Supplementary Data

Fig. S1: Complete step 2 dilution curve for endotoxin and non-endotoxin pyrogens
Peripheral blood mononuclear cells (PBMC) were stimulated with nine doses of RSE (from 0.006 to 1.6 EU/mL) (A), of FSL-1 (from 0.0002 to 0.078 ng/mL) (B) or R848 (from 0.004 to 0.9 mg/mL) (C), and IL-6 response, in terms of optical density values, was measured by ELISA. One representative experiment out of five experiments yielding similar results is shown.

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Fig. S2: Intermediate Precision Function
Intermediate precision (IP) of the method was calculated at 3 different RSE doses (0.1, 0.2 and 0.4 EU/mL) and arranged in a linear fitting. $R^2 = 0.841$. RSD, relative standard deviation; AS, assay sensitivity; RSE, reference standard endotoxin.

Scheme 1: Calculation of CLC and MVD
Scheme used for the calculation of contaminant limit concentration (CLC) and maximum valid dilution (MVD) for Encepur vaccine considering its use as a pediatric vaccine. K, threshold of pyrogenic dose per kilogram of body mass; M, maximum recommended dose of product per kilogram of body mass; LOD, limit of detection; AS, assay sensitivity

**MVD and CLC calculation**
- $K = 5$ EU/kg (as for any parenteral administration);
- $M =$ dose (mL)/body mass (kg) where dose = 0.25 mL and body mass = 5 kg (since it is a pediatric vaccine);
- LOD (single PBMC donor) = 0.04 EU/mL
- Assay sensitivity [AS] = 0.1 EU/mL (the lowest or one of the lower concentrations of RSE or NEPs close to the beginning of the linear part of the standard curve)

$CLC = \frac{K}{M} = 100$ EU/mL
$MVD^* = CLC/LOD = 2700$
$MVD^\circ = CLC/AS = 1000$

* As described in Ph. Eur.
° Proposed new calculation
Scheme 2: Plate layout for the application of Method A
Scheme of plate layout for testing one lot of Encepur by following Ph. Eur. guidance for Method A application. Three vaccine doses (1:100, 1:200 and 1:400) were tested alone or in combination with a fixed dose of the RSE (0.2 EU/mL); a RSE standard curve (from 0.025 to 0.8 EU/mL) was constructed for determining the equivalent of endotoxin units per mL (eEU/mL) present in the vaccine.

![Plate layout for Method A](image)

* Four doses of RSE are mandatory according to Ph. Eur. requirements. Given the variability among PBMC from different donors experienced during MAT optimization for Encepur, 6 RSE doses are strongly recommended.

Scheme 3: Plate layout for the application of Method B
Scheme of plate layout for testing one lot of Encepur following Ph. Eur. guidance for Method B application. Three vaccine doses (1:100, 1:200 and 1:400) were tested alone or in combination with a fixed dose of the RSE (0.1 EU/mL or 0.2 EU/mL); an RSE standard curve (from 0.025 to 0.4 EU/mL) was inserted.

![Plate layout for Method B](image)

* Four doses of RSE are mandatory according to Ph. Eur. requirements. Given the variability among PBMC from different donors experienced during MAT optimization for Encepur, 5 RSE doses are strongly recommended.