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Señor Director
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Superintendencia de Industria y Comercio
E. S. D.

SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO



No. 08-075690- -00007-0000

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Referencia : Expediente No. **08.075.690**
Asunto : Solicitud de patente para: **PRUEBA DE ACTIVACIÓN DE MONOCITO MEJORADA PARA TENER MEJOR CAPACIDAD PARA DETECTAR CONTAMINANTES PIROGÉNICOS SIN ENDOTOXINA EN PRODUCTOS MÉDICOS**
Solicitante : **BAXTER INTERNATIONAL INC., BAXTER HEALTHCARE S.A., NATIONAL BIOLOGICAL STANDARDS BOARD**
Fecha solicitud : 22 de julio de 2008

1. Yo, Luz Clemencia DE PÁEZ, en mi condición de apoderada de la sociedad solicitante de la patente de la referencia, me refiero al oficio No. **11421** notificado por fijación en lista del 29 de junio de 2012.
2. Mediante memorial radicado ante ese Despacho el 24 de septiembre de 2012, solicité un plazo adicional de treinta (30) días para atender el oficio No. **11421**.
3. Mediante oficio No. **11421** se puso en conocimiento del solicitante el informe que contiene las observaciones hechas por su Despacho sobre la presente solicitud de patente. Las observaciones realizadas en el oficio son las siguientes:

3.5 Análisis de patentabilidad

3.5.1 Novedad

El examinador considera que las características esenciales del anticuerpo de la reivindicación 1 habían sido previamente divulgadas en D1: "Evaluation of the in vitro pyrogen test system based on proinflammatory cytokine release from human monocytes: comparison with a human whole blood culture test system and with the rabbit pyrogen test". *Clinical and Diagnostic Laboratory Immunology*. (2002), el cual da a conocer un método para detectar pirógenos que se utiliza en el documento de la presente invención obteniendo de esta manera el mismo producto.

3.5.2 Nivel inventivo

El Examinador considera que la combinación de las enseñanzas de los documentos D2: "A rapid 'one-plate' in vitro test for pyrogens". *Journal of immunological methods*, Elsevier Science Publishers B.V. (2003) y D3: "A rapid 'one-plate' in vitro test for pyrogens". *Journal of immunological methods*, Elsevier Science Publishers B.V. (2005), afecta el nivel inventivo de la presente solicitud. Específicamente se afirma que la persona medianamente versada en la materia habría inferido sin mucho esfuerzo, el realizar el método que se conoce en D2 con las placas de fondo plano o fondo en U de polipropileno que se divulgan en D3, con el fin de llegar al objeto de la reivindicación 3.

4. Respuesta a las objeciones

De este modo y con el objeto de atender las objeciones arriba mencionadas, se desea presentar las siguientes modificaciones, aclaraciones y argumentos:

4.1 Modificación de una solicitud en trámite

De acuerdo con el artículo 34 de la Decisión 486 de la Comisión de la Comunidad Andina, donde se establece que la modificación de una solicitud es posible en cualquier momento del trámite mientras no implique una ampliación de la solicitud inicial, me permito presentar un nuevo pliego reivindicatorio conformado por 38 reivindicaciones.

A continuación se han introducido las siguientes modificaciones al pliego reivindicatorio:

Reivindicación No.	Reivindicación No. Correspondiente a la emitida por
1	Reivindicación 1 de US 7,736,863
2	Reivindicación 36 de US 7,736,863
3	Reivindicaciones 2 y 37 de US 7,736,863
4	Reivindicaciones 3 y 38 de US 7,736,863
5	Reivindicaciones 4 y 39 de US 7,736,863
6	Reivindicaciones 5 y 40 de US 7,736,863
7	Reivindicaciones 6 y 41 de US 7,736,863
8	Reivindicación 1 permitida en la solicitud Europea EP 06846727.3
9	Reivindicaciones 7 y 42 de US 7,736,863
10	Reivindicaciones 8 y 43 de US 7,736,863
11	Reivindicaciones 9 y 44 de US 7,736,863
12	Reivindicaciones 10 y 45 de US 7,736,863
13	Reivindicaciones 11 y 46 de US 7,736,863
14	Reivindicaciones 12 y 47 de US 7,736,863
15	Reivindicaciones 13 y 48 de US 7,736,863
16	Reivindicaciones 14 y 68 de US 7,736,863
17	Reivindicaciones 15 y 49 de US 7,736,863
18	Reivindicaciones 16 y 50 de US 7,736,863
19	Reivindicaciones 17-33 y 51-67 de US 7,736,863
20	Reivindicaciones 34 y 69 de US 7,736,863
21	Reivindicaciones 35 y 70 de US 7,736,863
22	Reivindicación 1 de US 8,058,021
23	Reivindicación 13 de US 8,058,021
24	Reivindicaciones 2 y 14 de US 8,058,021
25	Reivindicaciones 3 y 15 de US 8,058,021
26	Reivindicaciones 4 y 16 de US 8,058,021
27	Reivindicaciones 5 y 17 de US 8,058,021
28	Reivindicaciones 6 y 18 de US 8,058,021
29	Reivindicaciones 7 y 19 de US 8,058,021
30	Reivindicaciones 8 y 20 de US 8,058,021

31	Reivindicaciones 9 y 21 de US 8,058,021
32	Reivindicaciones 10 y 22 de US 8,058,021
33	Reivindicaciones 11 y 23 de US 8,058,021
34	Reivindicaciones 12 y 24 de US 8,058,021
35	Reivindicación 25 de US 8,058,021
36	Reivindicación 26 de US 8,058,021
37	Reivindicación 1 de US 8,058,021
38	Reivindicación 11 de US 8,058,021

La patente US 7,736,863 corresponde a la expedida para la solicitud provisional No. 60/752,970 de la cual se reivindicó prioridad en el trámite de la solicitud internacional No. PCT/US2006/062424, correspondiente a la presente fase nacional. Las solicitudes de continuación de esta solicitud de patente fueron otorgadas bajo los números 8,058,021 y 8,053,200. Por su parte la solicitud de patente EP 06846727.3, recientemente otorgada es la correspondiente a la presente solicitud. En consecuencia, las modificaciones al capítulo reivindicatorio fueron efectuadas teniendo en cuenta dichas solicitudes de patente que corresponden a la misma materia acá reivindicada y que cumplieron con los requisitos de patentabilidad.

Por lo anterior, se solicita atentamente tener en cuenta este nuevo pliego reivindicatorio para el análisis de patentabilidad.

4.2 Argumentos a favor de la novedad de la presente solicitud

El artículo 16 de la Decisión 486 señala lo siguiente:

“**Artículo 16.-** Una invención se considerará nueva cuando no está comprendida en el estado de la técnica.

El estado de la técnica comprenderá todo lo que haya sido accesible al público por una descripción escrita u oral, utilización, comercialización o cualquier otro medio antes de la fecha de presentación de la solicitud de patente o, en su caso, de la prioridad reconocida.

Sólo para el efecto de la determinación de la novedad, también se considerará dentro del estado de la técnica, el contenido de una solicitud de patente en trámite ante la oficina

nacional competente, cuya fecha de presentación o de prioridad fuese anterior a la fecha de presentación o de prioridad de la solicitud de patente que se estuviese examinando, siempre que dicho contenido esté incluido en la solicitud de fecha anterior cuando ella se publique o hubiese transcurrido el plazo previsto en el artículo 40”.

Siguiendo entonces los lineamientos de la Jurisprudencia aplicable al análisis de novedad de una solicitud de patente, se considera que cuando las características técnicas esenciales son idénticas a las características de la materia divulgada en cada documento del estado de la técnica, puede afectar dicho requerimiento de patentabilidad, en cuyo caso se considera que no tiene novedad. Adicionalmente se considera nueva la invención si se encuentra alguna diferencia o un sólo aspecto que no hubiese sido revelado en el arte previo.

Con base en lo anterior, respetuosamente manifestamos nuestro desacuerdo con el análisis llevado a cabo por el Examinador y con las conclusiones del mismo y solicitamos atentamente la reconsideración de las razones por las cuales se objeta la novedad, con base en la siguiente información:

Cada una de las reivindicaciones independientes (reivindicaciones 1 y 36) en el pliego reivindicatorio modificado requiere que un sistema de ensayo utilizado en el método reivindicado de detectar pirógenos sin endotoxina comprenda al menos un depósito de microtitulación formado de manera tal que, el reactivo que contiene monocitos se concentra para proporcionar un mayor contacto célula con célula en comparación con un depósito de microtitulación de fondo plano, en donde la superficie del depósito de microtitulación comprende un revestimiento de polipropileno de la totalidad del depósito de microtitulación que se compone de polipropileno.

En cuanto a la novedad del pliego reivindicatorio modificado presentado en el presente documento, D1 no divulga un sistema de ensayo que comprende las características antes mencionadas del sistema de ensayo. Limitadamente, D1 divulga una placa de 24 depósitos (Ejemplo, pág. 589, columna 1, secciones tituladas ‘Selection of a human monocytic cell line suitable for use as indicator cells’ y ‘Subcloning of MM6 cells and induction of proinflammatory cytokine release from MM6 cells by pyrogens’). Sin embargo, D1 no divulga el material del que está hecha la placa de 24 depósitos, y D1 tampoco enseña la forma de los depósitos de la placa de 24 depósitos. D1 también describe el uso de ‘tubos de polipropileno estériles’ en la página 589, sección titulada ‘Pyrogen-induced proinflammatory cytokine release from cultured

human whole blood cells'. Sin embargo, la forma del tubo no se divulga en D1 y, además, amablemente manifestamos que una persona medianamente versada en la materia puede apreciar que un tubo de polipropileno no es lo mismo que un depósito de microtitulación. Por lo tanto, no se puede afirmar que D1 destruye la novedad de las reivindicaciones modificadas en el presente pliego.

Ni D2 o D3 divulgan los métodos para detectar pirógenos sin endotoxina, como se reivindicó en las reivindicaciones modificadas 1 y 36. Ni D2 o D3 divulgan la detección de pirógenos usando un sistema de ensayo que comprende al menos un depósito de microtitulación formado de manera tal que el reactivo que contiene monocitos se concentra para proporcionar un mayor contacto célula con célula en comparación con un depósito de microtitulación de fondo plano, en donde la superficie del depósito de microtitulación comprende un revestimiento de polipropileno de la totalidad del depósito de microtitulación que se compone de polipropileno.

Limitadamente, D3 (pág. 164, secciones 2.4.2 y 2.6.2) divulga el uso de placas de 96 depósitos de cultivo de tejidos del Falcon Microtest, Beckton Dickinson Labware. Como se muestra en el Anexo A adjunto, estas placas están hechas de poliestireno y tienen pocillos de fondo plano. D3 (pág. 164, sección 2.4.3) divulga el uso de Nunc-Immuno 96-well plate MaxiSorp, F96 de Life Technologies. Sin embargo, como se muestra en el Anexo B, estas placas están hechas de poliestireno y tienen depósitos de fondo plano. Adicionalmente, D3 divulga, en la pág. 166, sección 2.9.2, el uso de Dynes PF microtiter 'flat bottom' styrene 96-well plates. Sin embargo, estas placas están hechas de estireno y son de fondo plano.

Adicionalmente, D2 es un artículo de una revista científica co-escrito por Stephen Poole, uno de los inventores de la presente solicitud. Como se ha indicado en el párrafo 3 de la Declaración por Stephen Poole como un archivo adjunto, ninguno de los experimentos descritos en D2 incluye el uso de placas de depósitos de microtitulación que tienen un recubrimiento de polipropileno o placas que fueron hechas en su totalidad de polipropileno.

En vista de lo anterior, no se puede afirmar que D2 o D3 pueden destruir la novedad de las reivindicaciones modificadas en el presente pliego.

4.3 Argumentos a favor del nivel inventivo de la presente solicitud

El artículo 18 de la Decisión 486 señala lo siguiente:

Artículo 18.- Se considerará que una invención tiene nivel inventivo, si para una persona del oficio normalmente versada en la materia técnica correspondiente, esa invención no hubiese resultado obvia ni se hubiese derivado de manera evidente del estado de la técnica.

Consecuentemente, una invención se considerará inventiva si la misma no hubiese resultado obvia a partir del arte previo, para una persona del oficio normalmente versada en la materia. Esto a su vez implica que el Examinador a cargo de esta evaluación debe colocarse en el momento exacto en el que el inventor buscaba resolver el problema técnico contemplado en la solicitud y para el cual poseía la literatura y el conocimiento disponibles en dicho momento.

Siguiendo entonces los lineamientos de la Jurisprudencia aplicable al análisis del nivel inventivo de una solicitud de patente, se considera que una anterioridad o una combinación de dos anterioridades puede afectar dicho requerimiento de patentabilidad, si una persona medianamente versada en la materia encuentra enseñanzas o sugerencias claras que, a partir de un conocimiento medio en dicho campo, le permitan llegar de manera obvia a la invención reivindicada. Adicionalmente se considera que una mejora sustancial, un efecto inesperado o una ventaja con respecto al arte previo es un indicio de nivel inventivo, por cuanto aporta un salto tecnológico en la regla técnica.

Respecto a lo anterior, el Tribunal de Justicia de la Comunidad Andina ha expuesto en la Interpretación Prejudicial del proceso 13-IP-2010:

“En este orden de ideas, se puede concluir que una invención goza de nivel inventivo cuando a los ojos de un experto medio en el asunto de que se trate, se necesita algo más que la simple aplicación de los conocimientos técnicos en la materia para llegar a ella, es decir, que de conformidad con el estado de la técnica el invento no es consecuencia clara y directa de dicho estado”.

[señalado fuera del texto]

Por lo anterior, se entiende que para que una persona medianamente versada en la materia pudiera derivar de manera obvia una invención, requeriría simplemente aplicar sus conocimientos técnicos. En contraste, todo aquello que va más allá de la simple aplicación de

los conocimientos técnicos y que no ha sido revelado en el estado de la técnica, debe ser considerado inventivo.

A este respecto, respetuosamente manifestamos nuestro desacuerdo con el análisis llevado a cabo por el Examinador y con las conclusiones del mismo y solicitamos atentamente la reconsideración de las razones por las cuales se objeta el nivel inventivo, con base en la siguiente información:

En cuanto al nivel inventivo de las reivindicaciones modificadas, una persona medianamente versada en la materia no habría sido motivada por ninguno de los documentos D1, D2 y D3 para llegar a los métodos para detectar pirógenos sin endotoxina de las reivindicaciones modificadas. La persona medianamente versada en la materia en el momento de presentación de la presente solicitud no habría sido motivada, porque el uso de polipropileno en ensayos de detección de pirógenos fue desalentado por los Laboratorios Charles River, Harlan Sera-Lab, y el European Dialysis and Transplant Nurses Association (EDTNA) (párrafo [0013] de la presente solicitud). Específicamente la sección 1.1.6.3 del 'EDTNA/ERCA Guidelines: Technical Section' (EDTNA/ERCA Journal 28(3): 107-115 (2002)), establece:

"Para la prueba de endotoxina, los contenedores de muestras debe ser estéril y libres de endotoxina, y hechos del material que no absorbe endotoxinas. El poliestireno normalmente no absorbe endotoxinas, mientras que el polipropileno debe ser evitado."

Similarmente, la pág. 4 del Endosafe Times 11(1): 1-6 (2004) establece:

"Sin embargo, Charles River ha recomendado evitar materiales de poliolefina (polipropileno y polietileno). Mientras que los materiales de poliolefina son extraordinariamente resistentes a los solventes y en sí mismas no lixivian residuos orgánicos, siempre hemos estado preocupados por sus propiedades superficiales hidrofóbicas. La escasez de restos cargados sobre una superficie de poliolefina podría absorber potencialmente endotoxina debido a los dominios hidrofóbicos asociados con el Lípido A."

Como se evidencia en lo anterior, los laboratorios Charles River y el EDTNA recomiendan evitar polipropileno al detectar endotoxina, ya que se cree que el polipropileno absorbe la endotoxina al ser detectada. En consecuencia, se puede decir que estas referencias

del estado de la técnica realmente divulgan enseñanzas que se encuentran lejos del uso del polipropileno en la detección de endotoxinas.

Alternativamente, aún si se supone hipotéticamente que la persona medianamente versada en la materia podría estar motivada para hacer esta sustitución, los resultados descritos en la presente solicitud fueron totalmente inesperados y sorprendentes.

Además, como se ha indicado en el párrafo 4 de la declaración de Stephen Poole presentada aquí, los resultados de D2 demostraron que ni la forma ni el revestimiento o la composición de los depósitos de las placas de microtitulación usadas en el ensayo tuvieron un efecto sobre la capacidad del ensayo de detectar pirógenos. Este hallazgo fue reportado en D2, en la página 213, párrafo 1 de la sección de Resultados, que establece: "Todos los tipos de placas utilizadas en los ensayos de una sola placa, por ejemplo 'fondo plano' y 'fondo en U', tanto en estireno y PVC, arrojaron resultados similares".

En contraste con lo que enseña y sugiere D2, la presente solicitud proporciona datos que demuestran que el material y la forma de los depósitos de microtitulación del sistema de ensayo, de hecho, hace una diferencia inesperada. Como se indica en el párrafo 5 de la declaración de Stephen Poole, el uso de placas de microtitulación de polipropileno con depósitos no planos proporcionaron una mejora significativa para la detección indirecta de pirógenos sin endotoxina. La mejora significativa se evidenció mediante, por ejemplo, la mayor diferencia en la detección de IL-6 unida a anticuerpos específicos IL-6 (como se representa por la absorbancia 450 nm) observada entre los controles Extraneal positivos y negativos cuando los depósitos no planos de polipropileno fueron utilizados en el ensayo (Figura 12A de la solicitud), en comparación con la diferencia entre los dos controles cuando se utilizaron depósitos planos de poliestireno en el ensayo (Figura 12B de la solicitud).

Los resultados obtenidos con el uso de los depósitos no planos de polipropileno fueron sorprendentes e inesperados, ya que ni la forma ni el revestimiento o la composición de los depósitos de placas de microtitulación usados en el ensayo hizo una diferencia en los estudios descritos en D2. Como se indica en el párrafo 5 de la declaración, el Dr. Poole no esperaba que el uso de placas de microtitulación de polipropileno con depósitos de fondo plano proporcionaría tal mejora en la capacidad del ensayo para detectar pirógenos sin endotoxina.

En vista de la significativa e inesperada ventaja de la invención reivindicada, proporcionada sobre el estado de la técnica, el objeto de las reivindicaciones dependientes es inventivo sobre las enseñanzas de D1 a D3.

4.4 Conclusión

Por todo lo anterior, el documento D1 no resulta una anterioridad válida capaz de afectar la novedad del producto de la presente solicitud. De la misma manera, el documento D1, el documento D2, el documento D3 o su combinación, no resultan anterioridades válidas capaces de afectar el nivel inventivo del producto de la presente solicitud.

Por lo tanto, consideramos que el Examinador está pasando por alto que una sola diferencia con respecto al estado de la técnica, permite concluir que el objeto reivindicado es nuevo. Además, está excluyendo el hecho de que el inventor en el momento de solucionar el problema técnico de la solicitud tuvo que emplear habilidad inventiva, pues la presente invención no resulta de la mera extrapolación de las enseñanzas del arte previo, como se afirma. Se está desconociendo el mérito investigativo empleado y que está aportando una ventaja al arte previo.

Dicho mérito investigativo ha sido reconocido por otras oficinas de patentes del mundo, las cuales han otorgado patentes para invenciones equivalentes. Tal es el caso de Estados Unidos, patente No. US7736863; Japón, patente No. JP4686611 y México, patente No. MX282528.

Si bien es cierto que cada país es autónomo en conceder o negar el privilegio de patente para una solicitud, también es cierto que los requisitos de concesión de dichos privilegios son universales, esto es, novedad, nivel inventivo y aplicación industrial, como también la forma de evaluarlos. Así las cosas, el hecho de que la misma solicitud cumpla con los mencionados requisitos en diferentes países, como se mencionó anteriormente, es un claro indicio del merecimiento de la patente en Colombia.

4. Puesto que se ha dado respuesta a todas las objeciones formuladas por el Examinador, se solicita atentamente proceder al otorgamiento de la solicitud de patente. En este sentido, respetuosamente recordamos al Examinador que la patente no podrá ser negada con base en objeciones diferentes a las expuestas en la acción oficial No. 11421, toda vez que al no haber sido trasladadas a mi representada, dicha decisión violaría tanto el tenor literal del artículo 45

de la Decisión 486 como el derecho constitucional al debido proceso. En este evento, y con base en el inciso 2 del artículo 45, respetuosamente solicitamos al Examinador expedir una nueva acción oficial que permita a mi representada contra argumentar las mismas o modificar la solicitud con el fin de cumplir los requisitos de patentabilidad.

5. Anexos

- Formulario de modificaciones en solicitud pendiente
- Nuevo pliego reivindicatorio conformado por 38 reivindicaciones
- Recibo por 840.000 correspondientes a veintiocho reivindicaciones en exceso sobre las primeras 10 de presentación de solicitud.
- Anexo A
- Anexo B
- EDTNA/ERCA Guidelines: Technical Section.
- Endosafe Times. Charles River Laboratories.
- Primera hoja de la solicitud de Estados Unidos US8053200 (B2)
- Primera hoja de la solicitud de Estados Unidos US7736863 (B2)
- Declaración de Stephen Poole, inventor en la presente solicitud
- Artículo 1: Stephen Poole *et al.* (2003). A rapid 'one-plate' in vitro test for pyrogens. Elsevier Science B.V. *Journal of Immunological Methods*. Vol 274: 209-220

Señor Director,


LUZ CLEMENCIA DE PAEZ

C.C. 35.456.344 de Usaquén

T.P.A. No. 23.555

COLO-16577-0841-001-0

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No. M-2012-229118\SGD



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**DIRECCIÓN DE NUEVAS CREACIONES
MODIFICACIONES Y/O CORRECCIONES****1. Identificación del Trámite**

- ☐ Error material ☒ Modificación a una Solicitud en trámite
- ☒ Patente de Invención
- ☐ Patente de Modelo de Utilidad
- ☐ Registro de Diseño Industrial
- ☐ Esquema de Trazado de Circuitos Integrados

2. Datos del solicitantePersona Natural ☐Persona Jurídica ☒**BAXTER INTERNATIONAL INC.,****BAXTER HEALTHCARE S. A.****Y****NATIONAL BIOLOGICAL STANDARDS BOARD**

Nombre o denominación / Nombre o razón social completa

Nombre representante legal

Tipo de empresa Micro ☐ Pequeña ☐ Mediana ☐ Otra ☐Documento de identificación: ☐ C.C. ☐ C.E. ☐ NIT ☐ Otro Número _____

Nacionalidad/País de constitución	Dirección y domicilio del titular	Ciudad
Estados Unidos de América,	One Baxter Parkway, Illinois 60015,	Deerfield,
Suiza	Y	8304 Zurich
Y	Hertistrasse 2, Wallisellen, Kanton,	Y
Reino Unido	Y	South Mimms,
Respectivamente	Blanche Lane, Potters Bar Hertfordshire EN6 3QG,	Respectivamente
	Respectivamente	
Dirección electrónica		No. Fax
clientes@cavelier.com		2 11 86 50
		Número telefónico
		3 47 36 11

☐ Autorizo expresamente a la Superintendencia de Industria y Comercio para que todas las comunicaciones sean enviadas a través de la dirección de correo electrónico

3.

Datos del apoderado:

Apellidos y Nombre De Páez Luz Clemencia		Documento de identificación C.C. 35.456.344	Tarjeta profesional T.P.A. 23.555
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Dirección y domicilio		Ciudad
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Dirección electrónica	No. Fax	Número telefónico
cavelier@cavelier.com	2 11 86 50	3 47 36 11

En caso de haber presentado poder general para asuntos que se adelanten ante la Delegatura de Propiedad Industrial, sírvase indicar el número de su radicación o protocolo de poder general

4.

Datos del trámite

Corrección:

Expediente No 08.075.690

Aparece

Debe ser

Modificación:

☒ Modificación al capítulo reivindicatorio.

Presento nuevo capítulo reivindicatorio compuesto por 38 reivindicaciones y pido respetuosamente que el examen de patentabilidad sea realizado sobre estas nuevas reivindicaciones presentadas. Respetuosamente manifiesto que a pesar de eliminar materia del pliego reivindicatorio originalmente presentado, mi representada no renuncia a la protección que pueda obtener sobre dicha materia y se reserva el derecho de presentar una solicitud divisional que la contenga durante el trámite de la presente solicitud, beneficiándose de la fecha de prioridad inicialmente reivindicada.

☐ Modificación al capítulo descriptivo.

☐ Modificación al resumen.

☐ Modificación a los dibujos.

☐ Modificación al solicitante.

5. Anexos

- ☐ Poder, si fuere el caso.
- ☒ Documento que contiene las modificaciones o correcciones.
- ☐ Copia de la solicitud y sus anexos en formato magnético.
- ☒ Comprobante de pago por 28 reivindicaciones adicionales después de la décima reivindicación por \$840.000

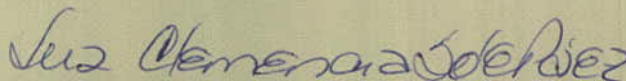
6. Firma

Nombre y apellidos

LUZ CLEMENCIA DE PAEZ

C.C. 35.456.344 DE Usaquén

Firma



Tarjeta Profesional 23.555

SGD\NCA\WPC16577\ID-229444

REIVINDICACIONES

1. Un método para detectar pirógenos sin endotoxina en una muestra, en donde el método comprende los pasos de:

combinar un reactivo que contiene monocitos y la muestra que será probada en un primer sistema de ensayo que comprende al menos un depósito de microtitulación formado de manera que el reactivo que contiene monocitos se concentre para proporcionar un mayor contacto célula con célula en comparación con un depósito de microtitulación de fondo plano, en donde la superficie del depósito de microtitulación comprende un revestimiento de polipropileno o la totalidad del depósito de microtitulación se compone de polipropileno;

incubar el reactivo que contiene monocitos y la muestra, en donde, cuando la muestra comprende un pirógeno, el reactivo que contiene monocito produce una citocina o un transmisor endógeno de la respuesta inflamatoria;

transferir los contenidos de primer sistema de ensayo a un segundo sistema de ensayo que comprende al menos una superficie tratada con un anticuerpo para una citocina o un transmisor endógeno de la respuesta inflamatoria; y

ensayar el segundo sistema de ensayo con respecto a la presencia de citocina o un transmisor endógeno enlazado al anticuerpo en la superficie;

mediante lo cual el nivel elevado de citocina o transmisor endógeno enlazado a la superficie indica la presencia de pirógenos en la muestra probada.

2. Un método para detectar pirógenos sin endotoxina en una muestra, en donde el método comprende los pasos de:

combinar un reactivo que contiene monocitos y la muestra que será probada en un sistema de ensayo que comprende (i) al menos un depósito de microtitulación formado de manera que el reactivo que contiene monocitos se concentre para proporcionar un mayor contacto célula con célula en comparación con un depósito de microtitulación de fondo plano, en donde la superficie del depósito de microtitulación comprende un revestimiento de polipropileno o la totalidad del depósito de microtitulación se compone de polipropileno, y (ii) al menos una superficie tratada con un anticuerpo para una citocina o un transmisor endógeno de la respuesta inflamatoria;

incubar el reactivo que contiene monocitos y la muestra, en donde, cuando la muestra comprende un pirógeno, el reactivo que contiene monocitos produce una citocina o un transmisor endógeno de la respuesta inflamatoria;

ensayar el sistema de ensayo para la presencia de citocinas o un transmisor endógeno enlazado al anticuerpo en la superficie;

mediante lo cual el nivel elevado de citocina o transmisor endógeno enlazado a la superficie indica la presencia de pirógenos en la muestra probada.

3. El método tal como se describe en cualesquiera de las reivindicaciones 1 o 2, caracterizado porque la citocina se selecciona del grupo que consiste de interleucina-1 (IL-1), interleucina-1ra(IL-1ra), interleucina-6 (IL-6), interleucina-8 (IL-8), y un factor de necrosis tumoral- α (TNF- α).

4. El método tal como se describe en la reivindicación 3, caracterizado porque la citocina es IL-6.

5. El método tal como se describe en cualesquiera de las reivindicaciones anteriores, caracterizado porque el transmisor endógeno es endotelina.

6. El método tal como se describe en cualesquiera de las reivindicaciones anteriores, caracterizado porque la muestra es un producto médico administrado en forma parenteral.

7. El método tal como se describe en la reivindicación 6, caracterizado porque el producto médico es un producto de sangre, solución de diálisis, vacuna y expansor de plasma.

8. El método tal como se describe en cualesquiera de las reivindicaciones anteriores, caracterizado porque el primer sistema de ensayo o el sistema de ensayo comprende una placa de microtitulación que comprende una pluralidad de depósitos de microtitulación, cada uno de los cuales tiene una forma de modo que el reactivo que contiene el monocito sea concentrado para proporcionar mayor contacto célula con célula en comparación a un depósito de microtitulación de fondo plano, en donde cada depósito de microtitulación comprende una superficie del depósito que comprende un revestimiento de polipropileno o cada depósito de microtitulación se compone enteramente de polipropileno.

9. El método tal como se describe en cualesquiera de las reivindicaciones anteriores, caracterizado porque el depósito de microtitulación comprende una región superior abierta, una región superior que se extiende hacia abajo desde la parte superior abierta, y una pared del fondo que se ahusa en diámetro desde la parte de arriba del punto más bajo hasta el punto más bajo.

10. El método tal como se describe en cualesquiera de las reivindicaciones anteriores, caracterizado porque el depósito de microtitulación comprende una región superior abierta, una región que se extiende hacia abajo desde la región superior que tiene un extremo superior y un extremo de fondo, y una región inferior que tiene un extremo superior y un punto más bajo, extendiéndose la región inferior desde el extremo inferior de la región superior y ahusándose en diámetro más rápidamente que la región superior del extremo superior de la región inferior hacia el punto más bajo.

11. El método tal como se describe en la reivindicación 9, caracterizado porque la pared inferior de dicho depósito de microtitulación no es plano, curvado, parabólico o se extiende hacia abajo, o los lados de dicho depósito de microtitulación están inclinados hacia el interior.

12. El método tal como se describe en cualesquiera de las reivindicaciones anteriores, caracterizado porque el sistema(s) de ensayo es un ensayo inmunoabsorbente enlazado por enzimas (ELISA) colorimétrico, un inmunoensayo radioetiquetado o un inmunoensayo etiquetado con fluorescencia.

13. El método tal como se describe en cualesquiera de las reivindicaciones anteriores, caracterizado porque el reactivo que contiene monocitos comprende células

mononucleares de sangre periférica o células de línea celular monolítica.

14. El método tal como se describe en la reivindicación 13, caracterizado porque la línea celular monolítica comprende una línea celular monolítica humana.

15. El método tal como se describe en la reivindicación 14, caracterizado porque la línea celular monocítica es MONOMAC-6, THP-1 o 28SC.

16. El método tal como se describe en la reivindicación 13, caracterizado porque la línea celular monocítica comprende una línea celular que tiene receptores tipo CD14 y Toll endógenos o que han sido transfectados con receptores tipo CD14 y/o Toll y/o genes reporteros para transmisores inflamatorios o pirogénicos.

17. El método tal como se describe en la reivindicación 13, caracterizado porque las células mononucleares de sangre periférica comprenden células mononucleares de sangre periférica humana.

18. El método tal como se describe en la reivindicación 13, caracterizado porque el reactivo que contiene monocitos se encuentra en dicho sistema de ensayo en una densidad

celular que es mayor a la densidad celular de los monocitos en la sangre completa.

19. El método tal como se describe la reivindicación 13, caracterizado porque el reactivo que contiene monocitos está presente en dicho sistema de ensayo en una densidad celular de (i) al menos o aproximadamente 250,000, (ii) al menos o aproximadamente 500,000, (iii) al menos o aproximadamente 600,000, (iv) al menos o aproximadamente 700,000, (v) al menos o aproximadamente 800,000, (vi) al menos o aproximadamente 900,000, (vii) al menos o aproximadamente 1,000,000, (viii) al menos o aproximadamente 1,100,000, (ix) al menos o aproximadamente 1,200,000, (x) al menos o aproximadamente 1,300,000, (xi) al menos o aproximadamente 1,400,000, (xii) al menos o aproximadamente 1,500,000, (xiii) al menos o aproximadamente 1,600,000, (xiv) al menos o aproximadamente 1,700,000, (xv) al menos o aproximadamente 1,800,000, (xvi) al menos o aproximadamente 1,900,000 ó (xvii) al menos o aproximadamente 2,000,000 células por depósito, en el que la densidad celular no excede un máximo de densidad celular a la que nutrientes celulares insuficientes existen en el volumen de reactivo dentro del depósito para poder mantener las células en el depósito.

20. El método tal como se describe en cualesquiera de las reivindicaciones anteriores, caracterizado porque el reactivo que contiene monocitos comprende la sangre completa.

21. El método tal como se describe en la reivindicación 20, caracterizado porque la sangre completa comprende la sangre completa humana.

22. Un equipo de diagnóstico que comprende:

a). un primer sistema de ensayo que incluye al menos un depósito de microtitulación formado de manera que el reactivo que contiene monocitos se concentre para proporcionar un mayor contacto célula con célula en comparación con un depósito de microtitulación de fondo plano, en donde la superficie del depósito de microtitulación comprende un revestimiento de polipropileno o la totalidad del depósito de microtitulación se compone de polipropileno;

b). un segundo sistema de ensayo que comprende al menos una superficie tratada con un anticuerpo para una citocina o un transmisor endógeno de la respuesta inflamatoria; y

c). instrucciones para incubar en el primer sistema de ensayo un reactivo que contiene monocitos y una muestra que será probada, transfiriendo los contenidos del primer sistema de ensayo a un segundo sistema de ensayo, y ensayando el segundo sistema de ensayo con respecto a la presencia de

citocina o un transmisor endógeno enlazado al anticuerpo en la superficie.

23. Un equipo de diagnóstico que comprende:

a). un sistema de ensayo que comprende (i) al menos un depósito de microtitulación formado de manera que el reactivo que contiene monocitos se concentre para proporcionar un mayor contacto célula con célula en comparación con un depósito de microtitulación de fondo plano, en donde la superficie del depósito de microtitulación comprende un revestimiento de polipropileno o la totalidad del depósito de microtitulación se compone de polipropileno, y (ii) al menos una superficie tratada con un anticuerpo para una citocina o un transmisor endógeno de la respuesta inflamatoria; y

instrucciones para combinar el reactivo que contiene monocitos y una muestra que será probada en un sistema de ensayos, incubando el reactivo que contiene los monocitos y la muestra, y ensayando el segundo sistema de ensayo con respecto a la presencia de citocina o un transmisor endógeno enlazado al anticuerpo en la superficie.

24. El equipo tal como se describe en cualesquiera de las reivindicaciones 22 o 23, que comprende un reactivo que contiene monocitos criopreservados.

25. El equipo tal como se describe en cualesquiera de las reivindicaciones 22 a 24, caracterizado porque cada depósito de microtutulación del primer sistema de ensayo o el sistema de ensayo comprende monocitos crioconservados.

26. El equipo tal como se describe en cualesquiera de las reivindicaciones 22 a 25, caracterizado porque el reactivo que contiene monocitos criopreservado se proporciona a un usuario final mediante el envío en hielo seco.

27. El equipo tal como se describe en cualesquiera de las reivindicaciones 22 a 26, caracterizado porque el reactivo que contiene monocitos criopreservado se proporciona a un usuario final en alícuotas en el número óptimo de número de células por depósito.

28. El equipo tal como se describe en cualesquiera de las reivindicaciones 22 a 27, caracterizado porque el reactivo que contiene monocitos criopreservado comprende células mononucleares de sangre periférica o células de línea celular monolítica crioconservada,

29. El equipo tal como se describe en la reivindicación 28, caracterizado porque la línea celular monolítica comprende una línea celular monolítica humana seleccionada del grupo que consiste de: MONOMAC-6, THP-1 o 28SC, y una línea celular que tiene receptores tipo CD14 y Toll

endógenos o que han sido transfectados con receptores tipo CD14 y/o Toll y/o genes reporteros para transmisores inflamatorios o pirogénicos.

30. El equipo tal como se describe en la reivindicación 24, caracterizado porque el reactivo que contiene monocitos criopreservado comprende la sangre completa.

31. El equipo tal como se describe en cualesquiera de las reivindicaciones 22 a 30, caracterizado porque la pared superior de dicho depósito de microtitulación no es plana, curva, parabólica o se extiende hacia abajo, o los lados de dicho depósito de microtitulación están inclinados hacia el interior.

32. El equipo tal como se describe en cualesquiera de las reivindicaciones 22 a 30, caracterizado porque el depósito de microtitulación comprende una región superior abierta, una región superior que se extiende hacia abajo desde la parte superior abierta, y una pared del fondo que se ahusa en diámetro desde la parte de arriba del punto más bajo hasta el punto más bajo.

33. El equipo tal como se describe en cualesquiera de las reivindicaciones 22 a 30, caracterizado porque el depósito de microtitulación comprende una región superior abierta, una región que se extiende hacia abajo desde la región superior

que tiene un extremo superior y un extremo de fondo, y una región inferior que tiene un extremo superior y un punto más bajo, extendiéndose la región inferior desde el extremo inferior de la región superior y ahusándose en diámetro más rápidamente que la región superior del extremo superior de la región inferior hacia el punto más bajo.

34. El equipo tal como se describe en cualesquiera de las reivindicaciones 22 a 33, caracterizado porque la citocina o transmisor endógeno de la respuesta inflamatoria se selecciona del grupo que consiste de interleucina-1 (IL-1), interleucina-1ra(IL-1ra), interleucina-6 (IL-6), interleucina-8 (IL-8), factor de necrosis tumoral - α (TNF- α), y endotelina.

35. Un equipo de diagnóstico que comprende:

(i) una placa de microtitulación que comprende una pluralidad de depósitos de microtitulación formados de modo que las células contenidas en cada depósito sean concentradas en comparación a un depósito de microtitulación de fondo plano, una superficie de dicho depósito que comprende polipropileno; y

(ii) un reactivo que contiene monocitos crioconservado el cual está contenido en los depósitos de dicha placa, en donde el reactivo que contiene monocitos comprende células mononucleares de sangre periférica crioconservadas o células de línea celular monocítica crioconservada, está en contacto con las superficies de dichos depósitos.

36. Un equipo de diagnóstico que comprende:

(i) un sistema de ensayo para detectar pirógenos sin endotoxina en un producto médico administrado en forma parenteral, dicho sistema de ensayo comprende una placa de microtitulación que comprende una pluralidad de depósitos de microtitulación formados de modo que las células contenidas en cada depósito sean concentradas en comparación con un depósito de microtitulación de fondo plano, una superficie de dicha pared comprendiendo polipropileno;

(ii) un reactivo que contiene monocitos crioconservado que está contenido en los depósitos de la placa, en donde el reactivo que contiene monocitos comprende sangre completa crioconservada y está en contacto con la superficie de dichos depósitos; y

(iii) un anticuerpo para IL-6.

37. Un método de análisis de una muestra, que comprende los pasos de:

combinar un reactivo que contiene monocitos y la muestra que será probada en un primer sistema de ensayo que incluye al menos un depósito de microtitulación formado de manera que el reactivo que contiene monocitos se concentre para proporcionar un mayor contacto célula con célula en comparación con un depósito de microtitulación de fondo plano, en donde la superficie del depósito de microtitulación comprende un revestimiento de polipropileno o

la totalidad del depósito de microtitulación se compone de polipropileno;

incubar el reactivo que contiene monocitos y la muestra;

transferir los contenidos del primer sistema de ensayo a un segundo sistema de ensayo que comprende al menos una superficie tratada con un anticuerpo para una citocina o un transmisor endógeno de la respuesta inflamatoria; y

ensayar el segundo sistema de ensayo con respecto a la presencia de citocina o un transmisor endógeno enlazado al anticuerpo en la superficie, mediante la cual la muestra es probada.

38. Un método de análisis de una muestra, que comprende los pasos de:

combinar un reactivo que contiene monocitos y la muestra que será probada en un primer sistema de ensayo que comprende (i) al menos un depósito de microtitulación formado de manera que el reactivo que contiene monocitos se concentre para proporcionar un mayor contacto célula con célula en comparación con un depósito de microtitulación de fondo plano, en donde la superficie del depósito de microtitulación comprende un revestimiento de polipropileno o la totalidad del depósito de microtitulación se compone de polipropileno, y (ii) al menos una superficie tratada con un anticuerpo para una citocina o un transmisor endógeno de la respuesta inflamatoria;

incubar el reactivo que contiene monocitos y la muestra;
y

ensayar el sistema de ensayo para la presencia de
citocinas o un transmisor endógeno enlazado al anticuerpo en
la superficie, mediante la cual la muestra es probada.

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EXHIBIT A



Ajudando as pessoas a
viverem vidas saudáveis

Product Catalog

Welcome to the BD (Becton, Dickinson and Company) Catalog of medical supplies, devices, laboratory equipment and diagnostic products.

Cell Cultureware
Product Number: 353936



[Safety Data Sheet](#)
[More information](#)
[Certificate of Analysis](#)

353936 - BD Falcon™ Clear 96-well Microtest™ Plate. Tissue-culture treated polystyrene, flat bottom, with lid. Packed in Ready-Stack bulk trays. (14/sp, 84/ca)

Key Product Features

Availability	US, Canada, Latin America, Europe, Australia, Asia Pacific, Japan
Product type	Plates
Brand	BD Falcon™
DNR (Latex) free?	Yes
Growth area	0.32 cm²
Growth surface	Tissue-culture treated
Lid	Yes
Material	Clear polystyrene
Notes	For assays not requiring cell attachment, see our Assay Plate products for additional plates and lids.
Shape	Flat-bottom
Size	96-well
Sterile?	Yes
Volume	370 µl

[Return to top of page](#)

353936 - BD Falcon™ Clear 96-well Microtest™ Plate. Tissue-culture treated polystyrene, flat bottom, with lid. Packed in Ready-Stack bulk trays. (14/sp, 84/ca)

Cross References

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353936 - BD Falcon™ Clear 96-well Microtest™ Plate. Tissue-culture treated polystyrene, flat bottom, with lid. Packed in Ready-Stack bulk trays. (14/sp, 84/ca)

Product Shipping and Handling Info.

Packaging Description	14/sp, 84/ca
Ordering / Contacts	Authorized Canadian distributors contact: BD 2100 Derry Road West Suite 100 Mississauga, Ontario L5N 0B3 Canada 866-979-9408 Outside US: contact the International Office nearest you

US and Puerto Rico Customers contact: BD Customer Service 1 Becton Drive Franklin Lakes, NJ 07417 1-888-237-2762	
Order Min	1 Case
Country of Origin	USA
Sterile?	Yes
How Sterilized	
Can be re-used?	No
Natural Rubber/Latex	Does not have Latex labeling Package does not have Latex Product has no Natural Rubber/Latex
Refrigerated shipping Information	Normal conditions
Batch Managed	Yes
Shelf Life	1825 Days
Return to top of page	

353936 - BD Falcon™ Clear 96-well Microtest™ Plate. Tissue-culture treated polystyrene, flat bottom, with lid. Packed in Ready-Stack bulk trays. (14/sp, 84/ca)

Product Packaging Info.			
Pkg level	EA	SP	CS
Quantity	1	14	84
Saleable?	No	Yes	Yes
EAN/UPC	382903539369	30382903539360	50382903539364
EAN Category	UC	IC	IC
Length	0.0 in	1.88 in	26.313 in
Width	0.0 in	1.022 in	14.313 in
Height	0.0 in	0.683 in	9.563 in
Weight	3.158 oz	1.184 lb	16.579 lb
Return to top of page			

BD
1 Becton Drive
Franklin Lakes, NJ USA 07417
201.847.6800

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EXHIBIT B

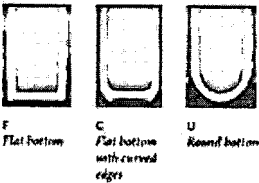
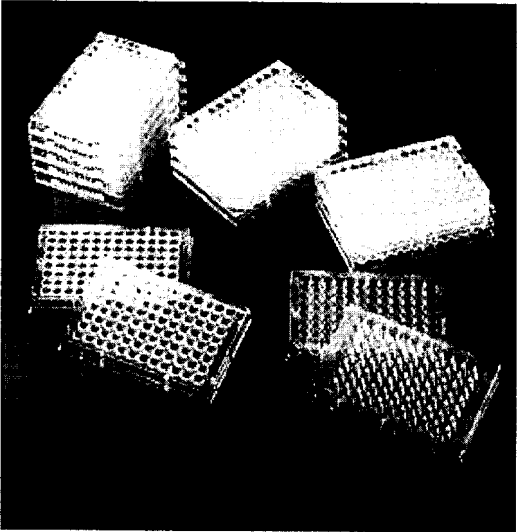
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Features
Details
Accessories
Links

Immuno 96 MicroWell™ Solid Plates
Clear

- Plates intended for quantitative and qualitative solid phase immuno assays (e.g. ELISA) and binding assays
- Choice of Surface
 - PolySorp™, with high affinity to molecules of a hydrophobic nature
 - MediSorp™, has a surface chemistry between PolySorp™ and MaxiSorp™. Provides low background signal with samples containing serum.
 - MaxiSorp™, with high affinity to molecules with mixed hydrophilic/hydrophobic domains
 - MultiSorp™, with high affinity to hydrophilic molecules
- Choice of F, C and U bottoms
- Raised rims on the wells
- Certified reproducibility of binding
- Alphanumeric orientation system
- Fit standard equipment
- Barcodes available on request



Details

Nunc-Immuno™ Plates
Polystyrene, without lids.
96 wells per plate.
Ext. dimensions 128 x 86 mm

Cat. No.	475094	456529	446140	475434	467320*	467340
Specifications	View	View	View	View	View	View
Configuration	F96	F96	C96	U96	F96	F96
Surface	PolySorp	PolySorp	PolySorp	PolySorp	MediSorp	MultiSorp
Design	Pinchbar	High Flange	Pinchbar	High Flange	High Flange	High Flange
Total volume, µl/well	400	400	350	300	400	400
Suggested working volume, µl/well	350	350	250	250	350	350
Total surface area, cm²/well	2.7	2.7	2.5	2.0	2.7	2.7
Barcode	-	-	-	-	-	-
Units per pack/case	5/60	10/180	5/60	5/60	5/60	5/60
Availability	Worldwide	Worldwide	Worldwide	Worldwide	Not Americas	Not Americas

* With certificate

Nunc-Immuno™ Plates

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EDTNA/ERCA Guidelines: Technical Section

1 Quality assurance for dialysis-quality water and dialysis fluid

1.1 Guidelines for the control and monitoring of microbiological contamination in water for dialysis

1.1.1 Scope

These guidelines apply to the control and monitoring of microbiological contamination in:

- water used for preparation of dialysis fluid in all settings, including patients' homes and acute care units
- water used for in-house production of liquid concentrates
- water used for reprocessing dialysers
- water used for preparation of ultrapure dialysis fluid by equipment fitted with additional point-of-use filtration

These guidelines should be taken into consideration when designing and modifying water treatment and distribution systems. Microbiological quality is affected by all components of the water treatment system, from the pre-treatment devices to the point of use. The suppliers of all components should ensure that their products are compatible with these guidelines. Where the complete installation is assembled using components from different suppliers, the renal unit staff must take overall responsibility for implementing the guidelines.

The microbiological quality of the water over the life of the system will depend on the maintenance. Responsibility for the specification and validation of the disinfection method and frequency may lie with a supplier or with the renal unit staff. This must be established before the system is put into use.

Although day-to-day management of the quality of the water used for dialysis is normally delegated to technical staff, ultimate responsibility for water quality may lie with the physician or pharmacist in charge of the dialysis service, or with a designated team, according to national regulations. To reflect this multidisciplinary process, these guidelines have been reviewed and approved by the European Society for Artificial Organs (ESAO) and by the European Renal Association/European Dialysis and Transplant Association (ERA-EDTA).

1.1.2 Exclusions

These guidelines are not applicable to water used in the preparation of commercially available dialysis fluid or substitution fluid supplied in sterile bags.

1.1.3 Applicable standards and guidelines

Monograph 1167:1997 (corrected 2000, republished 2001) Haemodialysis solutions, concentrated, water for diluting. *European Pharmacopoeia* Supplement 2001.

Monograph 0128:2000: Haemodialysis, solutions for. *European Pharmacopoeia*, Supplement 2001.

Circulaire DGS/DH/AFSSAPS No 311 relative aux spécifications techniques et à la sécurité sanitaire de la pratique de l'hémodiafiltration et de l'hémodiafiltration en ligne dans les établissements de santé. [Technical specifications and medical security of on-line haemofiltration and haemodiafiltration used in health care institutions] 7 June 2000 (French).

Tillverkning och hantering av hemodialysvätskor och hemofiltrationsvätskor inom sjukvården. [Manufacturing and handling of fluids for haemodialysis and haemofiltration in medical care] *Svensk läkemedelsstandard (SLS) 2001* (Swedish).

ERA-EDTA European Best Practice Guidelines for Haemodialysis: IV. Dialysis fluid purity. *Nephrology, Dialysis and Transplantation* 2002, Supplement (in press).

1.1.4 Glossary

For the purpose of these guidelines, the following definitions apply:

Biofilm: A coating on surfaces consisting of microcolonies of bacteria embedded in a protective extracellular matrix. The matrix, a slimy material secreted by the cells, protects the bacteria from antibiotics and disinfectants.

Colony forming units (CFU): An organism capable of replicating to form a distinct, visible colony on a culture plate. In practice, a colony may be formed by a group of organisms.

Dialysis fluid concentrate: A solution of chemicals which, when appropriately diluted, produces the dialysis fluid (definition from IEC 60601-2-16 2.107). Concentrates may be purchased as ready solutions or as powder that must be dissolved before use. They are subject to the requirements of EP Monograph 0128:2000.

Dialysis fluid: A solution that is intended to exchange solutes and/or water with blood during haemodialysis or

haemodiafiltration (definition from IEC 60601-2-16 2.107). 'Dialysate' is commonly used in place of dialysis fluid, although this term should be reserved for contaminated fluid leaving the dialyser.

Disinfection: The destruction of micro-organisms by physical (e.g. heat or UV radiation) or chemical means. Disinfection is a less lethal process than sterilization, since it is expected to destroy most recognized pathogenic micro-organisms, but not necessarily all those present. Disinfection does not eliminate endotoxin or other bacterial 'by-products', such as exotoxins.

Distribution system: The pipework used to transport treated water to the dialysis machine, together with any associated tanks, pumps, filters, irradiation units etc. The connections to the dialysis machines also form a part of the distribution system.

Endotoxin: A group of pyrogenic substances (lipopolysaccharides) that form a structural part of the outer cell wall of gram-negative bacteria. Under stress or during proliferation, endotoxin can be released from the bacteria. Endotoxins belong to the group of cytokine inducing substances and can cause fever, rigors, hypotension (in some cases followed by shock), multi-organ failure and even death if they enter the circulation.

Endotoxin units: Endotoxin levels are measured in terms of biological activity as Endotoxin Units (in the US) or International Units per ml. The units are related to the activity of a standard *E. coli* endotoxin. In 1997, the American and European Pharmacopoeias adopted a harmonised standard so that the EU and IU are now identical.

Exotoxin: Proteins secreted by micro-organisms to destabilise the function of their host. Exotoxins are small molecules with very specific actions, such blocking neural transmission or inhibiting protein synthesis. They may stimulate the body's immune system like endotoxins, but they cannot be detected by the LAL-test.

LAL (Limulus Amoebocyte Lysate) test: An assay used to detect endotoxins, which exploits the immune response of the horse shoe crab (*Limulus polyphemus*).

Microbiological contamination: Contamination with any form of micro-organism (e.g. bacteria, yeast, fungi and algae) and/or with the by-products of living or dead organisms such as endotoxins and exotoxins, such as microcystin, which is derived from blue-green algae.

Peptidoglycans: These pyrogenic substances are found in the cell walls of gram-positive bacteria in much higher quantities than in gram-negative bacteria. Peptidoglycans cannot be detected by the LAL-test but they can be assayed using Silkworm Larvae Reagent. Due to their ability to induce cytokines from blood cells they also can be analysed quantitatively by measuring the release of IL-1Ra (Interleukin-1-receptor-antagonist) in whole blood.

Point-of-use filtration: Filtration of the water and dialysis fluid at the dialysis machine to produce ultrapure dialysis fluid. Most current systems pass the dialysis fluid through at least two filters.

Pyrogen: A substance that can cause fever (elevation of body temperature to $> 37.8^{\circ}\text{C}$). Symptoms of a pyrogenic reaction may include chills, rigors, nausea, vomiting and hypotension.

Substitution (replacement) fluid: Very highly purified fluid produced from ultrapure dialysis fluid. Substitution fluid, which can be infused directly into the blood, must be sterile and non-pyrogenic. Guidelines on monitoring the quality of substitution fluid are given in Circulaire DGS/DH/AFSSAPS No 311 and SLS 2001 (see 1.1.3). The effects of treatment mode and patient size on allowable endotoxin levels are discussed in reference 1.

Ultrapure dialysis fluid: Highly purified dialysis fluid that can be used in place of conventional dialysis fluid. The definition of ultrapure dialysis fluid varies and probably reflects practical detection limits. The specification for ultrapure water used in the ERA/EDTA Guidelines, i.e. < 0.1 CFU/ml and < 0.03 IU/ml, is often used as a specification for ultrapure dialysis fluid. EDTNA/ERCA accept this specification, on the basis that ultrapure dialysis fluid must be further purified to produce substitution fluid.

Water treatment system: The entire system, including purification devices, pumps, monitoring systems and distribution systems, from the incoming water supply to the point of use.

1.1.5 Introduction

The ERA-EDTA Best Practice Guidelines for Dialysis Fluid Purity specify that water for the production of dialysis fluid should meet or exceed the requirements of the European Pharmacopoeia. References 2 to 6 of this document review the clinical arguments for the use of high quality dialysis fluid, a more extensive discussion and bibliography is provided in the ERA-EDTA Guidelines.

EP Monograph 1167 requires that water used for diluting concentrated haemodialysis solutions should have a total viable aerobic count of less than 100 CFU/ml and that the concentration of endotoxin should be less than 0.25 IU/ml.

The European Pharmacopoeia monograph for solutions for haemodialysis (EP 0128) does not give a clear requirement for the level of endotoxin in prepared dialysis fluid. The manufacturer of a concentrated solution must ensure that the solution has an endotoxin level of less than 0.5 IU/ml at the dilution used for dialysis. This could be interpreted as a limit of 1.25 IU/ml for bicarbonate dialysis fluid (where two concentrates are used), i.e. 0.5 IU/ml from each diluted concentrate plus 0.25 IU/ml from the water. This limit has not been widely adopted and, in practice, a much lower level

of contamination is easy to achieve so there is no reason why the dialysis fluid should not comply with the requirements of EP 1167 for microbiological contamination.

Lower levels for viable organisms and endotoxin in the water and/or dialysis fluid may be specified locally. Where ultrapure fluid is required, the limits will normally be < 0.1 CFU/ml and < 0.03 IU/ml.

This document provides practical guidance to help dialysis units produce water for dialysis that meets or exceeds the requirements for microbiological contamination in EP 1167, and for monitoring the quality of water and dialysis fluid. Although the guidelines are primarily intended for in-centre dialysis, the principles apply to home haemodialysis and self-care units, but the frequency of monitoring may have to be adapted.

The EP does not provide limits or suggest tests for other bacterial 'by-products', such as exotoxins or peptidoglycans. The Silkworm Larvae Reagent Assay (7) can be used to check for peptidoglycans if a substantial proportion of the bacteria in the water or dialysis fluid are gram positive. The growth of all micro-organisms should be minimised to reduce the levels of known and unknown harmful contaminants generated by living and dead organisms. Minimisation of bacterial growth is possible with good quality assurance (see Appendix).

For regulatory purposes, it is necessary to specify an 'acceptable' level of contamination, but there is mounting evidence to suggest that exposure to relatively low levels of endotoxin causes chronic inflammation and contributes to amyloidosis (8,9). There is increasing evidence linking inflammation to cardiovascular disease in the general public but the effect of inflammation on rates of mortality and morbidity in haemodialysis patients is still under debate. To be certain that the health of patients is not being compromised, dialysis units should aim to minimise their exposure to any detectable level of endotoxin.

Ideally, a water treatment and distribution system installed today should be designed to meet the requirements that will be in place in 7 to 10 years time. Whilst it is not possible to predict what the standard for dialysis-quality water will be in the future, it is likely that the maximum allowed levels of bacteria and endotoxin will be reduced. The ERA-EDTA guidelines advise dialysis units to work towards supplying ultrapure fluid routinely for all dialysis modalities.

Tighter control of microbiological contamination can only be achieved where the water distribution system is designed for very frequent automated disinfection. Frequent disinfection may be costly, but it will improve the water quality. Frequent monitoring to check for problems in a badly designed system is also costly, but testing does not improve quality (see Appendix). The extra cost of installing a system with automated daily heat disinfection is minimal when calculated as a cost per treatment over the lifetime of the plant.

1.1.6 Guidelines

1.1.6.1 Design and disinfection

Water treatment systems should be designed to be capable of producing water that meets the requirements for dialysis with a sustainable level of maintenance.

The water treatment system should be designed to minimise the formation of biofilm. Pipework with a smooth surface that resists corrosion and does not crack with age should be used for the distribution system. Internal ridges and/or grooves at the joints in the pipework should be minimised. Dead spaces and regions with low flow should be eliminated where possible. Where dead-end connectors to dialysis machines are used, they should be as short as possible.

Storage tanks in the distribution system provide a low flow zone where biofilm can form easily and should be avoided where possible. If treated water must be stored, the tank(s) should be designed to minimise bacterial growth and facilitate routine disinfection. An option to switch to direct feed by bypassing the tank is recommended.

The water treatment system should be designed to allow routine disinfection of the entire system, including the distribution pipework. An appropriate disinfection programme should be established when the system is installed or modified (see Appendix). The schedule may be based on information available for similar installations. Where no information is available, the reverse osmosis system and distribution pipework should be disinfected at least once a month.

The method of disinfection (heat or chemical) should be validated for use with the materials used in the distribution system. The disinfection programme should include the connections to the dialysis machines.

Rationale: Biofilm is the cause of persistent bacterial contamination of water and dialysis fluid (10). Connection and disconnection of dialysis machines and other devices mean that contamination of the distribution system with environmental bacteria cannot be avoided. If allowed to proliferate, the bacteria will form a biofilm. An established biofilm can be difficult, if not impossible to remove (11,12) [Evidence].

1.1.6.2 Frequency of microbiological testing

Water used for haemodialysis should be tested routinely to verify the efficacy of the disinfection programme, and to ensure that the water meets the requirements of the European Pharmacopoeia (total viable count < 100 CFU/ml and endotoxin level < 0.25 IU/ml) or the local specifications for purity.

Microbiological testing should be used to monitor the efficacy of the disinfection programme. When a water treatment system and/or distribution system is installed, the microbiological quality of the treated water should be checked at least once a week as part of the procedure for validating the disinfection programme. After validation, microbiological testing should be carried out at least once a month.

Trends in the test results should be monitored carefully, so testing must be frequent enough to pick up changes. If the trends show that the disinfection programme is not adequately controlling the proliferation of bacteria, the interval between disinfections should be reduced. Action should be initiated to reduce contamination before the allowed limits are reached (e.g. when the bacteria level exceeds 25 CFU/ml or the endotoxin level exceeds 0.125 IU/ml).

If a biofilm has become established, the test results should be treated with caution (see 1.1.8). The bacterial counts will drop after disinfection but may rebound soon after. High endotoxin levels and the appearance of fungi and yeasts in the water also indicate the presence of biofilm. Swabs taken from internal surfaces of the pipework can be cultured to confirm that a biofilm has formed. It may be possible to control the biofilm with extended disinfection times and/or more aggressive cleaning agents. If the levels of microbiological contamination remain high, the cause should be determined and the components responsible replaced.

Rationale: Good manufacturing, laboratory and clinical practices separate monitoring from control methods. In this case, the control of bacteria levels is the disinfection that takes place at regular intervals. Microbiological testing is the independent monitoring method [Opinion].

1.1.6.3 Sampling location, timing and techniques

Samples should be taken from the distribution system at the time and location expected to give the highest level of contamination, using a port designed for taking microbiological samples, and tested within 6 hours.

Microbiological contamination may vary with the consumption of water. Where treated water is stored, the levels may be higher following non-dialysis days. Samples should be taken at a consistent time, which should be when the levels are expected to be high.

A study of the variation of contamination with time should be carried out in order to establish the most appropriate sampling schedule. It may be necessary to repeat this study if the system is modified or the pattern of water consumption changes.

Samples should be taken from the point(s) expected to have the highest bacterial load, normally the end of the distribution loop, or the last machine point in a dead-end system, where the flow is lowest. If there are dead-end connections to the dialysis machines, samples should also be taken from the tubing to the machine. A sampling port designed for collecting samples for microbiological testing (e.g. an in-line septum port allowing mid-stream samples to be taken with a syringe) should be installed at this location. The manufacturer of the port should provide a validated procedure for taking samples.

If samples are taken from taps, the tap should be flame sterilised or chemically disinfected (e.g. by soaking with 70% ethanol or 80-90% isopropyl alcohol and allowing the alcohol to evaporate). When the tap is opened, at least 2 litres of water should be run off at a high flow before taking the sample. The sample should be taken without touching the tap as any movement of the valve may release biofilm.

Samples of dialysis fluid should also be taken from ports designed for collecting samples for microbiological testing, ideally a septum port in the line between the machine and the dialyser.

For endotoxin tests, the sample containers must be sterile and endotoxin-free, and made from material that does not adsorb endotoxin. Polystyrene does not normally adsorb endotoxin, while polypropylene should be avoided. Advice on containers should be sought from the laboratory carrying out the LAL tests or from the suppliers of LAL reagent.

Ideally, water samples should be tested immediately for bacteria and endotoxin. If the samples cannot be tested immediately they should be kept cool (< 10 °C), but not frozen. Where testing has to be delayed for more than 6 hours, the storage conditions should be validated. If necessary, dialysis units should consider training staff to test samples in-house. The LAL gel-clot test can be carried out relatively easily in a dialysis unit. The suppliers of the LAL reagent will normally provide training in the testing and validation procedures. Any in-house procedure for culturing bacteria should be validated by an accredited Microbiology Laboratory.

Rationale: Variation of microbiological contamination with consumption is more likely to occur in systems with storage tanks. It is important to establish the normal variability in levels to avoid misinterpreting results [Opinion].

Samples taken from taps may have poor reproducibility. In an EDTNA/ERCA study, several units measured higher endotoxin levels in samples taken from taps in the distribution circuit than from septum ports in the tubing to the dialysis machine (13). If samples must be stored, they should be kept cool to stabilise the level of bacteria. Repeated freezing can reduce the measurable endotoxin level. If it is necessary to transport samples on dry ice, the storage conditions should be validated [Opinion].

1.1.6.4 Techniques for culturing water borne micro-organisms

A culturing technique that is proven to give a good recovery of bacteria from purified water should be used. Levels of fungi and yeast should be monitored periodically using an appropriate technique.

Bacteria levels in water and dialysis fluid should be monitored using a culturing technique that is proven to give good recovery of bacteria from clean water.

The general technique for "microbiological examination of non-sterile product" described in the European Pharmacopoeia (Section 2.6.12) is not appropriate for dialysis water. The evidence suggests that a low nutrient agar, such as Tryptone Glucose Extract Agar or Reasoner's 2A, should be used (14-16) and that samples should be incubated for at least 7 days at room temperature (17). These conditions have been shown to give good recovery for most environmental bacteria found in purified water. Some species are better adapted for growth at a higher temperature and/or on richer media, but the long incubation time will allow most of these to grow.

If local regulations require testing for specific organisms (e.g. *Pseudomonas aeruginosa*) with a specially adapted agar, a separate test should be conducted.

The inoculation volume should be chosen according to the allowed limit and the expected count. When testing to the requirements of EP 1167, a spread or pour plate inoculated with 1 to 5 ml of water is recommended. If the water is highly contaminated it can be diluted with water for injection to give a count. When testing ultrapure water, a sample of at least 100 mls should be filtered using a sterile 0.2 micron filter. The filter should then be cultured using the conditions above.

The number of colony forming units should be counted in a consistent way. Careful visual inspection under bright lighting is sufficient.

The European Pharmacopoeia does not give a specific limit for fungi and yeasts, as they are grouped in the total viable aerobic count. The Svensk läkemedelsstandard 2001 specifies a limit of 10 CFU/ml.

To monitor levels of fungi and yeasts, a large sample volume is normally required because these organisms adhere well to surfaces and appear at very low levels in the flowing water. The sample should be filtered through a 0.2 micron filter. Fungi and yeasts need more nutrients than bacteria and grow better on a richer agar (such as Malt Extract Agar or Sabouraud's Dextrose Agar). Incubation should be for 7 days at room temperature. It can be of interest to incubate plates for longer as some organisms, such as fungi, may take more than 7 days to grow. Testing for yeasts and fungi may be less frequent than for bacteria where levels of these organisms are consistently very low. They should not appear at all in a well-maintained system and are rarely seen in any system in the first year or so of operation.

Rationale: Comparisons of different test methods indicate that the recovery of bacteria from water used for dialysis is highest when a low nutrient agar, a long incubation time and a low temperature is used [Evidence].

The Swedish and French Standards listed in 1.1.3, and the ERA-EDTA Best Practice Guidelines, recommend testing for bacteria by incubating on Tryptone Glucose Extract Agar (TGEA) or Reasoner's 2A at 20-22 °C. The Swedish Standard recommends incubation for 5 days, the other documents prefer 7 days. The 7 day incubation is generally more convenient (as counts over the weekend can be avoided) and gives better recovery.

The above method should be regarded as the standard for water. Additional methods that are in current use, e.g. Cled agar or blood agar at 35-37 °C for at least 5 days, may be of interest but should not replace the standard. New techniques that give comparable or better recovery with a shorter incubation time must be validated against the standard method [Evidence and opinion].

1.1.6.5 Measuring endotoxin levels in water and dialysis fluid

Endotoxin levels should be measured using Limulus Amoebocyte Lysate (LAL) by the gel-clot, turbidimetric or chromogenic method, or an equivalent test.

Bacterial endotoxin levels should be monitored using the Limulus Amoebocyte Lysate (LAL) assay, as recommended in the European Pharmacopoeia (Section 2.6.14). All the established techniques (gel-clot, turbidimetric and chromogenic) for LAL testing can be used for testing water for haemodialysis. Alternative techniques may be used provided they have been validated at endotoxin levels of 0.25 IU/ml or below.

The simplest form of LAL assay is the gel-clot test, which can be performed in the dialysis unit, provided staff have received the necessary training. It gives a pass-fail result, which is sufficient for testing water with a stable endotoxin level, but it can be made semi-quantitative by diluting the sample with endotoxin-free water.

The kinetic LAL tests (chromogenic, turbidimetric) measure the quantity of endotoxin in each sample directly, provided it is within the calibrated range. The kinetic tests are not often used in dialysis units as the equipment required is expensive and has significant operating overheads.

Dialysis fluid should be tested routinely for endotoxin to monitor the efficacy of the dialysis machine disinfection procedure. If the endotoxin levels in the dialysis fluid are significantly higher than in the water feeding the machine, the

disinfection schedule or the method used should be modified. If all machines are subject to the same procedures then a representative sample of machines can be tested each month.

Dialysis fluid samples should also be tested if a patient experiences a pyrogenic reaction during dialysis. A gel-clot test can be performed in about 1 hour to clarify the origin of the reaction.

When the LAL test is used to monitor endotoxin in dialysis fluid, the sample should be diluted at least 1:1 with endotoxin-free water to prevent interference with the clotting mechanism due to the bicarbonate (13). Note that LAL test works over a limited pH range so that it cannot be used to test the acid component of the dialysis fluid.

Rationale: From a clinical viewpoint, it is more important to test water and dialysis fluid for endotoxin than for bacteria, as bacteria do not pass through an intact dialyser membrane. There is still controversy over the transmembrane passage of bacteria-derived substances, but smaller bacterial endotoxins have been shown to cross all types of membrane (2,18-21). Hydrophobic high-flux membranes (such as polysulphone) can adsorb low molecular weight endotoxins (22), but the backfiltration zone that usually occurs in high flux dialysers could allow these molecules to pass into the blood if there is any damage to the membrane [Evidence].

The LAL assay is the method recommended for endotoxin testing by the European Pharmacopoeia, and is widely used to test non-pyrogenic products in the pharmaceutical industry. However, staff should be aware that the LAL assay will not detect all pyrogenic substances derived from bacteria (7,23).

1.1.7 Outcome measures

The implementation of this guideline should be audited by reviewing the record of bacterial counts and endotoxin measurements over time. The record should show planned test dates, actual test dates and the results obtained. Adherence to the disinfection schedule should also be audited.

If routine measurements of pre and post dialysis body temperature and/or markers of inflammation such as C-reactive protein (CRP) are available for unselected groups of patients, these should be audited periodically to look for trends that may be related to water quality. Non-routine measurements, and measurements made only on selected ('at-risk') patients must not be used for this purpose.

The dialysis unit should have documented procedures that come into effect when the European Pharmacopoeia (or local) limits for contamination are exceeded. The ERA-EDTA Best Practice Guidelines state that the procedures should include temporary closure of the dialysis unit when the safe limits for contaminants are exceeded. The content of these procedures must be reviewed regularly. The ability of staff to implement the procedures effectively should also be checked.

1.1.8 References

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23. Bonnie-Schorn E, Grassmann A, Uhlenbusch-Körwer I, Weber C, Vienken J. Clinical consequences of microbial contamination. In "Water Quality in Haemodialysis", Lengerich: Pabst Science Publishers 1998; 29-34.
A review of the methods for detecting pyrogens and other biologically active substances. For more details about this book see reference 4.

1.1.9 Appendix: Quality assurance or Quality control?

Quality assurance (QA), applied to production of water for dialysis, means designing and maintaining the entire system for the production and distribution of purified water to a standard that ensures that the water meets or exceeds the requirements of the European Pharmacopoeia. This will include preventing the proliferation of bacteria. In the context of quality assurance, bacteria levels are monitored for the purpose of audit only.

QA is provided by modern water distribution systems with high quality pipework, minimal dead space and automated heat disinfection. Daily or twice weekly disinfection is required to prevent bacteria growth on the surfaces of the distribution system. In most units this is feasible only with an automated disinfection procedure that is easy for dialysis unit personnel to use. Heat disinfection is amenable to automation and can be left unattended. It also eliminates the need to handle chemicals and carries no risk for the patients as all the dialysis machines have high temperature alarms.

Internal quality control (QC) involves the analysing of samples to find out if they meet the criteria for acceptability. If a sample fails the QC test, appropriate action is taken. When monitoring water for dialysis, QC measurements showing an increasing trend in counts may be used to adapt the maintenance schedule, for example by initiating a disinfection.

Most water distribution systems in current use were designed with no provision for routine disinfection or for cold chemical disinfection at long intervals. With such systems, bacteria are allowed to proliferate and QC tests are required to check that the water quality is acceptable. When contamination exceeds the allowed limits, the bacteria must be destroyed. The disinfection processes required are time consuming (and have to be undertaken outside normal operating hours) and are not without risk to the staff and patients.

These systems are difficult to keep clean because bacterial biofilm can build up during the interval between disinfections, and because the disinfectant cannot penetrate biofilm and destroy bacteria that are deeply embedded. They are also very difficult to monitor effectively because bacteria levels obtained by culturing the viable organisms in a mid-stream sample gives a very limited indication of the number of bacteria in the system.

Even with the best incubation techniques, there will be some bacteria that do not grow and some colony forming units that contain more than one viable organism. And even if the culture technique could recover every organism in the flowing water, it cannot show the extent to which the distribution system is colonised. To understand this, it is necessary to gain some insight into the life of micro-organisms and the difficult job of the microbiologist.

Nutrients are mainly organic molecules with electrical charges that are attracted to surfaces. Bacteria will try to adhere to surfaces to be where their food is – the ones that fall off are the losers. Species that adhere well will rarely be seen in the flowing water. Pieces of biofilm that come unstuck will contain many cells, but will appear as '1 CFU'. So the microbiologist performing the QC tests sees only the colonies formed from the pieces of biofilm that have been lost from the surface that will grow in his cultivation conditions. This is a tiny fraction (perhaps 1/100000) of the actual number in the system.

Internal QC can be used to keep the measured level of microbiological contamination below the EP limits it relies on the ability of staff to keep the growth of bacteria under control. Over the lifetime of a system this will inevitably become more difficult. With the QA approach, there is no build-up of resistant biofilm, so there is no reason why the system should not continue to produce high quality water as long as the maintenance schedule is followed.

1.1.10 Acknowledgements

This document was prepared by the Research Board and the Technicians Special Interest Group of EDTNA/ERCA with advice from Dr Rolf Nystrand, Microbiology Advisor to EDTNA/ERCA.

A panel of experts, including representatives from industry and regulatory bodies, as well technical and clinical staff from dialysis units in Europe and North America, provided comments and criticism as the document passed through three revisions during 2000-01.

EDTNA/ERCA thanks all those involved for their help in producing a document that will help our members improve the quality of the care they provide to dialysis patients throughout the world, especially:

Matthew J. Arduino, Environmental and Applied Microbiology Section, Centers for Disease Control & Prevention, USA; Bernard Canaud, Department of Nephrology, Lapeyronie Hospital, Montpellier, France, President-elect of the French Dialysis Society and Expert member of AFFSAPS on behalf of the *European Renal Association/European Dialysis and Transplant Association*; Jim Cooper, Charles River Endosafe, Charleston, USA; Jean-Yves De Vos, Werken Glorieux, Ronse, Belgium; David J Evans, Fresenius Medical Care GmbH, Huthwaite, UK; LeRoy J Fischbach, Minntech Corporation, Minneapolis, USA and AAMI Renal Disease and Detoxification Committee Co-Chair*; Alois Gorke, Kreis-krankenhaus, Murnau, Germany; Roger Greenwood, Renal Unit, Lister Hospital, Stevenage, UK; Maurice Harrington, Renal Unit, Hope Hospital, Salford, UK; Dan Jonsson, Gambro, Lund, Sweden; Stephan Krietemayer, Lauer Membran Wassertechnik, Germany; Elizabeth Lindley, Dept of Renal Medicine, St James's University Hospital, Leeds, UK; Franta Lopot, Dept of Internal Medicine, Charles University Medical School, Prague, Czech Republic; Thomas Ludewig, TÜV Product Service, Munich, Germany; Ian Morgan, Renal Unit, King's College Hospital, London, UK; Rolf Nystrand, Bio-TeQ Nystrand Consulting, Malmö, Sweden; Hans Polaschegg, Medical Devices Consultant, Köstenberg, Austria; Bernd Stegmayr, Nephrology Division, Norrlands Universitets Sjukhus, Umeå, Sweden on behalf of the *European Society for Artificial Organs*; André Stragier, Clinique Saint-Luc, Brussels, Belgium; James Tattersall, Basildon District Hospital, UK; Hans Träger, Werner GmbH, Leverkusen, Germany; Richard A. Ward, University of Louisville, Kentucky, USA, and AAMI Water for Hemodialysis Working Group Chair*

* Input from Lee Fischbach and Dick Ward was in their role as individuals and should not be seen as representing the views of AAMI.

ENDOSAFE TIMES



IN THIS ISSUE:

■ Perspective

■ PTS in Space

- Introduction
- Planetary Protection
- Life Detection

■ Laboratory Notebook

- Endotoxin and Storage Containers
- Methods
- Results
- Conclusions

■ LAL Pointers

■ What's New

PERSPECTIVE

Last year Charles River Laboratories introduced a revolutionary new platform for LAL testing, the Endosafe®-PTS. This new Portable Test System (PTS) represents a radical departure from traditional LAL testing methodologies. It is designed to be an accurate, sensitive and rapid LAL test that is also very user friendly. PTS has provided customers with real-time data for point-of-use analyses. While we are confident and thrilled that this new platform will greatly facilitate LAL testing in both pharmaceutical and non-pharmaceutical laboratory settings, our collaboration with NASA has been most exciting. NASA is investigating PTS as a means of augmenting their Planetary Protection Program. This program assesses the microbial contamination of space craft and space craft assembly areas. PTS is also being considered as an on-board component of life detection instrumentation for future spaceflight missions to look for evidence of life on other planets in our solar system. We are taking LAL where it's never gone before.

The Laboratory Notebook section of this issue of the Endosafe Times also presents a summary of a study our Technical Service department performed that assessed the stability of purified endotoxins (CSE) in a variety of containers. The type of containers used to prepare and store CSE dilutions can have a profound effect on LAL activity. As such, the type of glass or plastic can be a source of LAL assay interference.

PTS IN SPACE

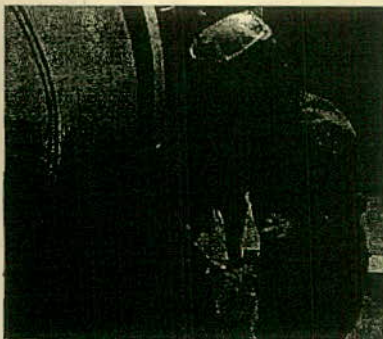
INTRODUCTION

Whether one is concerned with collection of data to support spacecraft microbial contamination, or life detection, major desirable criteria include reagent systems that are accurate and sensitive. The ability to report data near the point of collection and in near real-time is also highly desirable. PTS satisfies these criteria. The polystyrene cartridge is loaded with a licensed LAL reagent and endotoxin controls. The LAL enzyme system, with its inherent amplification, is unquestionably the most sensitive means of detecting lipopolysaccharides from Gram negative bacteria. Our ability to control the Factor C or Factor G activation


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A technician swabs the surface of spacecraft hardware for LAL testing.

pathway allows us to render LAL specificity towards Gram negative bacteria or fungi. Other rapid microbiological and biochemical tests will be available in the future. That PTS represents a truly miniaturized LAL instrument capable of performing kinetic spectrophotometric analyses for microbial detection in a compact "microfluidic" chamber is advantageous to NASA for obvious reasons.

PLANETARY PROTECTION

As with most pharmaceutical bioburden testing, the current approved NASA Standard Assay as stipulated in the NASA Standard Procedure for The Microbiological Examination of Space Hardware (NHB 5340.1C) requires three days to determine the microbiological bioburden. Utilizing the PTS LAL system, we were to determine if an LAL assay could yield useful information to enable near real-time decisions during space craft assembly. Swabbing methods mimic the NASA Standard Procedure with the cotton swab replaced with ultra clean polyester (Texwipe). Table 1 shows data from representative samples taken at Kennedy Space Center, Florida. A 25 cm² area was swabbed and the swab extracted with ultra clean water. Aliquots of each sample were analyzed by LAL with results expressed as EU/ml or plated on TSA plates and allowed to grow in an incubator at 37 degrees C for three days. Any organisms that grew on the plate were Gram stained and classified as Gram positive, Gram negative or mold.

LIFE DETECTION

NASA's commitment to an Exobiology / Astrobiology Program supports research in topics related to the detection of life in the universe and to the characterization of prebiotic organic chemistry. The prime mission of the Viking landers in 1976 was to search for life. In 1996, discovery of possible evidence for past life in Martian meteorite ALH84001 (McKay, et al., 1996) gave new impetus to the search for life on Mars and the restructuring of the Mars robotic program that ensued had a strong and ultimate goal of searching for life.

Sample	CFU / plate	LAL EU/ml	Gram stain
Floor by airlock	1	0.007	1 mold
Wall by airlock	0	< 0.005	NA
Floor (center)	1	0.12	1 Gram +
Wall vent	8	9.73	2 Gram - 3 Gram + 1 mold
Metal dolly	14	4.44	1Gram - 1Gram +

Table 1. Comparison of samples taken at KSC assembly area and analyzed by LAL or conventional microbiology.

A theoretical test of LAL sensitivity with mineral samples was carried out by testing pure silica which had been cleaned by exposure to dry heat (500 degrees C, 12 hours) and subsequently spiked with known concentrations of bacteria. Stainless steel tubes were first cleaned by the same dry heat, then inoculated with 10⁷ cells. Clean silica was added and mixed for one hour. Contaminated silica was extracted with ultra pure water. The limit of detection was shown to be 10⁻¹³ g/g. The results of that study are

Sample	Weight (g)	EU/ml	LPS(ng)	LPS/g sample
49	0.316	0.69	0.031	9.93E-11
53	0.336	6.0	0.272	8.10E-10
54	0.349	6.8	0.309	8.86E-10
negative	0.320	<0.01	<0.0005	<1.42E-12
negative	0.352	<0.01	<0.0005	<1.29E-12
negative	0.343	<0.01	<0.0005	<1.33E-12

Table 2. Calculated LAL values for silica samples exposed to bacterial contamination.

shown in Table 2. Samples # 49, 53 and 54 all reached onset time in less than 17 minutes and had calculated values shown. Negatives received no bacterial contamination and showed no LAL signal.

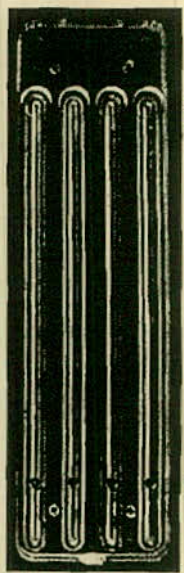
A team has been assembled to design and construct instrumentation to detect multiple biological signatures of life on other planetary bodies, including amino acids, nucleotides, polycyclic aromatic hydrocarbons, alkanes, whole cells, LPS, DNA, proteins

and other organics. Evolution of the instrument concept has led to MASSE (Modular Assay for Solar System Exploration) headed by Andrew Steele of the Carnegie Institute and David McKay of the Johnson Space Center in Houston. The LAL assay module will contribute analysis of extracts for microbial cell wall materials in this system.

Extreme environments on Earth are used to test the technology of life detection that might one day be used to test for signs of microbial life on Mars and other planets. An expedition organized by Hans Amundsen of PGP Norway, was meant to test instrumentation for life detection in one of the world's most northerly hot springs. Troll springs had not been previously characterized for microbial diversity. Techniques tested were ATP luminometry, field PCR, and field LAL assay unit (PTS produced by Charles River Laboratories). The future of PTS technology is all about bringing Charles River technology close to the source of needed tests and providing near real-time results - whether that is on the production floor of a biotech company, the clinical lab of the future, extreme environments in the field or perhaps, on the surface of another world.

LABORATORY NOTEBOOK
ENDOTOXIN AND STORAGE CONTAINERS

Accessory materials can have an impact on meeting the suitability requirements for LAL testing. The effects of endotoxin incompatible pipettes, LRW, and storage containers may be as subtle as changes in reaction time for standards in quantitative testing methods or as consistent half lambda or 2 lambda end points in gel clot testing. Conversely, the effect may also be profound such as a complete failure in linearity requirements, a complete failure in the recovery of Positive Product Controls (PPCs), or quarter lambda or 4 lambda end points in gel clot testing. As such, one of the most important accessories in LAL testing is the dilution tubes used to prepare the endotoxin standards. There are a variety of glass and plastic containers available for laboratory applications; Charles River has always touted depyrogenated borosilicate test tubes as the "gold" standard.



Sample well
(25microliter)

Optical Chamber

The PTS microcuvette contains pre-dispensed reagents necessary to run an LAL assay.



Dr. Andrew Steele of the Carnegie Institute of Washington field tests the PTS during the Arctic Mars Analogue Svalbard Expedition (AMASE).

In addition to the capacity to subject this material to validated depyrogenation processes, this Type 1 USP glass possesses excellent properties with respect to alkaline leachables. Indeed, it is used in most LVP and SVP pharmaceutical preparations. We have also found that certain plastic test tubes work very well in the LAL laboratory. Our QC and Technical Service laboratories have had excellent results when using polystyrene test tubes. Charles River has, however, recommended the avoidance of polyolefin materials (polypropylene and polyethylene). While polyolefin's materials are extraordinarily resilient to solvents and in and of themselves do not leach organics, we have always been concerned with their hydrophobic surface properties. The paucity of charged moieties on a polyolefin surface could potentially adsorb endotoxin due to the hydrophobic domains associated with Lipid A. In the following experiment, we examined the stability of a CSE preparation in a variety of glass and plastic containers.

METHODS

In this study, we used CSE lot EM31652. Stock solutions of this CSE were prepared at two concentrations. A concentration of 0.5 EU/ml was used to represent a mid-range CSE standard. A second concentration of 0.005 EU/ml was also prepared. This concentration was used to represent the practical sensitivity limit of most LAL testing laboratories. Additionally, we believed that the 0.005 EU/ml standards would exaggerate any difference between storage materials. Once prepared, aliquots from both solutions were transferred to test containers of the following compositions: (1) borosilicate glass; (2) flint glass (USP Type 2); (3) polystyrene; (4) polyethylene terephthalate glycol (PETG); and (5) polypropylene. The test tubes were assayed immediately and then held at refrigerated temperatures for one week. All LAL examination utilized a single KTA LAL lot (S3442E). All examinations were quantitative. Our results have been summarized and illustrated as Figures 1 and 2.

RESULTS

Figure 1 illustrates the relative CSE stability of the 0.5 EU/ml standards. The standard concentration stored in the borosilicate glass test tube exhibited the fastest reaction time. On the other hand, the polystyrene test tube demonstrated the most consistent response over the 7 day storage period. The activity and the apparent stability of the CSE stored in the container was also very good. The flint and polypropylene containers were, in every respect, the least suitable container types.

Figure 2 illustrates the stability of the 0.005 EU/ml CSE. As expected, this endotoxin standard presented the data with the most variability. The borosilicate test tube did produce reaction times that were slightly slower and the apparent stability began to wane at 4 days. However, the long term stability of the 0.005 EU/ml endotoxin standards in polystyrene and PETG is quite remarkable. Flint glass tubes exhibited the worst LAL activity characteristics.

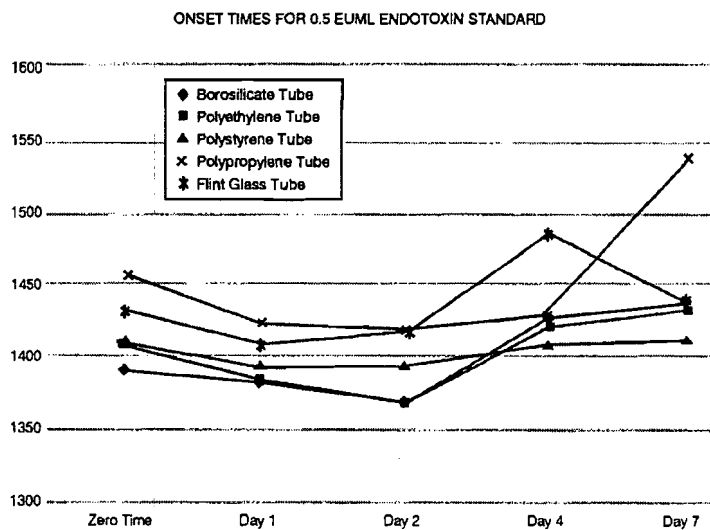


Figure 1

however, provide some general information that supported some standard LAL practices and recommendations that have been made in our industry.

The data in this study support our recommendation for the use of both borosilicate and polystyrene test tubes. Polystyrene test tubes demonstrated the most consistent endotoxin response - even after 7 days of storage. Borosilicate test tubes did begin to show some loss in endotoxin activity at 7 days. The PETG container also demonstrated data that suggests it too would be acceptable in LAL laboratories as an endotoxin storage material.

With both of the endotoxin standard concentrations illustrated in this study, the endotoxin activity measured in flint glass and polypropylene test tubes exhibited considerable variation and an apparent loss in activity over time. The data from

the flint tubes are particularly interesting in that this glass test tube (10 x 75 mm) is often used as a reaction tube for gel clot testing. However, as a reaction tube, samples or standards are mixed with LAL, a reagent which Endosafe buffers to minimize interference. Alkaline extractables that could potentially interfere with endotoxin detections are subsequently neutralized when LAL is added to the sample reaction reaction mixture. The polypropylene and flint glass containers, however, can not be recommended for use as endotoxin dilution and/or storage tubes.

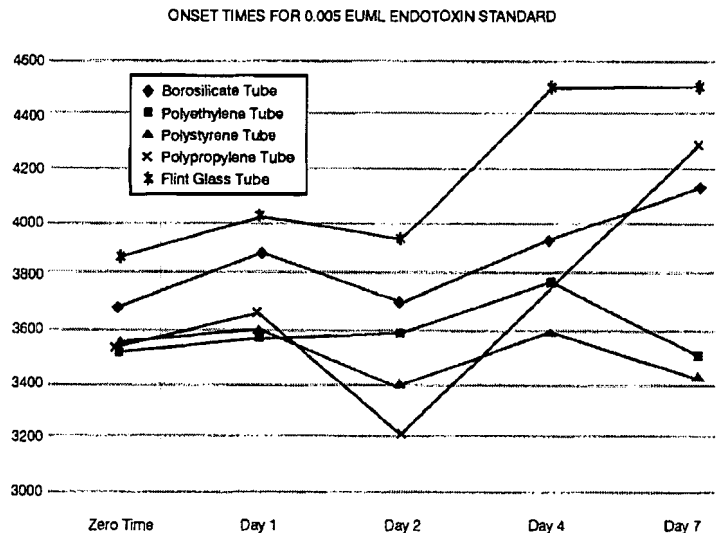


Figure 2

LAL POINTERS

The success story associated with the discovery of LAL and its replacement of the rabbit pyrogen test is nothing short of extraordinary. LAL, and the series of enzymes associated with the Gram negative bacterial endotoxin activation pathway, has enhanced pyrogen (endotoxin) testing programs for pharmaceutical companies around the world. Our new endotoxin portable test system (PTS) utilizes LAL. In fact, the formulation that we use is one that has already been licensed by the FDA for another methodology. The good news is that PTS utilizes a reagent (LAL) that has proven its utility. However, because PTS is still LAL, it is subject to all of the interferences typically associated with LAL. Therefore, the chemical composition of any given sample as well as the pH and ionic strength are some of the issues that must be considered during the development of PTS testing methods. The method development / product screening approach for PTS applications would be identical to those used for other LAL methodologies. Remember to call our Technical Services staff whenever you have questions or problems with the testing of any product with any LAL method. We can be reached at 800-762-7016.

WHAT'S NEW

NEW PRODUCT INFORMATION

Now available - Endosafe™ - Biological Indicators for monitoring gas and steam sterilization processes for health care products. Please call Technical Service at 1-800-762-7016 for more detailed product information or visit us on the web at www.criver.com.

Introducing a Sales Representative in the Mid-Atlantic region of the United States - Caralyn Andres, 856-218-7729 and a new Sales Representative in the Southeastern region of the United States - Austin Caudle, 919-332-2165.

The LAL User Group has been revived. For more information, please go to www.LALUserGroup.org.

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(12) **United States Patent**
Poole et al.

(10) **Patent No.:** **US 8,053,200 B2**
(45) **Date of Patent:** ***Nov. 8, 2011**

(54) **MONOCYTE ACTIVATION TEST BETTER ABLE TO DETECT NON-ENDOTOXIN PYROGENIC CONTAMINANTS IN MEDICAL PRODUCTS**

(58) **Field of Classification Search** None
See application file for complete search history.

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7,736,863	B2 *	6/2010	Poole et al.	435/7.21
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(Continued)

Primary Examiner — Cherie M Woodward

(74) *Attorney, Agent, or Firm* — Marshall, Gerstein & Borun LLP

ABSTRACT

An improved monocyte activation test is described that is better able to detect non-endotoxin pyrogens in medical products, in which a sample is incubated with a monocyte-containing reagent in an assay system comprising at least one surface comprising polypropylene. The invention also concerns assay systems for use in these tests that include at least one microtiter well having at least one interior surface comprising polypropylene and having a shape such that monocyte-containing reagent is concentrated in the well to provide greater cell to cell contact. The invention also relates to a diagnostic kit that can be used to test for the presence of non-endotoxin pyrogens in a sample.

20 Claims, 16 Drawing Sheets

(75) **Inventors:** **Stephen Poole**, London (GB); **Mehul Patel**, Lafayette, CO (US)

(73) **Assignees:** **Baxter International Inc.**, Deerfield, IL (US); **Baxter Healthcare S.A.**, Glattpark (Opfikon) (CH); **National Institute for Biological Standards and Control**, Blanche Lane, South Mimms, Potters Bar

(*) **Notice:** Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 0 days.

This patent is subject to a terminal disclaimer.

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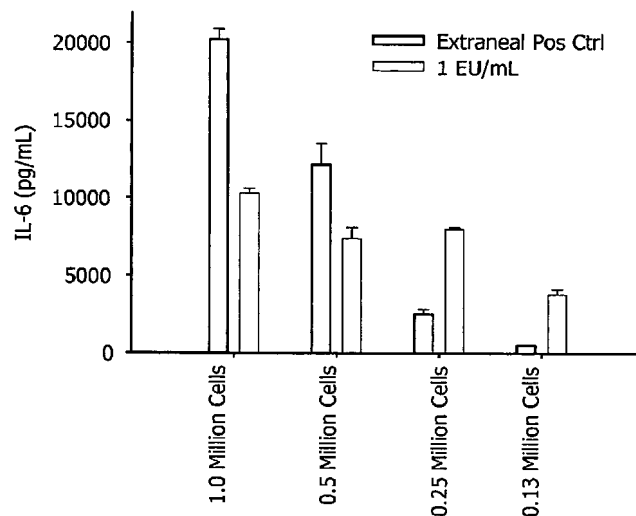
Related U.S. Application Data

(62) Division of application No. 11/613,866, filed on Dec. 20, 2006, now Pat. No. 7,736,863.

(60) Provisional application No. 60/752,970, filed on Dec. 22, 2005.

(51) **Int. Cl.**
G01N 33/567 (2006.01)
C12M 1/34 (2006.01)

(52) **U.S. Cl.** **435/7.1; 435/7.21; 435/287.2**





US007736863B2

(12) **United States Patent**
Poole et al.

(10) **Patent No.:** **US 7,736,863 B2**
(45) **Date of Patent:** **Jun. 15, 2010**

(54) **MONOCYTE ACTIVATION TEST BETTER
ABLE TO DETECT NON-ENDOTOXIN
PYROGENIC CONTAMINANTS IN MEDICAL
PRODUCTS**

6,696,261 B2 2/2004 Patel et al.
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OTHER PUBLICATIONS

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(51) **Int. Cl.**
G01N 33/567 (2006.01)
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(52) **U.S. Cl.** **435/7.21; 435/287.2**

(58) **Field of Classification Search** None
See application file for complete search history.

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asp?ID=298&lang=GB](http://web.archive.org/web/2003101901343/muncbrand.com/page.asp?ID=298&lang=GB) (NuncBrand FAQ for Immunology Products,
published on the Internet at least since Jan. 2, 2004) (hereinafter
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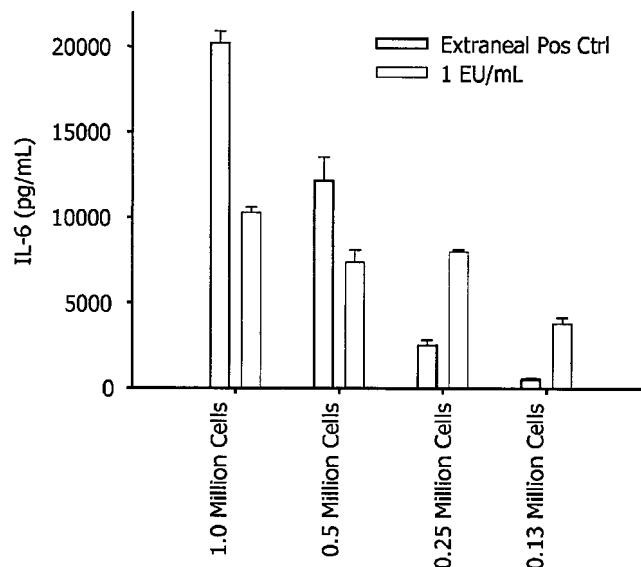
(Continued)

Primary Examiner—Cherie M Woodward
(74) *Attorney, Agent, or Firm*—Marshall, Gerstein & Borun
LLP

(57) **ABSTRACT**

An improved monocyte activation test is described that is
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ucts, in which a sample is incubated with a monocyte-con-
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greater cell to cell contact. The invention also relates to a
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70 Claims, 16 Drawing Sheets



52

Docket No.: 31203/30021A
(PATENT)

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent Application of:
Stephen Poole et al.

Application No.: 11/613,866

Confirmation No.: 8436

Filed: December 20, 2006

Art Unit: 1647

For: MONOCYTE ACTIVATION TEST BETTER
ABLE TO DETECT NON-ENDOTOXIN
PYROGENIC CONTAMINANTS IN
MEDICAL PRODUCTS

Examiner: C. M. Woodward

DECLARATION UNDER 37C.F.R. 1.132 OF STEPHEN POOLE

I, Stephen Poole, do hereby declare as follows:

1. I am a co-inventor of the subject patent application. Currently, I am the Parenteral Sections Leader of the Biotherapeutics Group of the National Institute for Biological Standards and Control (NIBSC). I have authored over 200 scientific journal articles. My curriculum vitae is appended hereto as Exhibit A.

2. I am a co-author of the scientific journal article entitled, "A rapid 'one-plate' in vitro test for pyrogens" which published in the *Journal of Immunological Methods* Vol. 274 (2003): 209-220 (a copy of which is appended hereto as Exhibit B; referred to hereinafter as "Poole et al."). Poole et al. describes an ELISA-based pyrogen detection assay in which pyrogen-free 96-well plates coated with pyrogen-free anti-cytokine antibodies or antibodies specific for endogenous mediators of the inflammatory response are used in conjunction with monocytes. In this assay, a test sample is contacted with the monocytes and, if the test sample includes a pyrogen, a cytokine or endogenous mediator is released by the monocytes and subsequently binds to the antibodies on the surface of the plate. The cytokine or endogenous mediator bound to the antibody is subsequently detected in the assay.

3. In the experiments described in Poole et al., a variety of microtiter

plates were used in the assay, including Dynex PF microtiter 'flat bottom' styrene 96 well plates, Dynex pyrogen-free (PF) microtiter 'U-bottom' styrene 96-well plates, Dynex (non PF) polyvinylcarbonate (PVC) microtiter 'U bottom' 96-well plates, Falcon Microtest tissue culture plate 96-well, Falcon Microtest III flexible 96-well assay plates, and Nunc-Immuno (nonPF) 96-well plates. However, none of the experiments described in Poole et al. included the use of microtiter plates with wells having a polypropylene coating or plates which were entirely made of polypropylene.

4. The results of Poole et al. demonstrated that neither the shape nor the coating or composition of the wells of the microtiter plates used in the assay had an affect on the ability of the assay to detect pyrogens. We reported this finding by stating (at page 213, first paragraph of the Results section of Poole et al.): "All of the different plate types used in the one-plate assays, e.g., 'flat bottom', 'U-bottom', styrene and PVC gave similar results."

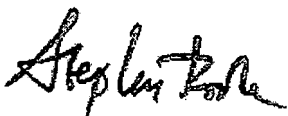
5. After Poole et al. published and before the priority date of the subject patent application, we carried out a pyrogen detection assay similar to the one described in Poole et al. but changed the assay, such that polypropylene microtiter plates with non-flat wells were used. The use of the polypropylene microtiter plates with non-flat wells provided a significant improvement for the indirect detection of non-endotoxin pyrogens. The significant improvement is evidenced by, for example, the greater difference in the detection of IL-6 bound to IL-6-specific antibody (as represented by the absorbance at 450 nm) seen between the Extraneal positive and negative controls when polypropylene, non-flat wells were used in the assay (Figure 12A of the application), as compared to the difference among the two controls when polystyrene, flat wells were used in the assay (Figure 12B of the application). Given that neither the shape nor the coating or composition of the wells of the microtiter plates used in the assay made a difference in the studies described in Poole et al., we did not expect that the use of polypropylene microtiter plates with non-flat bottomed wells would provide such an improvement in the ability of the assay to detect non-endotoxin pyrogens.

6. I declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like are

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Docket No.: 31203/30021A

punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the above-identified application or any patent issuing thereon.

Signed: 
Stephen Poole

Date: 12 January 2010

CURRICULUM VITAE

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1972-1973	Research Assistant, Department of Pharmacology, Royal College of Surgeons of England
1974-1982	Research Scientist, Department of Pharmacology, Institute of Psychiatry, London
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POSTS HELD AND OTHER RESPONSIBILITIES:

Member of:-

- HPA R&D Group
- MHRA Expert Advisory Group on Clinical Trials (of "*higher risk*" medicines and vaccines)
- EDQM Group MAT (Monocyte Activation Test for pyrogens)
- EDQM Group BET (Bacterial Endotoxins Test)
- EMA Group drafting guideline for pre-clinical cytokine release tests
- USP/EDQM/Japanese Pharmacopoeia/WHO Endotoxin Standardization Group

PUBLICATIONS

Some 200 peer-reviewed, PubMed-cited, publications, including two in the Journal *NATURE*. Numerous review articles and book chapters and co-authorship of: *Bacteria - Cytokine Interactions in Health and Disease* (Portland Press, 1998).

PATENTS

1. Peptides and pharmaceutical composition thereof in the treatment of pain.
Patent number: US538961 1995-02-14
2. Pharmaceutical preparation for the treatment of inflammatory processes.
Patent number: EP1027894 2000-08-16; US6399572 2002-06-04.
3. Pyrogenicity test for use with automated immunoassay systems.
Patent number: US6696261 2004-02-24.

SUMMARY OF SCIENTIFIC WORK:

A background in neuro, cardiovascular and immunopharmacology and cell biology and responsible for the evaluation of biological medicines and vaccines for more than 25 years. Has developed and applied novel cell-based assays for measuring pro-inflammatory activities of medicines and vaccines and their contaminants. One of these tests, the Monocyte Activation Test for pyrogens, will be introduced into the *Ph Eur* in April 2010.

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Responsible for the distribution of a variety of standards and European batch release of parenteral medicines.

A rapid 'one-plate' in vitro test for pyrogens

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Abstract

A rapid, 'one-plate' monocyte-activation test is described for detecting endotoxin and non-endotoxin pyrogens in parenteral medicinal products. The one-plate test offers useful gains over conventional 'two-plate' (cell culture plate + ELISA plate) tests in terms of its limit of detection, robustness, speed and cost. The 'one-plate' test is likely to be applicable to a wide range of products because it allows less time for product interference in the test. The 'one-plate' test utilises pyrogen-free anti-cytokine (interleukin (IL)-6 or tumour necrosis factor α (TNF α)) antibodies (Ab), coated and stabilised onto (pyrogen-free) 96-well plates. Monocytes/monocytic cells, endotoxin (lipopolysaccharides, LPS) standard or sample and (pyrogen-free) second (labelled) Ab are cultured together (usually for 2–4 h) on the Ab-coated plate and then the plate is washed and the ELISA completed. There is no transfer from one plate to another and no (further) incubations of (released) cytokine with, first, coating Ab and, then, developing Ab since these steps have already taken place during the initial cell culture. The rapid, 'one-plate' test is readily automated. The preferred readout is IL-6, which gives a limit of detection of 0.015 endotoxin units (EU)/ml with peripheral blood mononuclear cell (PBMC), 0.03 EU/ml with diluted whole blood and 0.05 EU/ml with a monocytic cell line (MONO MAC 6).

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Keywords: One plate; In vitro test for pyrogens; Monocyte-activation test; Endotoxin; Interleukin-6; Tumour necrosis factor α

1. Introduction

Parenteral pharmaceutical products are required to be free from pyrogenic contamination. While a pyrogen may be defined as any substance that causes fever, the pyrogens of concern to the pharmaceutical industry are bacterial endotoxins (lipopolysaccharides, LPS) from Gram-negative bacteria since it is endotoxin that most frequently contaminates parenteral medicinal products (Mascoli and Weary, 1979a,b). Currently one of two Pharmacopoeial tests for pyrogenic contamination is applied to

Abbreviations: BET, bacterial endotoxins test; ELISA, enzyme-linked immunosorbent assay; EU, endotoxin units; HRP, horse radish peroxidase; HSA, human serum albumin; IL, interleukin; IU, international unit; LAL, Limulus amoebocyte lysate; LPS, lipopolysaccharide; OPD, 1, 2-phenylenediamine dihydrochloride orthophenylene diamine; PBMC, peripheral blood mononuclear cell; S.E.M., standard error of the mean; TNF α , tumour necrosis factor α ; Tween 20, polyoxyethylene-sorbitan monolaurate; w/v, weight per volume.

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parenteral medicinal products. These are the rabbit pyrogen test and the bacterial endotoxins test (BET). As its name suggests, the rabbit pyrogen test detects endotoxin and other pyrogens, and involves measuring the rise in body temperature evoked in rabbits by the intravenous injection of a sterile solution of the substance to be examined. Again, as its name suggests, the bacterial endotoxins test (BET) detects only endotoxin. The principle of the BET is that endotoxin causes extracellular coagulation of the blood (haemolymph) of the horseshoe crab, *Limulus polyphemus*. (Levin and Bang, 1964). Although the BET, which is also known as the *Limulus* amoebocyte lysate (LAL) test, is gradually superseding the rabbit pyrogen test, thousands of rabbit pyrogen tests are still carried out each year around the world, in instances where the BET cannot be applied. Although generally reliable, both the rabbit pyrogen test and BET have shortcomings. The rabbit test is costly, not quantitative and uses experimental animals. The BET gives false negatives with certain products, can overestimate the pyrogen content of other products, does not detect pyrogens other than bacterial endotoxin (LPS), such as Gram-positive exotoxins, viruses and fungi (Dinarello et al., 1984; Poole et al., 1988; Ray et al., 1990; Taktak et al., 1991; Fennrich et al., 1999) and also uses animals, albeit only as 'involuntary' blood donors.

The basis of the rabbit pyrogen test is the in vivo stimulation by exogenous pyrogens (usually endotoxin) of rabbit peripheral blood monocytes to produce the endogenous pyrogens, which cause fever. The major endogenous pyrogens are the cytokines tumour necrosis factor α (TNF α), interleukin-1 (IL-1 α and IL-1 β , two separate gene products), IL-6 and IL-8 (Dinarello et al., 1999). Given the shortcomings of the rabbit pyrogen test and the BET, alternative in vitro tests for pyrogens based upon monocyte activation have been proposed. The basis of these 'monocyte-activation tests' or 'monocyte tests' is the exquisite sensitivity to exogenous pyrogen of human monocytes. In such tests, products are incubated with human peripheral blood monocytes or, more usually, the (monocyte-containing) peripheral blood mononuclear cell fraction (PBMNC) and the cell-conditioned media assayed for pyrogenic cytokines (Duff and Atkins, 1982;

Dinarello et al., 1984; Poole et al., 1988, 1989a,b; Hansen and Christensen, 1990; Taktak et al., 1991; Bleeker et al., 1994).

Unfortunately, the isolation of monocytes/PBMNC from whole blood is labour-intensive, time-consuming, technically sophisticated, requires expensive reagents and does not guarantee the isolation of cells in a non-activated state. This prompted the evaluation of various 'monocytic' cell lines, including MONO MAC 6 cells (Ziegler-Heithrock et al., 1988) and THP-1 cells (Tsuchiya et al., 1980), and various cytokine readouts (Poole, 1989; Poole et al., 1989a,b; Taktak et al., 1991). Taktak et al. (1991) favoured a MONO MAC 6/IL-6 test and applied it to therapeutic human serum albumin (HSA) that had caused adverse reactions in recipients and detected pyrogenic contamination in the HSA that had not been detected in either the rabbit pyrogen test or the LAL test. Subsequently, sub-clones of the MONO MAC 6 and THP-1 cell lines have been used in in vitro test for pyrogens (Eperon and Jungi, 1996; Eperon et al., 1997; Peterbauer et al., 1999, 2000; Nakagawa et al., 2002).

Although cell lines avoid the need to make primary cultures of white blood cells, an in vitro test for pyrogens utilising a monocytic cell line has a number of disadvantages. These include: (i) the weeks/months required to establish, starting from a frozen aliquot, a sufficient number of cells of sufficient sensitivity (to pyrogen/endotoxin) to use in a test, (ii) inconsistencies in the sensitivities of cells of different passage numbers and different periods of time spent frozen, (iii) the need to co-stimulate with agents such as interferon- γ , or vitamin D (Taktak et al., 1991; Eperon and Jungi, 1996; Eperon et al., 1997; Peterbauer et al., 1999, 2000), (iv) verification of the continued presence on the cell line of the various receptor elements (e.g. CD14 and Toll-like receptors) necessary for the transduction of responses to endotoxin and other pyrogens (Means et al., 1999; Ulevitch, 1999; Takeuchi et al., 1999).

To avoid some of the disadvantages of test systems requiring the isolation of white blood cells or maintenance of monocytic cell lines, diluted whole blood may be used as the source of monocytes, since the monocytes in diluted whole blood respond to pyrogen/endotoxin by releasing pyrogenic cytokines (Desch et al., 1989; Finch-Arietta and

Cochran, 1991; Nerad et al., 1992; De Groote et al., 1992; Hartung and Wendel, 1996; Schins et al., 1996; Pool et al., 1998; Nakagawa et al., 2002). A monocyte test utilising diluted whole blood as the source of monocytes offers the considerable advantage of not requiring the isolation of monocytes/white blood cells and of culturing the monocytes in the presence of all of the constituents of the blood with which they usually reside. However, in common with all of the earlier variants of the *in vitro* test for pyrogens ('monocyte test'), the 'whole-blood *in vitro* test for pyrogens' suffers from the disadvantage of requiring two incubation steps, on two separate plates: an incubation of monocytes (as diluted blood) on one plate, followed by a second incubation of the monocyte-conditioned diluted blood on the ELISA plate used for the quantification of the cytokine measured.

The aims of the study were to assess the value of the one 'one-plate' *in vitro* monocyte-activation test for pyrogens to determine whether or not it offered significant gains over the conventional 'two-plate' (culture plate+ELISA plate) tests in terms of its quantification limit, robustness, speed and cost.

2. Materials and methods

2.1. Reagents

The following materials were obtained from the sources indicated. The WHO international standards for bacterial endotoxin (LPS, ampoule code 94/580, identical to EC6 [US FDA], lot G [USP] and BRP3 [EP]), IL-1 β (86/680), IL-6 (89/548), and TNF α (87/650) were provided by NIBSC. The specific activities of these standards are endotoxin: 10000 IU (international units)=10000 EU (endotoxin units, FDA/USP)=1 μ g *E. coli* endotoxin/vial, IL-1 β : 100000 IU/1 μ g/ampoule, IL-6: 100000 IU/1 μ g/ampoule, TNF α : 40000 IU/1 μ g/ampoule. Matched pairs (for ELISA) of monoclonal antibodies (MAbs) to human IL-1 β and to human IL-6 (R&D Systems). Fragmin (Dalteparin, 10000 IU/ml, Pharmacia). Anti-human TNF α monoclonal antibody (MAb) 101-4 and sheep anti-TNF α serum were a gift from Dr. T Meager, Division of Immunobiology, NIBSC.

2.2. Consumables

Dynex PF microtiter 'flat bottom' styrene 96-well plates (#9305 Dynex Tech.); Dynex PF microtiter 'U-bottom' styrene 96-well plates (#2305); Dynex (non-PF) PVC microtiter 'U bottom' 96-well plates (#2401); Falcon Microtest tissue culture plate 96-well (#353072); Falcon Microtest III flexible 96-well assay plates (#3911, Beckton Dickinson Labware: 353072); Nunc-Immuno (non-PF) 96-well plates; centrifuge tubes (Falcon 2070 Blue Max); DEAE Trisacryl M, Dulbecco's phosphate buffered saline (Sigma, D-703); cell culture grade glycine (G-6388); hydrochloric acid, 0.1 M, sterile-filtered (Sigma, H-9892); cell culture grade polyoxyethylene-sorbitan monolaurate (Tween 20, P-2287); cell culture grade sucrose (S-1888); cell culture grade trehalose dihydrate (T-0167, Sigma Chemical); Gyrocent-L protein concentrators (Triple red Lab. Tech., #1433400); Nalgene cryogenic vials (#5000-0020); RPMI-1640 (GIBCO, Life Technologies), prepared sterile and pyrogen-free; sterile HSA (a dilution to 1% of clinical grade HSA, 4.5% w/v, Blood Products Laboratory, Elstree, Herts); pyrogen-free, sterile water for irrigation and 0.9% w/v NaCl (Baxter Healthcare). Other reagents/materials were purchased as sterile and pyrogen-free and glassware baked at 250 °C for 1 h.

2.3. Preparation of pyrogen-free anti-cytokine antibodies

Anti-IL-6 polyclonal antibodies were purified from a goat-anti-IL-6 serum by affinity chromatography on IL-6 (Taktak et al., 1991) using pyrogen-free wash buffer and elution buffer. The fractions were neutralised with pyrogen-free 1M sodium bicarbonate. The antibodies were concentrated on a Gyrocent-L centrifugal concentrator. Pyrogen-free biotinylated anti-IL-6 antibodies were prepared and purified as described previously (Taktak et al., 1991) except that pyrogen-free buffer was used. HRP-labelled anti-IL-6 antibodies, anti-TNF α monoclonal antibody (MAb) 101-4 (Meager et al., 1987), purified by FPLC, and sheep polyclonal anti-TNF α IgG (purified by HPLC or on DEAE Trisacryl M, Sigma) were made pyrogen-free using End-X (Associated of Cape Cod, USA). The solutions of pyrogen-free antibodies contained <0.03

EU (= IU)/ml of endotoxin contamination as measured in a LAL test.

2.4. Preparation and stabilisation of 96-well plates (pre)coated with pyrogen-free anti-cytokine antibodies

Non-sterile plates were rinsed extensively with pyrogen-free PBS. Ninety-six well plates of each type (see above) were coated with pyrogen-free anti-cytokine antibodies: goat anti-human IL-6 polyclonal antibodies, MAb 101-4 against human TNF α (1 μ g/ml, in either sterile PBS, pH 7.4, or sodium bicarbonate buffer, pH 8.2–9.6, 100 μ l/well, +4 °C, overnight). Plates were washed twice with sterile PBS + 0.1% Tween 20, then once with sterile PBS (without Tween). When the plates were not required immediately, a blocking/glazing solution (100 μ l/well of either sterile trehalose, 2% + sterile HSA, 1% or sterile sucrose, 5% + sterile HSA, 1%) was added for 1 h at room temperature. After 1 h, the blocking/glazing solution was carefully decanted and the plates were air-dried in a Class II 'sterile' cabinet for at least 4 h (usually overnight), sealed in plastic bags and stored at 4 °C.

2.5. Collection of blood

Twenty blood donors were included in the study. Seven were male and thirteen were female. The ages of the males ranged from 27 to 47 years with a mean of 36 years. The ages of the females ranged from 23 to 49 years with a mean of 34 years. All of the donors appeared to be in good health. None were taking NSAIDs, immunosuppressants, glucocorticoids or any other drugs known to influence the production of cytokines. Using a 20-ml syringe and a 21-gauge needle, 2–10 ml blood was drawn from the median cubital or cephalic vein of the left or right arm of donors and transferred immediately into 50 ml sterile, pyrogen-free centrifuge tubes (Falcon 2070 Blue Max) containing sufficient heparin (Fragmin, 10000 IU/ml, Pharmacia, diluted 1/10 with sterile, pyrogen-free saline) to give a concentration of 10 IU/ml in the blood. The blood was collected in the morning, held at room temperature and used within 1 h (typically 30 min) of its collection. The duration of short-term blood storage does not significantly affect IL-6 release (Schins et al., 1996).

2.6. Isolation of PBMNC and culture of PBMNC and MONO MAC 6 and THP-1 cells

Isolation of PBMNC and culture of PBMNC, MONO MAC 6 and THP-1 cells were as described previously (Taktak et al., 1991). Counts of viable cell numbers were made by taking an aliquot of 100 μ l of a cell suspension and adding 850 μ l RPMI and 50 μ l 0.4% w/v Trypan blue solution. The number of viable cells in this solution was counted using a haemocytometer. This procedure is described in detail in the Sigma (Biochemical and Reagents For Life Science Research) Catalogue 2000/2001, pp. 1848–1849.

2.7. Protocol for 'two-plate' in vitro test for pyrogens using PBMNC, MONO MAC 6 or THP-1 cells

Cultures (250 μ l) were set up in quadruplicate using 96-well plates. The 250 μ l volume comprised: samples/standards diluted in RPMI containing 2% AB serum (125 μ l) and cells at an initial concentration of two million cells/ml in RPMI containing 2% AB serum (125 μ l), giving a final concentration (in the wells) of one million cells/ml. The contents of the wells were mixed by swirling gently (without cross-contaminating wells) and incubated without vibration (to allow the cells to settle) at 37 °C for 2–24 h in an atmosphere of 5% CO₂ in air. At the end of the tissue culture incubation, the cell-conditioned culture medium above the cells was made homogeneous by aspirating and expelling 100 μ l three times before transferring 100 μ l aliquots of the medium from each of the wells of the tissue culture plate into the corresponding wells of the ELISA plate. Aliquots were taken for ELISA determinations of IL-6 (Taktak et al., 1991), or using a matched pair of MAbs to human IL-6 from R&D Systems, both assays giving similar results). TNF α (Meager et al., 1987), IL-1 β (ELISA developed using the antibodies described by Rafferty et al., 1991, or using a matched pair of MAbs to human IL-1 β from R&D Systems, or an IL-1 β ELISA kit kindly provided by DPC Biemann, all of which gave similar results). All of the ELISAs employed detected 62.5 pg/ml cytokine or less and showed good regression on dose over the range 62.5–1000 pg/ml, with CVs of the order of 7–10%.

2.8. Protocol for 'two-plate' *in vitro* test for pyrogens using human whole blood

Cultures (250 µl) of 20% human whole blood were carried out in quadruplicate using 96-well plates. To distribute cells evenly throughout the blood, the blood donation was swirled gently prior to the withdrawal of aliquots (5 ml) into a smaller tube (7 ml) from which aliquots (50 µl) were taken for addition to the culture plate. The additions per well were: RPMI (75 µl), blood (50 µl), endotoxin sample/standard in RPMI or saline (125 µl). The contents of the wells were mixed by swirling gently (without cross-contaminating wells) and incubated without vibration (to allow the cells to settle) at 37 °C for 2–24 h in an atmosphere of 5% CO₂ in air. At the end of the tissue culture incubation, 100 µl aliquots of clear supernatant from each well were carefully transferred into the corresponding wells of the ELISA plate. Aspiration of cells was avoided by supporting the assay plate such that it sloped down at an angle of about 45° from columns 1 to 12. The ELISAs were as described above in Section 2.7.

2.9. Protocol for 'one-plate' *in vitro* test for pyrogens using PBMNC, MONO MAC 6 or THP-1 cells

Cultures (250 µl) were carried out in quadruplicate using 96-well plates previously coated with pyrogen-free anti-IL-6 antibodies. The 250 µl volume comprised: samples/standards diluted in RPMI containing 2% AB serum (125 µl), pyrogen-free biotinylated anti-IL-6 antibodies (10 µl), cells at an initial concentration of two million cells/ml in RPMI containing 2% AB serum (115 µl), giving a final concentration (in the wells) of one million cells/ml. The contents of the wells were mixed by swirling gently (without cross-contaminating wells) and incubated without vibration (to allow the cells to settle) at 37 °C for 2–24 h in an atmosphere of 5% CO₂ in air. At the end of the incubation, cultures were aspirated and discarded (in accordance with local safety procedures) and plates were washed four times with wash/dilution buffer (Taktak et al., 1991). Subsequent steps were as described previously (Taktak et al., 1991). Briefly, avidin-HRP (in 100 µl) was added for 15 min at room temperature. OPD (in 100 µl) was added for 10–20 min (until sufficient colour devel-

oped). 1 M H₂SO₄ (150 µl/well) was added to stop the reaction. Optical densities were read at 490 nm. In some experiments, HRP-labelled anti-IL-6 antibodies were substituted for biotinylated anti-IL-6 antibodies to eliminate the avidin-HRP step and so reduce the number of steps required for the ELISA. The ELISA for IL-6 detected 62.5 pg/ml cytokine or less and showed good regression on dose over the range 62.5–4000 pg/ml, with CVs of the order of 7–10%.

2.10. Protocol for 'one-plate' *in vitro* test for pyrogens using human whole blood

Cultures (250 µl) of 20% human whole blood were carried out in quadruplicate using 96-well plates previously coated with pyrogen-free anti-cytokine (IL-6 or TNFα) antibodies. To distribute cells evenly throughout the blood, the blood donation was swirled gently prior to the withdrawal of aliquots (5 ml) into a smaller tube (7 ml) from which aliquots (50 µl) were taken for addition to the culture plate. The additions per well were: RPMI or saline (65 µl), blood (50 µl), biotinylated anti-cytokine (IL-6 or TNFα) antibodies (10 µl) and endotoxin sample/standard in RPMI or saline (125 µl). The contents of the wells were mixed by swirling gently (without cross-contaminating wells) and incubated without vibration (to allow the cells to settle) at 37 °C for 2–24 h in an atmosphere of 5% CO₂ in air. The steps following the incubation were as described above in Section 2.9. The ELISAs for IL-6 and TNFα detected 62.5 pg/ml cytokine or less and showed good regression on dose over the range 62.5–4000 pg/ml, with CVs of the order of 7–10%.

3. Results

All of the different plate types used in the one-plate assays, e.g. 'flat bottom', 'U-bottom', styrene and PVC gave similar results. Similarly, the different coating/blocking buffers gave similar results.

3.1. Comparison of one-plate and two-plate tests for pyrogens using PBMNC/IL-6

Stimulation of PBMNCs (10⁶ cells/ml) with endotoxin (LPS, 0–0.25 EU/ml=0–25 pg/ml) for 4 h evoked the dose-dependent release of immunoreactive

IL-6 into the PBMC-conditioned medium (Fig. 1). With the one-plate format, the endotoxin–IL-6 dose–response curve was steeper and the limit of detection (0.015 EU/ml) lower than with the two-plate format (0.03 EU/ml). The limit of detection was calculated as $3.3 \times$ the standard deviation of the response divided by the slope of the calibration curve, as described in the ICH Harmonised Tripartite Guideline, Validation of Analytical Procedures: Methodology, 1996 (<http://www.mcdurenet.com/EMEA/PDFs/Q2a.pdf>). Stimulation of cells for 24 h gave similar results except that the IL-6 values were increased approximately two-fold (data not shown).

3.2. Comparison of one-plate and two-plate tests for pyrogens using monocytic cell lines

Stimulation of MONO MAC 6 cells (10^6 cells/ml) with endotoxin (LPS, 0–0.8 EU/ml = 0–80 pg/ml) for 24 h evoked the dose-dependent release of immunoreactive IL-6 into the cell-conditioned medium (Fig. 2).

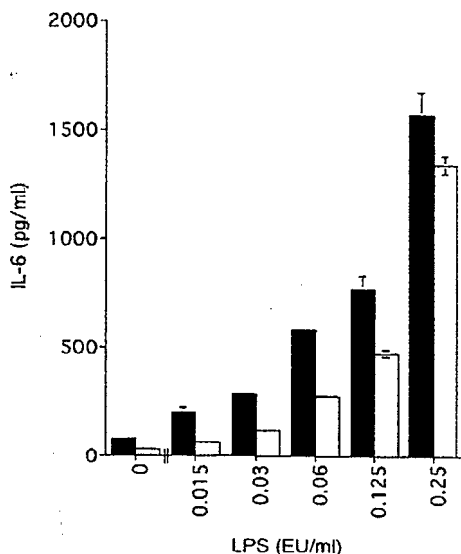


Fig. 1. Comparison of 'one-plate' (filled columns) and 'two-plate' (open columns) in vitro tests for pyrogens using PBMCs (10^6 cells/ml) stimulated with endotoxin (LPS, 0–0.25 EU/ml = 0–25 pg/ml) for 4 h. Values for IL-6 are means of quadruplicates $\pm$ S.E.M. The experiment is representative of three independent experiments, each using PBMCs from a different donor.

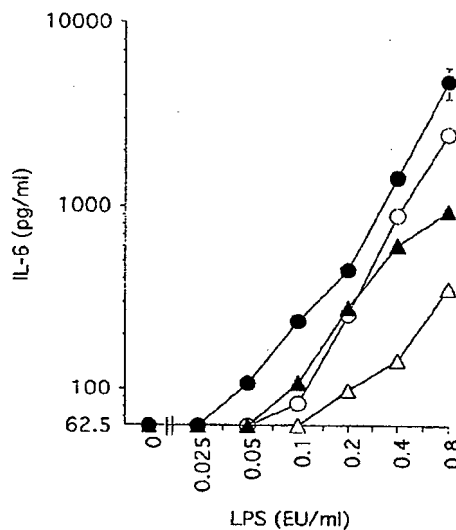


Fig. 2. Comparison of 'one-plate' and 'two-plate' in vitro tests for pyrogens using MONO MAC 6 cells and THP-1 cells. Release of IL-6 by MONO MAC 6 cells (circles) and THP-1 cells (triangles) stimulated with endotoxin (LPS, 0–0.8 EU/ml = 0–80 pg/ml) using either the 'one-plate' format (filled symbols) or the 'two-plate' format (open symbols). Values for IL-6 are means of quadruplicates $\pm$ S.E.M. The experiment is representative of three independent experiments, each using cells of a different passage number.

With the one-plate format, the endotoxin–IL-6 dose–response curve was steeper and the limit of detection (0.05 EU/ml) lower than with the two-plate format (0.1 EU/ml). Similar results were obtained with THP-1 cells: stimulation of THP-1 cells (10^6 cells/ml) with endotoxin (LPS, 0–0.8 EU/ml = 0–80 pg/ml) for 24 h evoked the dose-dependent release of immunoreactive IL-6 into the cell-conditioned medium (Fig. 2). Again, with the one-plate format, the endotoxin–IL-6 dose–response curve was steeper and the limit of detection (0.1 EU/ml) lower than with the two-plate format (0.2 EU/ml).

3.3. Release of TNF α , IL-1 β and IL-6 by whole blood (20%) stimulated with endotoxin in a two-plate test

Stimulation of whole blood (diluted 1/5 to 20% with saline) with endotoxin (LPS, 0–4 EU/ml = 0–400 pg/ml) for 4 h evoked the release of immunoreactive TNF α , IL-1 β , and IL-6 into the blood (Fig. 3).

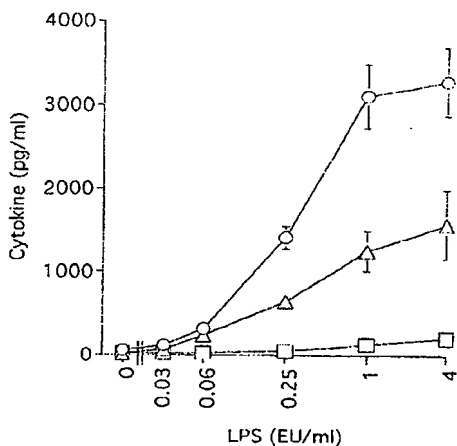


Fig. 3. Release of cytokines by whole blood stimulated with endotoxin (LPS) in a one-plate test. Release of IL-1 β (squares), TNF α (triangles), and IL-6 (circles) by whole blood (diluted 1/5 to 20% with RPMI) stimulated with endotoxin (LPS, 0–4 EU/ml=0–400 pg/ml) for 4 h. Values for cytokines are means $\pm$ S.E.M.s of six independent experiments, each using blood from a different donor.

The responses for each cytokine were dose-dependent and the relative concentrations of the cytokines (pg/ml) released for a given dose of endotoxin had the relation IL-6>TNF α >IL-1 β . A similar pattern of cytokine release was obtained with 24-h stimulation with endotoxin (data not shown).

3.4. Comparison of one-plate and two-plate whole blood tests for pyrogens with IL-6 as the readout

Stimulation of human whole blood (diluted 1/5 to 20% with saline) with endotoxin (LPS, 0–4 EU/ml=0–400 pg/ml) for 4 h evoked the dose-dependent release of immunoreactive IL-6 into the blood (Fig. 4). With the one-plate format, the endotoxin–IL-6 dose–response curves were steeper and the limit of detection (0.03 EU/ml) lower than with the two-plate format (0.06 EU/ml).

3.5. Comparison of one-plate and two-plate whole blood tests for pyrogens with TNF α as the readout

Stimulation of human whole blood (diluted 1/5 to 20% with saline) with endotoxin (LPS, 0–4 EU/ml=0–400 pg/ml) for 2 h evoked the dose-dependent

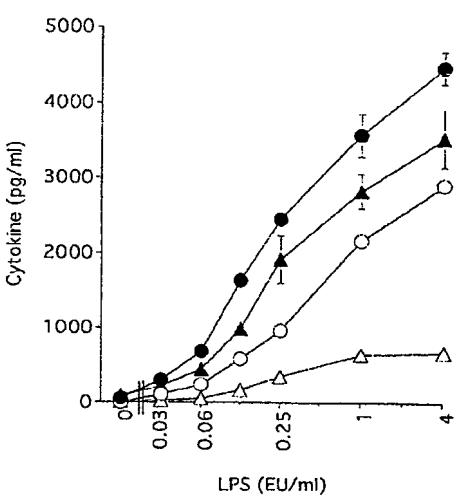


Fig. 4. Comparison of 'one-plate' and 'two-plate' whole blood tests for pyrogens with IL-6 and TNF α as the readouts. Release of IL-6 (circles) and TNF α (triangles) by whole blood (diluted 1/5 to 20% with saline) stimulated with endotoxin (LPS, 0–4 EU/ml=0–400 pg/ml) for 2 h (TNF α) or 4 h (IL-6) using either the 'one-plate' format (filled symbols) or the 'two-plate' format (open symbols). Values for TNF α and IL-6 are means of quadruplicates $\pm$ S.E.M. The experiment is representative of three independent experiments, each using blood from a different donor.

release of immunoreactive TNF α (Fig. 4). With the one-plate format, the endotoxin–TNF α dose–response curve was steeper and the limit of detection (0.03 EU/ml) lower than with the two-plate format (0.06 EU/ml).

3.6. Spike recoveries for HSA spiked with endotoxin

Spike recoveries (%) for LPS (2.5 EU/ml) added to HSA (4.5%) and diluted 1/10 for testing with whole blood (20%, 4-h culture) are given in Table 1. In two-

Table 1			
Spike recoveries (%) for LPS added to HSA (4.5%) and diluted 1/10 for testing with whole blood (20%, 4-h culture)			
Test type	Readout	LPS spike (EU/ml)	Spike recovery %
Two-plate	TNF α	2.5	73 $\pm$ 6 (n=11)
Two-plate	IL-1 β	2.5	49 $\pm$ 13 (n=6)
Two-plate	IL-6	2.5	64 $\pm$ 8 (n=13)
One-plate	IL-6	2.5	87 $\pm$ 13 (n=8)

Values are means $\pm$ S.E.M.

plate tests with TNF α as readout, LPS spike recovery was $73 \pm 6\%$, compared with $64 \pm 8\%$ with IL-6 as the readout and just $49 \pm 13\%$ with IL-1 β as the readout. In one-plate tests with IL-6 as readout, spike recovery increased markedly to $87 \pm 13\%$.

4. Discussion

In the 20 years since an in vitro test for pyrogens ('monocyte test') was first suggested (Duff and Atkins, 1982), several different test systems have been described. The sources of monocytes/monocytic cells have included purified monocytes, PBMNC, monocytic cell lines and diluted whole blood and the chosen readouts have included TNF α , IL-1 β , IL-6, and neopterin (reviewed by Poole and Gaines Das, 2001). With monocytes, PBMNC and monocytic cells, the preferred readout is usually IL-6 because IL-6, unlike IL-1 β and TNF α , is secreted entirely into the cell-conditioned medium in large quantities, thereby permitting its complete estimation (Poole et al., 1989a,b; Taktak et al., 1991). In contrast to IL-6, immunoreactive TNF α and IL-1 β remain largely intracellular, raising the possibility that test preparations affecting cell-permeability might be more likely to interfere in the tests with (immunoreactive) TNF α or IL-1 β as the readout, rather than IL-6. Also, certain pro-inflammatory bacterial components stimulate the production of IL-6 but not TNF α and IL-1 β (Reddi et al., 1996).

In a recent study using whole blood (Nakagawa et al., 2002), it was reported that the structurally diverse pyrogens endotoxin, peptidoglycan, *Staphylococcus aureus* Cowan 1 and poly(I.C) all stimulated the release of more IL-6 than either TNF α or IL-1 β (values given in pg/ml). Moreover, IL-6 was induced by lower concentrations of each pyrogen than were required to induce TNF α and IL-1 β . Consistent with these data, the concentrations of IL-6 detected in peripheral blood following administration of endotoxin in man were greater than the concentrations of TNF α and IL-1 β (Luheshi and Rothwell, 1996; Bruunsgaard et al., 1999; Suffredini et al., 1999). Indeed, there is good evidence to suggest that IL-6 is the principal circulating endogenous pyrogen in man (Dinarello, 1999) and experimental animals (Dinarello et al., 1999; Cartmell et al., 2000) and best

correlates with pyrogenic activity in the blood (Rothwell, 1990; Kluger, 1991; Buck et al., 1994; Rothwell and Hopkins, 1995; Lin and Huang, 1998; Otto et al., 1999; Dinarello, 1999). The pyrogenic activity of TNF α and IL-1 appears to be largely IL-6-dependent (Chai et al., 1996; Luheshi and Rothwell, 1996; Sundgren-Andersson et al., 1998; Zetterstrom et al., 1998; Cartmell et al., 2000).

With IL-6 as the readout, the one-plate format offered a lower limit of detection than conventional two-plate assays. With PBMNC, the limit of detection was 0.015 EU/ml even when the duration of the cell culture was just 4 h, and limits of detection of 0.05 and 0.1 EU/ml were obtained with the monocytic cell lines MONO MAC 6 and THP-1, respectively, with cell cultures of 24-h duration. The advantage of these lower limits of detection is that they permit larger dilutions of test samples to overcome interference by the samples in the test.

The use of (diluted) human whole blood as a source of monocytes has been described by a number of groups, including Desch et al., 1989; Finch-Arietta and Cochran, 1991; Nerad et al., 1992; De Groote et al., 1992; Hartung and Wendel, 1996; Schins et al., 1996; Pool et al., 1998; Fennrich et al., 1999; Jahnke et al., 2000; Nakagawa et al., 2002. The major differences between the different protocols utilising whole blood are the cytokine chosen as the readout (measured variable), the duration of the blood culture, and the inclusion/omission of interferon- γ as co-stimulus (priming agent). The preferred readout is IL-1 β (Hartung and Wendel, 1996; Fennrich et al., 1999; Jahnke et al., 2000) or IL-6 (Pool et al., 1998; Nakagawa et al., 2002), although TNF α also would appear to be suitable (Desch et al., 1989; Finch-Arietta and Cochran, 1991; De Groote et al., 1992; Hartung and Wendel, 1996). The duration of incubation is determined to some extent by convenience but also by the choice of readout. A short (4 h) incubation of (diluted) blood with a very large dose of endotoxin (10 μ g/ml) stimulated the production of IL-1 β (and TNF α), whereas a longer incubation (24 h) of (diluted) blood was required for much smaller doses of endotoxin (1 pg/ml and above) to stimulate the production of IL-1 β (and TNF α , Hartung and Wendel, 1996). A lengthy incubation (18 h), in the presence of interferon- γ , was also favoured when IL-6 was the readout (Pool et al., 1998).

In the present study, the concentrations of cytokines (pg/ml) produced by (diluted) whole blood stimulated with endotoxin for 4–24 h had the rank order IL-6>TNF α >IL-1 β , consistent with the recent study of Nakagawa et al. (2002). Also consistent with the data of the Japanese group was the finding that IL-6 was induced by smaller concentrations of LPS than was required to induce TNF α and IL-1 β . To ensure that the values for IL-1 β were not being underestimated, measurements were made using three independent ELISA systems, all of which gave similar results. Regarding the recovery of an endotoxin spike of 2.5 EU/ml in HSA (4.5% w/v), the best endotoxin spike recoveries in two-plate assays using whole blood (20%, 4 h) were obtained with TNF α as readout, followed by IL-6 and IL-1 β . In 4-h whole blood tests with IL-6 as readout, the one-plate format offered a useful improvement in endotoxin spike recovery (from 64% to 87%) for the endotoxin spike of 2.5 EU/ml in HSA.

The rabbit pyrogen test can be completed in less than 4 h, whereas a 'two-plate' *in vitro* test for pyrogens involves a 4-h incubation of monocytes/monocytic cells and product followed by an ELISA requiring a second incubation of cytokine on the capture antibody. Since the *in vitro* test for pyrogens is intended as a replacement for the rabbit pyrogen test, we sought to develop a version of this test, the 'one-plate' test, that could be completed in about the time taken to carry out a rabbit pyrogen test. The one-plate test utilises pyrogen-free anti-cytokine (IL-6 or TNF α) antibodies (Ab) coated and stabilised onto (pyrogen-free) 96-well plates. Diluted blood, endotoxin standard/sample and (pyrogen-free) biotinylated Ab are cultured together (usually for 2–4 h, depending upon the readout) on the Ab-coated plate and then the plate is washed and the ELISA completed. There is no transfer from one plate to another and no (further) incubations of (released) cytokine with, first, coating Ab and, then, developing Ab since these steps have already taken place during the initial culture. The one-plate *in vitro* test for pyrogens with whole blood as the source of monocytes and IL-6 as the readout can be carried out in 5 h from the time when the blood is drawn and, with TNF α as the readout, in just 3 h. With IL-6 as the readout, there is a 4-h culture of blood + product and with TNF α , this can be reduced to 2 h.

In addition to improved robustness (endotoxin spike recovery), the one-plate format also offered improvements in the limit of detection with whole blood as the source of monocytes. With whole blood/IL-6/4 h and whole blood/TNF/2 h combinations, the limit of detection was 0.03 EU/ml. With IL-6 as the readout, the improved limit of detection of the one-plate format is likely to be a consequence of the removal/neutralisation of IL-6 (as it is produced) by the capture Ab and second Ab. In monocytes, endotoxin-stimulated TNF α and IL-1 β stimulate the production of IL-6, which inhibits further production of TNF α and IL-1 β (Schindler et al., 1990). Consequently, the removal/neutralisation of the IL-6 removes the negative feedback exerted by the released IL-6, which leads to the production of more TNF α and IL-1 β , which stimulate the production of more IL-6. With TNF α as the readout, the improvement may be a consequence of the capture by the anti-TNF α (coating) antibody (on the plate) of monocytes expressing the transmembrane form of TNF α (Grell et al., 1995), permitting the measurement of both cell-associated and released TNF α .

TNF α has an advantage as the readout in a one-plate test for pyrogens because of the shorter incubation time required for its release (2 vs. 4 h for IL-6). This makes the TNF readout particularly suitable for 'in process' pyrogen testing. However, the choice of IL-6 as the readout, in addition to the advantages noted above, has the advantage, with whole blood as the source of monocytes, to 'screen out' donors not in good health who may have aberrant responses. Blood cultures in the absence of added endotoxin contained concentrations of immunoreactive TNF α and IL-1 β close to or below the detection limits of the ELISAs used to detect them. In contrast, concentrations of immunoreactive IL-6 were detectable in these cultures. For healthy donors, concentrations of immunoreactive IL-6 were below 200 pg (20 IU)/ml (typically 50 pg/ml or less) but in donors who had recently recovered from minor viral or bacterial infections basal (i.e. unstimulated), concentrations of IL-6 sometimes exceeded 200 pg/ml and responses to added endotoxin were generally smaller for a given dose of endotoxin than for the blood of healthy donors (Poole, unpublished data). These findings accord with reports that plasma concentrations of immunoreactive IL-6 (unlike those of TNF α and IL-1 β) are likely to be increased in indi-

viduals not in good health (Buck et al., 1994; Lin and Huang, 1998; Otto et al., 1999). Consequently, the choice of IL-6 as the measured variable for a test using whole blood as the source of monocytes permits the identification and 'screening out' (albeit retrospectively) of donors who are not in good health, even though they may feel well enough to serve as a blood donor for the test. Based on the data collected for this study, a 'cut-off' value of 200 pg (20 IU)/ml for basal (unstimulated) immunoreactive IL-6 release is proposed for the rejection of test data.

The one-plate format will facilitate both the acceptance and automation of the in vitro test for pyrogens. While the pyrogens that contaminate parenteral medicinal products are almost invariably endotoxins, which rapidly stimulate cytokine production, some 'non-endotoxin' pyrogens can take longer to stimulate cytokine production (Fennrich et al., 1999; Hartung et al., 2001). Although these non-endotoxin pyrogens are unlikely contaminants of parenteral medicinal products, a culture of longer duration than 4 h may be required to ensure freedom from contamination with 'non-endotoxin' pyrogens. However, even for tests requiring a >4-h culture, many of the advantages (robustness, improved limit of detection, convenience, cost) of the one-plate format will still apply. The next challenge for scientists working in this area is to find an alternative to assays requiring human blood, which has the disadvantage of requiring laboratory workers to collect and work with a material that may contain infectious agents, and to cell lines that, starting from a frozen aliquot, can require weeks/months to be suitable for use in a test.

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