Appropriate computer-assisted data reduction programs may also be used for the calculation of results.

The use of the absorbance wavelength at A 620 nm and A450/620 nm allows for two ways to calculate sample results. It is recommended to get sample results by using the primary standard curve at A 450/620 nm for samples within the standard level 5. For samples with calprotectin value above standard level 5, it is recommended to use the secondary standard curve at A 620 nm.

**EXAMPLE DATA AND STANDARD CURVE (low)**

A typical absorbance data and the resulting standard curve from this fecal human calprotectin ELISA are represented. This curve should not be used in lieu of standard curve run with each assay.

<table>
<thead>
<tr>
<th>Well I.D.</th>
<th>OD 450 nm Absorbance</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Readings</td>
<td>Average</td>
</tr>
<tr>
<td>Std-1: 0 µg/g (0 ng/mL)</td>
<td>0.026</td>
<td>0.027</td>
</tr>
<tr>
<td>Std-2: 25 µg/g (69.5 ng/mL)</td>
<td>0.061</td>
<td>0.059</td>
</tr>
<tr>
<td>Std-3: 56.2 µg/g</td>
<td>0.305</td>
<td>0.292</td>
</tr>
</tbody>
</table>

**INTERPRETATION OF RESULTS**

1. Calculate the average absorbance for each pair of duplicate test results.
2. Subtract the average absorbance of the level 1 standard (0 ng/mL) from the average absorbance of all other readings to obtain corrected absorbance.
3. The standard curve is generated by the corrected absorbance of all standard levels on the ordinate against the standard concentration on the abscissa using point-to-point or log-log paper. Appropriate computer-assisted data reduction programs may also be used for the calculation of results.

**PROCEDURAL NOTES**

1. It is recommended that all standards, controls, and unknown samples be assayed in duplicate. The average absorbance reading of each duplicate should be used for data reduction and the calculation of results.
2. Keep light-sensitive reagents in the original amber bottles.
3. Store any unused antibody-coated strips in the foil zipper bag with desiccant to protect from moisture.
4. Careful technique and use of properly calibrated pipetting devices are necessary to ensure reproducibility of the test.
5. Incubation times or temperatures other than those stated in this insert may affect the results.
6. An orbital mixer with a larger orbit radius (e.g., > 1 cm) may be used at speeds of 150 to 200 rpm.
7. Avoid air bubbles in the microwell as this could result in lower binding efficiency and higher CV% of duplicate reading.
8. All reagents should be mixed gently and thoroughly prior to use. Avoid foaming.
9. If adapting this assay to automated ELISA system such as DS-2, a procedural validation is necessary if there is any modification of the assay procedure.

**2. Patient Sample Preparation**

If the Epitope Diagnostics Fecal Sample Collection Tube (Cat. 30356) is used, there is no sample preparation required.

**3. Assay Procedure:**

1. **Add 50 µL of Assay Buffer (Cat. 30485) into the designated microwells. Gently tap the plate to coat the wells evenly.**
2. **Add 50 µL of Standards, Controls and extracted patient samples into the designated microwells.**
3. **Seal the plate wells securely, cover with foil or other material to protect from light, and rotate on an ELISA plate shaker (small orbit radius) for 1 hr. ± 5 minutes at 400 to 450 rpm.**
4. **Just prior to the end of the incubation time, dilute the proper amount of Tracer Antibody for the assay.**
5. **Wash each well 5 times by dispensing 350 µL of working wash solution into each well and then completely aspirating the contents. Alternatively, an automated microplate washer can be used.**
6. **Add 100 µL of diluted Tracer Antibody to each well.**
7. **Seal the plate wells securely, cover with foil or other material to protect from light, and rotate on an ELISA plate shaker (small orbit radius) for 45 minutes ± 5 minutes at 400 to 450 rpm.**
8. **Wash each well 5 times by dispensing 350 µL of working wash solution into each well and then completely aspirating the contents. Alternatively, an automated microplate washer can be used.**
9. **Add 100 µL of ELISA HRP Substrate (Cat. 10020) into each of the wells.**
10. **Cover the plate with aluminum foil to or other material to avoid exposure to light. Incubate plate static, at room temperature, for 12 minutes (Optional 8 - 15 minutes).**
11. **Remove the aluminum foil. Read the absorbance at 620 nm (optional wavelengths from 595 nm to 650 nm depending on available filters) immediately. Note: please shake the plate to reach a homogenous blue color distribution in the well right before reading!**
12. **Immediately add 100 µL of ELISA Stop Solution (Cat. 30559) into each of the wells. Mix gently.**
13. **Read the absorbance at 450 nm with reference filter at 620 nm or 650 nm.**

**Note:** This antibody working solution should be freshly prepared just before pipetting the tracer antibody to the washed wells.
EXAMPLE DATA AND STANDARD CURVE (high)
A typical absorbance data and the resulting standard curve from this fecal human calprotectin ELISA are represented. This curve should not be used in lieu of standard curve run with each assay.

<table>
<thead>
<tr>
<th>Well I.D.</th>
<th>OD 620 nm Absorbance</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Readings</td>
<td>Average</td>
</tr>
<tr>
<td>Std-1: 0 µg/g (0 ng/mL)</td>
<td>0.043</td>
<td>0.042</td>
</tr>
<tr>
<td></td>
<td>0.041</td>
<td></td>
</tr>
<tr>
<td>Std-3: 56.2 µg/g (156 ng/mL)</td>
<td>0.132</td>
<td>0.126</td>
</tr>
<tr>
<td></td>
<td>0.120</td>
<td></td>
</tr>
<tr>
<td>Std-4: 145 µg/g (403 ng/mL)</td>
<td>0.494</td>
<td>0.457</td>
</tr>
<tr>
<td></td>
<td>0.420</td>
<td></td>
</tr>
<tr>
<td>Std-5: 321 µg/g (892 ng/mL)</td>
<td>1.368</td>
<td>1.374</td>
</tr>
<tr>
<td></td>
<td>1.380</td>
<td></td>
</tr>
<tr>
<td>Std-6: 669 µg/g (1860 ng/mL)</td>
<td>1.945</td>
<td>1.948</td>
</tr>
<tr>
<td></td>
<td>1.950</td>
<td></td>
</tr>
<tr>
<td>Std-7: 2000 µg/g (5560 ng/mL)</td>
<td>2.415</td>
<td>2.432</td>
</tr>
<tr>
<td></td>
<td>2.448</td>
<td></td>
</tr>
<tr>
<td>Control 2</td>
<td>1.145</td>
<td>1.147</td>
</tr>
<tr>
<td></td>
<td>1.149</td>
<td></td>
</tr>
<tr>
<td>Control 3</td>
<td>1.778</td>
<td>1.779</td>
</tr>
<tr>
<td></td>
<td>1.779</td>
<td></td>
</tr>
</tbody>
</table>

EXPECTED VALUES
Stool samples from normal healthy adults with age of 24 – 58 were collected and measured with this ELISA. The recommended normal cut-off for fecal Calprotectin concentration by using this ELISA and sample collection system is 120 ng/mL or 43.2 µg/g directly read from assay standard curve. We strongly recommend that each clinical laboratory to establish its own normal cut-off level by measuring normal stool samples with this ELISA and sample collection system.

Please be aware that patients with recent diarrhea would give a much higher level of fecal Calprotectin. Taking spicy food or alcohol may also cause intestinal irritation resulting in an abnormal fecal Calprotectin level.

Note: Calprotectin ng/mL X 0.36 = Calprotectin µg/g
Calprotectin µg/g X 2.78 = Calprotectin ng/mL

Please program ELISA reader by selecting assay standards concentration either in “µg/g” or “ng/mL to avoid manual calculation!

LIMITATION OF THE PROCEDURE
1. A strong positive of fecal calprotectin is likely to indicate a more significant clinical pathological condition of a patient. However, a low positive of fecal calprotectin does not indicate a lesser possibility of inflammation.
2. A normal fecal calprotectin level does not rule out the presence of any gastrointestinal diseases such as IBD.
3. For sample values reading greater than the highest standard, it is recommended to re-assay samples with dilution (i.e. 1:10 or 1:100 with Extraction Buffer).
4. Water deionized with polyester resins may inactivate the horseradish peroxidase enzyme.

QUALITY CONTROL
To assure the validity of the results each assay should include adequate controls.

PERFORMANCE CHARACTERISTICS
Sensitivity
The analytical sensitivity (LLOD) of the human calprotectin ELISA as determined by the 95% confidence limit on 12 duplicate determination of zero standard is approximately 2.5 ng/mL. A LLOQ was determined by dilution of assay standards and it is about 5 ng/mL.
High Dose “hook” effect
This assay has showed that it did not have any high dose “hook” for calprotectin level up to 40,000 ng/mL in extraction buffer.

Precision
The intra-assay precision was validated by measuring three sample extracts in a single assay with 12 replicate determinations.

<table>
<thead>
<tr>
<th>Mean Calprotectin Value (µg/g)</th>
<th>CV (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.74</td>
<td>2.9</td>
</tr>
<tr>
<td>26.59</td>
<td>3.5</td>
</tr>
<tr>
<td>54.70</td>
<td>2.5</td>
</tr>
</tbody>
</table>

The inter-assay precision was validated by measuring two samples in duplicate in 4 individual assays.

<table>
<thead>
<tr>
<th>Mean Calprotectin Value (µg/g)</th>
<th>CV (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>21.64</td>
<td>8.6</td>
</tr>
<tr>
<td>70.31</td>
<td>2.0</td>
</tr>
</tbody>
</table>

The precision of inter-sample collection was performed by collecting five specimens from one bowel movement. These grouped samples are measured in an assay according to the assay procedure. The results of Calprotectin concentration in the value of ng/mL indicate that there are very satisfactory agreements of the five samples collected from one bowel movement.

<table>
<thead>
<tr>
<th>Donor</th>
<th>Sample 1</th>
<th>Sample 2</th>
<th>Sample 3</th>
<th>Sample 4</th>
<th>Sample 5</th>
<th>CV%</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>57.0</td>
<td>65.0</td>
<td>59.2</td>
<td>56.2</td>
<td>49.8</td>
<td>9.5</td>
</tr>
<tr>
<td>B</td>
<td>60.4</td>
<td>55.3</td>
<td>58.8</td>
<td>71.7</td>
<td>81.1</td>
<td>16.3</td>
</tr>
<tr>
<td>C</td>
<td>72.3</td>
<td>69.3</td>
<td>51.5</td>
<td>65.7</td>
<td>65.6</td>
<td>12.3</td>
</tr>
</tbody>
</table>

Linearity
One sample was diluted with assay buffer and tested. The results of Calprotectin concentration in the value of ng/mL are as follows:

<table>
<thead>
<tr>
<th>DILUTION</th>
<th>OBSERVED VALUE</th>
<th>EXPECTED VALUE</th>
<th>RECOVERY %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neat</td>
<td>195.84</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>1:2</td>
<td>87.88</td>
<td>97.92</td>
<td>89.7</td>
</tr>
<tr>
<td>1:4</td>
<td>46.58</td>
<td>48.96</td>
<td>95.1</td>
</tr>
<tr>
<td>1:8</td>
<td>24.53</td>
<td>24.48</td>
<td>100.2</td>
</tr>
<tr>
<td>1:16</td>
<td>13.77</td>
<td>12.24</td>
<td>112.5</td>
</tr>
</tbody>
</table>

Spike Recovery
Three fecal extracts and three assay standards were spiked together in various volume combinations and tested. The results Calprotectin concentration in the value of ng/mL are as follows:

<table>
<thead>
<tr>
<th>#</th>
<th>Orig. Value</th>
<th>Amount Spiked</th>
<th>Observed Value</th>
<th>Expected Value</th>
<th>Recovery %</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>30.0</td>
<td>37.1</td>
<td>61.9</td>
<td>67.1</td>
<td>92.2</td>
</tr>
<tr>
<td>2</td>
<td>73.0</td>
<td>12.7</td>
<td>85.7</td>
<td>89.3</td>
<td>104.2</td>
</tr>
<tr>
<td>3</td>
<td>217.7</td>
<td>30.3</td>
<td>248.0</td>
<td>256.9</td>
<td>96.5</td>
</tr>
</tbody>
</table>

**WARRANTY**
This product is warranted to perform as described in its labeling and literature when used in accordance with all instructions. Epitope Diagnostics, Inc. DISCLAIMS ANY IMPLIED WARRANTY OF MERCHANTABILITY OR FITNESS FOR A PARTICULAR PURPOSE, and in no event shall Epitope Diagnostics, Inc. be liable for consequential damages. Replacement of the product or refund of the purchase price is the exclusive remedy for the purchaser. This warranty gives you specific legal rights and you may have other rights, which vary from state to state.

**REFERENCES**

**TECHNICAL ASSISTANCE AND CUSTOMER SERVICE**
For technical assistance or place an order, please contact Epitope Diagnostics, Inc. at (858) 693-7877 or fax to (858) 693-7676. www.epitopediagnostics.com

This product is developed and manufactured by Epitope Diagnostics, Inc.
7110 Carroll Road
San Diego, CA 92121, USA

**Calprotectin ELISA: Condensed Assay Protocol**

1. 50 µl Assay Buffer per well

2. 50 µl Calibrators, controls and extracted patient samples
   Incubate @ RT for 60 min on ELISA plate shaker
   Wash 5 x

3. 100 µl Tracer Antibody
   Incubate @ RT for 45 min on ELISA plate shaker
   Wash 5 x

4. 100 µl TMB Substrate
   Incubate @ RT for 12 min static

5. Read absorbance at 620 nm
   Immediately

6. 100 µl Stop Solution

7. Read absorbance at 450/620 or 450/650 nm