Instructions for use
Plasma Metanephrine ELISA
Metanephrine Plasma ELISA Fast Track

1. Introduction

1.1 Intended use and principle of the test

Enzyme Immunoassay for the quantitative determination of free Metanephrine in plasma.

**Related Products:**

<table>
<thead>
<tr>
<th>2-MET Plasma ELISA Fast Track</th>
<th>2-MET Plasma RIA Fast Track</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normetanephrine Plasma ELISA Fast Track</td>
<td>Normetanephrine Plasma RIA Fast Track</td>
</tr>
</tbody>
</table>

Alternatively the assay can also be run automatically on an ELISA processor such as the Gemini instrument from Stratec Biomedical. The Gemini protocol is available upon request.

Metanephrine (Metadrenaline) is first extracted using an ion exchange matrix followed by an acylation process. The subsequent competitive ELISA uses the microtiter plate format. The antigen is bound to the solid phase of the microtiter plate. The acylated standards, controls and samples and the solid phase bound analytes compete for a fixed number of antibody binding sites. After the system is in equilibrium, free antigen and free antigen-antibody complexes are removed by washing. The antibody bound to the solid phase is detected by an anti-rabbit IgG-peroxidase conjugate using TMB as a substrate. The reaction is monitored at 450 nm.

Quantification of unknown samples is achieved by comparing their absorbance with a reference curve prepared with known standards.

⚠️ The antibodies used in this test kit only recognise the biologically relevant L-forms of Metanephrines. Commercially available synthetic Normetanephrine or Metanephrine is always a mixture of the D- and L-form. The ratio between both forms differs widely from lot to lot. This has important implications if synthetic Metanephrines are used to enrich native samples. As only about 50% of the synthetic Metanephrines - the L-portion - will be detected by use of this kit, spiked samples will be underestimated. Therefore native samples containing solely the L-form should be used.

1.2 Clinical application

Metanephrine and Normetanephrine are the metabolites of the catecholamines Epinephrine and Norepinephrine, respectively. Cells derived from neuroendocrine tumors (e.g. pheochromocytoma) are known to produce catecholamines which are secreted episodically via vesicles into the blood stream. But beside this, a small portion of the catecholamines is metabolized inside the cells to the corresponding catecholamines metabolites – namely Metanephrine, Normetanephrine and 3-Methoxytyramine – which are secreted at low levels continuously into the blood stream.

This kit is for research use only and should not be used as a diagnostic tool.

2. Procedural cautions, guidelines, warnings and limitations

2.1 Procedural cautions, guidelines, warnings and warnings

(1) This kit is intended for professional use only. Users should have a thorough understanding of this protocol for the successful use of this kit. Only the test instruction provided with the kit is valid and has to be used to run the assay. Reliable performance will only be attained by strict and careful adherence to the instructions provided.

(2) This assay was validated for a certain type of sample as indicated in Intended Use (please refer to Chapter 1). Any off-label use of this kit is in the responsibility of the user and the manufacturer cannot be held liable.

(3) Reagents of this kit which contain human serum or plasma have been tested and confirmed negative for HIV 1/II, HBsAg and HCV by approved procedures. All reagents, however, should be treated as potential biohazards in use and for disposal.

(4) The principles of Good Laboratory Practice (GLP) have to be followed.

(5) In order to reduce exposure to potentially harmful substances, wear lab coats, disposable protective gloves and protective glasses where necessary.

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(6) All kit reagents and specimens should be brought to room temperature and mixed gently but thoroughly before use. Avoid repeated freezing and thawing of reagents and specimens.

(7) For dilution or reconstitution purposes, use deionized, distilled, or ultra-pure water.

(8) The microplate contains snap-off strips. Unused wells must be stored at 2 °C to 8 °C in the sealed foil pouch with desiccant and used in the frame provided. Microwell strips which are removed from the frame for usage should be marked accordingly to avoid any mix-up.

(9) Duplicate determination of sample is highly recommended to be able to identify potential pipetting errors.

(10) Once the test has been started, all steps should be completed without interruption. Make sure that the required reagents, materials and devices are prepared ready at the appropriate time.

(11) Incubation times do influence the results. All wells should be handled in the same order and time intervals.

(12) To avoid cross-contamination of reagents, use new disposable pipette tips for dispensing each reagent, sample, standard and control.

(13) A standard curve must be established for each run.

(14) The controls should be included in each run and fall within established confidence limits. The confidence limits are listed in the QC-Report.

(15) Do not mix kit components with different lot numbers within a test and do not use reagents beyond expiry date as shown on the kit labels.

(16) Avoid contact with Stop Solution containing 0.25 M H$_2$SO$_4$. It may cause skin irritation and burns. In case of contact with eyes or skin, rinse off immediately with water.

(17) TMB substrate has an irritant effect on skin and mucosa. In case of possible contact, wash eyes with an abundant volume of water and skin with soap and abundant water. Wash contaminated objects before reusing them.

(18) For information on hazardous substances included in the kit please refer to Safety Data Sheet (SDS). The Safety Data Sheet for this product is made available directly on the website of the manufacturer or upon request.

(19) The expected reference values reported in this test instruction are only indicative. It is recommended that each laboratory establishes its own reference intervals.

(20) This kit is for research use only and should not be used as a diagnostic tool.

(21) Kit reagents must be regarded as hazardous waste and disposed according to national regulations.

2.2 Limitations

Any inappropriate handling of samples or modification of this test might influence the results.

2.2.1 Interfering substances

Samples containing precipitates or fibrin strands or which are haemolytic or lipemic might cause inaccurate results.

2.2.2 Drug interferences

Please refer to point "Sample collection and storage".

2.2.3 High-Dose-Hook effect

No hook effect was observed in this test.

3. Storage and stability

Store the unopened reagents at 2 - 8 °C until expiration date. Do not use components beyond the expiry date indicated on the kit labels. Once opened, the reagents are stable for 1 month when stored at 2 - 8 °C. Once the resealable pouch has been opened, care should be taken to close it tightly with desiccant again.

4. Materials

4.1 Content of the kit

**BA D-0090**  | **FOILS**  | **Adhesive Foil** - Ready to use  
Content: Adhesive Foils in a resealable pouch  
Volume: 1 x 4 foils

**BA E-0030**  | **WASH-CONEC 50x**  | **Wash Buffer Concentrate** - Concentrated 50x  
Content: Buffer with a non-ionic detergent and physiological pH  
Volume: 1 x 20 ml/vial, light purple cap
**BA E-0040 Enzyme Conjugate** - Ready to use
Content: Goat anti-rabbit immunoglobulins conjugated with peroxidase
Volume: 1 x 12 ml/vial, red cap

**BA E-0055 Substrate** - Ready to use
Content: Chromogenic substrate containing tetramethylbenzidine, substrate buffer and hydrogen peroxide
Volume: 1 x 12 ml/vial, black cap

**BA E-0080 Stop-Solin** - Ready to use
Content: 0.25 M sulfuric acid
Volume: 1 x 12 ml/vial, light grey cap

**BA E-0131 Metanephrine Microtiter Strips** - Ready to use
Content: 1 x 96 well (12x8) antigen precoated microwell plate in a resealable blue pouch with desiccant

**BA E-8110 Metanephrine Antiserum** - Ready to use
Content: Rabbit anti-Metanephrine antibody, blue coloured
Volume: 1 x 6 ml/vial, blue cap

**Standards and Controls** - Ready to use

<table>
<thead>
<tr>
<th>Cat. no.</th>
<th>Component</th>
<th>Colour/Cap</th>
<th>Concentration pg/ml</th>
<th>Concentration pmol/l</th>
<th>Volume/Vial</th>
</tr>
</thead>
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<tr>
<td>BA E-8301</td>
<td>STANDARD A</td>
<td>white</td>
<td>0 0</td>
<td></td>
<td>4 ml</td>
</tr>
<tr>
<td>BA E-8302</td>
<td>STANDARD B</td>
<td>light yellow</td>
<td>36 183</td>
<td></td>
<td>4 ml</td>
</tr>
<tr>
<td>BA E-8303</td>
<td>STANDARD C</td>
<td>orange</td>
<td>120 608</td>
<td></td>
<td>4 ml</td>
</tr>
<tr>
<td>BA E-8304</td>
<td>STANDARD D</td>
<td>dark blue</td>
<td>360 1 825</td>
<td></td>
<td>4 ml</td>
</tr>
<tr>
<td>BA E-8305</td>
<td>STANDARD E</td>
<td>light grey</td>
<td>1 200 6 084</td>
<td></td>
<td>4 ml</td>
</tr>
<tr>
<td>BA E-8306</td>
<td>STANDARD F</td>
<td>black</td>
<td>3 600 18 252</td>
<td></td>
<td>4 ml</td>
</tr>
<tr>
<td>BA E-8351</td>
<td>CONTROL 1</td>
<td>light green</td>
<td>Refer to QC-Report for expected value and acceptable range!</td>
<td>4 ml</td>
<td></td>
</tr>
<tr>
<td>BA E-8352</td>
<td>CONTROL 2</td>
<td>dark red</td>
<td>Refer to QC-Report for expected value and acceptable range!</td>
<td>4 ml</td>
<td></td>
</tr>
</tbody>
</table>

Conversion: Metanephrine (pg/ml) x 5.07 = Metanephrine (pmol/l)
Content: Acidic buffer with non-mercury stabilizer, spiked with a defined quantity of Metanephrine

**BA E-8327 Adjustment Buffer** - Ready to use
Content: Tris-Buffer
Volume: 1 x 10 ml/vial, yellow cap

**BA R-8313 Assay Buffer** - Ready to use
Content: 25% organic solvent
Volume: 1 x 30 ml/vial, orange cap

**BA R-8312 Acylation Concentrate** – Concentrated
Content: Acylation reagent in DMSO
Volume: 1 x 1.5 ml/vial, dark grey cap

**Hazards identification:**
H290 May be corrosive to metals.
H314 Causes severe skin burns and eye damage.

**BA E-8351**
Content: Acidic buffer with non-mercury stabilizer, spiked with a defined quantity of Metanephrine
Volume: 1 x 10 ml/vial, yellow cap

**BA R-8313**
Content: 25% organic solvent
Volume: 1 x 30 ml/vial, orange cap

**BA R-8312**
Content: Acylation reagent in DMSO
Volume: 1 x 1.5 ml/vial, dark grey cap

**Hazards identification:**
H302 Harmful if swallowed.
BA R-8318  Extraction Plate - Ready to use
Content: 1 x 96 well plate, precoated with ion-exchanger in a resealable pouch

BA R-8325  Cleaning Concentrate - Concentrated 25x
Content: Buffer with sodium acetate
Volume: 1 x 20 ml/vial, brown cap

BA R-8326  Elution Buffer - Ready to use
Content: 0.1 M Sodium hydroxide, dark purple coloured
Volume: 1 x 14 ml/vial, dark green cap

BA R-8828  Equalizing-Reagent - Ready to use
Content: Human serum, negative for HIV I/II, HBsAg and HCV
Volume: 1 x 14 ml/vial, white cap

4.2 Additional materials and equipment required but not provided in the kit
− Calibrated precision pipettes to dispense volumes between 20 - 350 µl; 3 ml
− Microtiter plate washing device (manual, semi-automated or automated)
− ELISA reader capable of reading absorbance at 450 nm and if possible 620 - 650 nm
− Microtiter plate shaker (shaking amplitude 3 mm; approx. 600 rpm)
− Absorbent material (paper towel)
− Water (deionized, distilled, or ultra-pure)
− Vortex mixer

5. Sample collection and storage
Medications like Serotonin-noradrenaline reuptake inhibitors, trycyclic antidepressants, MAO inhibitors, antihypertensive drugs and L-DOPA can influence Metanephrine and Normetanephrine level. People who are taking such medication should consult with their doctor before specimen collection.
Sympathomimetic agents, sport and smoking can also influence Metanephrine and Normetanephrine level. Alcohol and caffeinated drinks should be avoided the day before and including the day of sample collection.
EDTA- or Heparin-Plasma
Whole blood should be collected into centrifuge tubes (Monovette™ or Vacuette™) containing EDTA or heparin as anti-coagulant and centrifuged (according to manufacturer’s instructions) immediately after collection.
Haemolytic and lipemic samples should not be used for the assay.
Storage: up to 6 hours at 2 - 8 °C, for longer period (up to 6 month) at -20 °C.
Repeated freezing and thawing should be avoided.

6. Test procedure
The ELISA can be run using an overnight incubation without shaking (results within approx. 24 hours) or alternatively as a fast version with shortened antiserum incubation times with shaking (results within approx. 6 hours).
Allow all reagents to reach room temperature and mix thoroughly by gentle inversion before use. Number the Extraction Plate and microwell plate (microtiter strips which are removed from the frame for usage should be marked accordingly to avoid any mix-up).
Duplicate determinations are recommended.
The binding of the antibodies, the enzyme conjugates, and the activity of the enzyme used are temperature dependent, the absorption values may vary if a thermostat is not used. The higher the temperature, the higher the absorption values will be. The absorption values also depend on the incubation times. The optimal temperature for the Enzyme Immunoassay is between 20 – 25 °C.

6.1 Preparation of reagents
Wash Buffer
Dilute the 20 ml of Wash Buffer Concentrate with water (deionized, distilled, or ultra-pure) to a final volume of 1000 ml.
Storage: 1 month at 2 – 8 °C

Cleaning Buffer
Dilute the 20 ml Cleaning Concentrate with water (deionized, distilled, or ultra-pure) to a final volume of 500 ml.
Storage: 1 month at 2 – 8 °C
**Acylation Solution**
As the Acylation Solution is only stable for a maximum of 3 minutes, it should not be prepared before starting the assay. Therefore its preparation is described in the protocol in chapter 6.3, step 3. Discard after use!

**Metanephrine Microtiter Strips**
In rare cases residues of the blocking and stabilizing reagent can be seen in the wells as small, white dots or lines. These residues do not influence the quality of the product.

**6.2 Preparation of samples Extraction**

1. Pipette 20 µl of standards and controls into the respective wells of the Extraction Plate.
2. Add 20 µl Standard A to all wells containing plasma samples.
3. Add 200 µl of Equalizing Reagent to the wells with standards and controls.
4. Pipette 200 µl of plasma samples to the respective wells.
5. Incubate plate for 2 hours at RT (20 – 25 °C) on a shaker (approx. 600 rpm).
6. Empty plate and blot dry by tapping the inverted plate on absorbent material.
7. Pipette 250 µl of Assay Buffer into all wells. Incubate the plate for 5 min at RT (20 – 25 °C) on a shaker (approx. 600 rpm). Empty plate and blot dry by tapping the inverted plate on absorbent material.
8. Wash the plate 3 x by adding 350 µl of Cleaning Buffer, discarding the content and blotting dry each time by tapping the inverted plate on absorbent material.
9. Pipette 100 µl of Elution Buffer into all wells. Please note: the colour changes caused by the elution buffer can vary between standards and samples.
10. Cover plate with adhesive foil. Incubate 15 min at RT (20 - 25 °C) on a shaker (approx. 600 rpm). Remove the foil. Do not decant the supernatant thereafter!

The following volumes of the supernatant are needed for the subsequent ELISA:

| Metanephrine 50 µl |

**6.3 Metanephrine ELISA**

1. Pipette 25 µl of Adjustment Buffer into all wells of the Metanephrine Microtiter Strips.
2. Pipette 50 µl of the extracted standards, controls and samples into the respective wells.
3. Preparation of Acylation Solution:
Pipette 80 µl Acylation Reagent Concentrate (BA R-8312) to 3 ml water (deionized, distilled, or ultra-pure) and mix thoroughly.
4. Pipette 25 µl of the freshly prepared Acylation Solution into all wells.
5. Incubate for 15 min at RT (20 – 25 °C) on a shaker (approx. 600 rpm).
6. Pipette 50 µl of the Metanephrine Antiserum into all wells.
7. Cover the plate with Adhesive Foil, shake for 1 min at RT (20 – 25 °C) on a shaker and incubate for 15 - 20 h (overnight) at 2 – 8 °C without shaking. Alternatively incubate for 2 h at RT (20 - 25 °C) on a shaker (approx. 600 rpm).
8. Remove the foil. Discard or aspirate the contents of the wells. Wash the plate 4 x by adding 300 µl of Wash Buffer, discarding the content and blotting dry each time by tapping the inverted plate on absorbent material.
9. Pipette 100 µl of the Enzyme Conjugate into all wells.
10. Incubate for 30 min at RT (20 – 25 °C) on a shaker (approx. 600 rpm).
11. Discard or aspirate the contents of the wells. Wash the plate 4 x by adding 300 µl of Wash Buffer, discarding the content and blotting dry each time by tapping the inverted plate on absorbent material.
12. Pipette 100 µl of the Substrate into all wells and incubate for 20 - 30 min at RT (20 – 25 °C) on a shaker (approx. 600 rpm). Avoid exposure to direct sunlight!
13. Add 100 µl of the Stop Solution to all wells and shake the microtiter plate to ensure a homogeneous distribution of the solution.
14. Read the absorbance of the solution in the wells within 10 minutes, using a microplate reader set to 450 nm (if available a reference wavelength between 620 nm and 650 nm is recommended).
7. Calculation of results

<table>
<thead>
<tr>
<th>Measuring range</th>
<th>Metanephrine</th>
</tr>
</thead>
<tbody>
<tr>
<td>(overnight ELISA)</td>
<td>15.1 – 3 600 pg/ml</td>
</tr>
</tbody>
</table>

The standard curve is obtained by plotting the absorbance readings (calculate the mean absorbance) of the standards (linear, y-axis) against the corresponding standard concentrations (logarithmic, x-axis).

Use a non-linear regression for curve fitting (e.g. spline, 4- parameter, akima).

⚠️ This assay is a competitive assay. This means: the OD-values are decreasing with increasing concentrations of the analyte. OD-values found below the standard curve correspond to high concentrations of the analyte in the sample and have to be reported as being positive.

The concentrations of the samples and controls can be read directly from the standard curve.

Samples found with concentrations higher than the highest standard (Standard F) should be diluted accordingly with the included Equalizing Reagent and have to be re-assayed.

**Conversion**

Metanephrine (pg/ml) x 5.07 = Metanephrine (pmol/l)

**Expected reference value**

It is strongly recommended that each laboratory should determine its own reference value.

The expected reference values indicated below are based on method comparison studies to LC-MS/MS (1) with blood samples taken in the sitting position.

| Metanephrine | < 65 pg/ml |

For the interpretation of the results, a grey area has to be considered. This grey area does not depend on the methodology used and is reflected in a slight to mediate increase in Metanephrine and Normetanephrine up to 4 times the upper cut-off (Eisenhofer et al. 2003). Approx. 20 % of the tumors are found in this grey area, especially in the case of the Hereditary Syndrome, incidental tumors and in sporadic cases of Pheochromocytomas with a diameter less than 1 cm.

In case of a result in the grey area, it is recommended to collect a new sample together with an anamnesis concerning especially influences like the medication and age of the patient.

7.1 Quality control

It is recommended to use control samples according to national regulations. Use controls at both normal and pathological levels. The kit or other commercial controls should fall within established confidence limits. The confidence limits of the kit controls are indicated in the QC-Report.

7.2 Typical standard curve

⚠️ Example, do not use for calculation!
8. Assay characteristics (overnight ELISA)

<table>
<thead>
<tr>
<th>Analytical Sensitivity</th>
<th>Metanephrine</th>
</tr>
</thead>
<tbody>
<tr>
<td>LOD (pg/ml)</td>
<td>14.9</td>
</tr>
<tr>
<td>LOQ (pg/ml)</td>
<td>15.1</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Substance</th>
<th>Cross Reactivity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Metanephrine</td>
<td></td>
</tr>
<tr>
<td>Derivatized Metanephrine</td>
<td>100</td>
</tr>
<tr>
<td>Derivatized Normetanephrine</td>
<td>0.05</td>
</tr>
<tr>
<td>3-Methoxytyramin</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>Adrenaline</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>Noradrenaline</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>Dopamin</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>Vanillic mandelic acid</td>
<td>&lt; 0.01</td>
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<tr>
<td>Homovanillic acid</td>
<td>&lt; 0.01</td>
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<tr>
<td>L-DOPA</td>
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<tr>
<td>L-Tyrosin</td>
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<td>Tyramine</td>
<td>&lt; 0.01</td>
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<tr>
<td>Normetanephrine</td>
<td>&lt; 0.01</td>
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<tr>
<td>Acetaminophen</td>
<td>&lt; 0.01</td>
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</table>

<table>
<thead>
<tr>
<th>Precision</th>
<th>Substances</th>
<th>Sample 1 Mean (pg/ml)</th>
<th>CV (%)</th>
<th>Sample 2 Mean (pg/ml)</th>
<th>CV (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intra-Assay</td>
<td>Metanephrine</td>
<td>66.3</td>
<td>11.4</td>
<td>122</td>
<td>13.5</td>
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<tr>
<td></td>
<td></td>
<td>308</td>
<td>10.6</td>
<td>783</td>
<td>9.2</td>
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<tr>
<td>Inter-Assay</td>
<td>Metanephrine</td>
<td>67.8</td>
<td>17.6</td>
<td>134</td>
<td>12.7</td>
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<tr>
<td></td>
<td></td>
<td>319</td>
<td>11.0</td>
<td>847</td>
<td>7.5</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Linearity</th>
<th>Serial dilution up to</th>
<th>Mean (%)</th>
<th>Range (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Metanephrine</td>
<td>1:64</td>
<td>107</td>
<td>101 - 124</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Recovery</th>
<th>Sample Mean (%)</th>
<th>Range (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Metanephrine</td>
<td>88</td>
<td>80 - 99</td>
</tr>
</tbody>
</table>

Method Comparison: ELISA vs. LC-MS/MS\(^{(1)}\)

\[ y = 0.91x + 1.8; \quad r^2 = 0.96; \quad n = 46 \]

9. References/Literature

Warranty Information
Eagle Biosciences, Inc. warrants its Product(s) to operate or perform substantially in conformance with its specifications, as set forth in the accompanying package insert. This warranty is expressly limited to the refund of the price of any defective Product or the replacement of any defective Product with new Product. This warranty applies only when the Buyer gives written notice to the Eagle Biosciences within the expiration period of the Product(s) by the Buyer. In addition, Eagle Biosciences has no obligation to replace Product(s) as result of a) Buyer negligence, fault or misuse, b) improper use, c) improper storage and handling, d) intentional damage, or e) event of force majeure, acts of God, or accident. Eagle Biosciences makes no warranties, either expressed or implied, except as provided herein, including without limitation thereof, warranties as to marketability, merchantability, fitness for a particular purpose or use, or non-infringement of any intellectual property rights. In no event shall the company be liable for any indirect, incidental, or consequential damages of any nature, or losses or expenses resulting from any defective product or the use of any product. Product(s) may not be resold, modified, or altered for resale without prior written approval from Eagle Biosciences, Inc.

For further information about this kit, its application or the procedures in this kit insert, please contact the Technical Service Team at Eagle Biosciences, Inc. at info@eaglebio.com or at 866-411-8023.

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⚠️ For updated literature or any other information please contact your local supplier.

Symbols:

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<tr>
<th>Symbol</th>
<th>Description</th>
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<tr>
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<tr>
<td>![ruo]</td>
<td>For research use only!</td>
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