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54 Título: **Uso de sondas para el diagnóstico del linfoma esplénico de la zona marginal.**

57 Resumen:

Uso de sondas para el diagnóstico del linfoma esplénico de la zona marginal. La invención se refiere al uso de la secuencia correspondiente al fragmento del cromosoma 7 humano contenido en el BAC denominado RP11-44M6, perteneciente a la región 7q22.1-7q22.2, para el diagnóstico del LEZM mediante la detección de la existencia de deleciones en dicha zona, así como al uso opcional de sondas pertenecientes a la región 7q32-7q33 en el mismo ensayo para aumentar el porcentaje de pacientes de LEZM que pueden diagnosticarse en la misma prueba, prefiriéndose que la sonda opcional se elija entre las correspondientes a los insertos de los BACs RP11-36B6, RP11-329I5 y RP11-193I17. La invención se refiere igualmente a métodos de diagnóstico de LEZM en seres humanos en los que se utiliza como sonda el inserto de RP11-44M6, sola o en combinación con otra u otras, así como a los kits que incluyen dicha sonda.

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Aviso: Se puede realizar consulta prevista por el art. 37.3.8 LP.

## DESCRIPCIÓN

Uso de sondas para el diagnóstico del linfoma esplénico de la zona marginal.

5 **Campo técnico**

La invención se refiere al uso de sondas para el diagnóstico de la enfermedad neoplásica conocida como linfoma esplénico de la zona marginal (LEZM) en seres humanos. Concretamente, la invención se refiere al uso de la secuencia correspondiente al fragmento del cromosoma 7 humano contenido en el BAC denominado RP11-44M6, perteneciente a la región 7q22.1-7q22.2, para el diagnóstico del LEZM mediante la detección de la existencia de deleciones en dicha zona, así como al uso opcional de sondas pertenecientes a la región 7q32-7q33 en el mismo ensayo para complementar el estudio y aumentar el porcentaje de pacientes de LEZM que pueden ser diagnosticados en la misma prueba. La invención se refiere igualmente a métodos de diagnóstico de LEZM en los que se utiliza dicha sonda, sola o en combinación con otra, así como a los kits que incluyen dicha sonda.

15 **Estado de la técnica**

El Linfoma Esplénico de la Zona Marginal (LEZM), es una enfermedad tumoral que puede afectar a cualquier órgano (aunque los más comunes son el bazo y los ganglios linfáticos), que ha sido considerado como una entidad clínico-patológica distinta desde 1991 (Hollema H, Visser I, Poppema S: "Small lymphocytic lymphomas with predominant splenomegaly: a comparison of immunophenotypes with cases of predominant lymphadenopathy", *Modern Pathol* 1991, 4: 712-717; Schmid C, Kirkham N, Diss T, Isaacson PG: "Splenic marginal zone cell lymphoma", *Am J Surg Pathol* 1992, 16: 455-466). En la clasificación Europea-Americana de neoplasias linfoides, el LEZM era categorizado como una entidad separada, pero de manera provisional (Harris NL, Jaffe ES, Stain H y col: "A revised European-American classification of lymphoid neoplasms: a proposal from the International Lymphoma Study Group", *Blood* 1994, 84: 1361-92). Más recientemente, la clasificación de enfermedades hematológicas de la Organización Mundial de la Salud (OMS) (Jaffe ES, Harris NL, Stein H, Vardiman JW: "World Health Organization classification of tumours: pathology and genetics of tumours of haematopoietic and lymphoid tissues", Lyon: IARC Press 2001), ha designado a los LEZM como un subtipo distinto de linfomas no-Hodgkin (Harris NL, Jaffe ES, Diebold J, Flandrin G, Muller-Hermelink HK, Vardiman J, Lister TA, Bloomfield CD: "The World Health Organization classification of neoplastic diseases of the haematopoietic and lymphoid tissues: report of the clinical advisory committee meeting", *airlie house, Virginia, November, 1957, Histopathology* 2000, 36: 69-86) (LNH), en base a su morfología, inmunofenotipo y características clínicas (Mollejo M, Meránguez J, Lloret E, Sánchez A, Campo E, Algara P, Cristóbal E, Piris MA: "Splenic Marginal Zone Lymphoma: A distinctive type of low-grade B-cell lymphoma. A clinicopathological study of 13 cases", *Am J Surg Pathol*, 1995, 19: 1146-1157) junto con los linfomas asociados a mucosas (MALT) y los linfomas de la zona marginal nodal (LZMN). Estos tres subtipos de linfomas están incluidos en los linfomas de la zona marginal porque tienen su origen común en el compartimento marginal de las células B de los nódulos linfáticos y del bazo (Maes B, De Wolf-Peeters C.: "Marginal zone cell lymphoma: an update on recent advances", *Histopathology* 2002, 40: 117-126; Catherine Thieblemont, Pascale Felman, Evelyne Callet-Bauchu, Alexandra Traverse-Glehen, Gilles Salles, Françoise Berger, Bertrand Coiffier: "Splenic marginal-zone lymphoma: a distinct clinical and pathology entity", *Oncology*, 2003, Vol. 4).

El diagnóstico de la existencia de un linfoma no es difícil para los expertos. Sin embargo, es importante clasificar cada linfoma dentro del subtipo al que pertenece para poder dar al paciente el tratamiento adecuado, ya que cada tipo de linfoma tiene un tratamiento distinto. Así, por ejemplo, en el caso del LEZM no se debe aplicar quimioterapia, siendo el tratamiento más adecuado la extirpación del órgano afectado, generalmente el bazo, por lo que es muy importante diagnosticarlo correctamente. En el momento actual el diagnóstico de los LNH y la diferenciación entre cada uno de ellos se basa en las características morfológicas, inmunohistoquímicas y moleculares, siendo las alteraciones genéticas en general, y las traslocaciones específicas en particular, una de las características que más está ayudando para distinguir unos linfomas de otros. La mayoría de los LNH se caracterizan por la presencia de traslocaciones específicas, que sirven para su diagnóstico. Sin embargo, los LEZM carecen de un marcador molecular conocido pues, a diferencia de otros diversos linfomas, no se ha identificado en ellos una traslocación que les sea característica, lo que dificulta su diagnóstico. Si bien los análisis citogenéticos muestran que en este tipo de tumores pueden detectarse pérdidas en las que están involucrados los cromosomas 1, 3, 7 y 8 (Dierlamm J, Pittaluga S, Wlodarska I, Stull M, Thomas J, Boogaerts M, Michaux L, Driessen A, Mecucci C, Cassiman JJ: "Marginal zone B-cell lymphomas of different sites share similar cytogenetic and morphologic features", *Blood* 1996, 87: 299-307; Sole F, Woessner S, Florensa L, Espinet B, Mollejo M, Martín P, Piris MA: "Frequent involvement of chromosomes 1, 3, 7 y 8 in splenic marginal B-cell lymphoma", *Br J Haematol* 1997; 98: 446-449), no se había encontrado hasta ahora una alteración que fuera característica de los LEZM y permitiera la identificación de una alta proporción de los pacientes que la padecen. Los cambios cromosómicos en 3q, por ejemplo, se han detectado en varios tipos de LNH, siendo del 52% la incidencia global de ganancias en el cromosoma 3 observada en los linfomas de la zona marginal. Algunos estudios de citogenética convencional pusieron de manifiesto que una de las alteraciones más frecuentes en los LEZM eran las pérdidas en el brazo largo del cromosoma 7 (del(7q)), lo que podía sugerir que la del(7q) está asociada con los LEZM (Brynes RK, Almaguer PD, Leathery KE, McCourty A, Aubert DA, Medeiros LJ, Nathwani BN: "Numerical cytogenetic abnormalities of chromosomes 3, 7, and 12 in marginal B-cell lymphoma", *Mod Pathol* 1996, 9: 995-1000; Wotherspoon AC, Finn TM, Isaacson PG: "Trisomy 3 in low-grade B-cell lymphomas of mucosa-associated lymphoid tissues", *Blood* 1995, 85: 2000-2004; Dierlamm J, Michaux L, Wlodarska I, Pittaluga S, Zeller W, Stull M, Criel A, Thomas J, Boogaerts M, Delaere P, Cassiman JJ, De Wolf-Peeters C, Mecucci C, Van den Berghe H: "Trisomy 3 in

marginal zone B-cell lymphoma: a study based on cytogenetic analysis and fluorescence *in situ* hybridization”, *Br J Haematol* 1996, 93: 242-249; Hernández JM, Mecucci C, Criel A, Meeus P, Michaux I, Van Hoof A, Verhoef G, Louwagie A, Scheiff JM, Michaux JL: “Cytogenetic analysis of B-cell chronic lymphoid leukemias classified according to morfologic and immunophenotypic (FAB) criteria”, *Leukemia* 1995, 9: 2140-2146). Hasta ahora, sin embargo, se estimaba que las deleciones en el brazo largo del cromosoma 7 no se presentaban en más de un 25% de los pacientes aquejados de LEZM (Franco V, Florensa AM, Llanito: “Splenic marginal zone lymphoma”, *Blood* 2003; 101:2464-2472), por lo que no se consideraba que el desarrollo de métodos diagnósticos basados en su detección pudiera tener interés, al permitir identificar sólo una proporción poco significativa del total de pacientes aquejados de LEZM.

Recientemente, estudios de pérdida de heterocigosidad demostraron que la presencia de pérdida alélica en LEZM (40%) es mayor que la observada en otros síndromes linfoproliferativos de células-B. El microsatélite más frecuentemente delecionado fue D7S487 (Mateo M, Mollejo M, Villuendas R, Algara P, Sánchez-Beato M, Martínez P, Piris MA: “7q32-7q33 allelic loss is a frequent finding in splenic marginal zone lymphoma”, *Am J Pathol* 1999, 154: 1583-1589). Los estudios de Hibridación Genómica Comparada (CGH) realizados por el grupo de los inventores confirmaron que hay distintas regiones implicadas en estos linfomas, tales como 5q, 7q 9q, 12q y 20q (Hernández J.M., García J.L., Gutiérrez N.C., Mollejo M., Martínez-Climent J.A., Flores T., González M.B., Piris M.A., & San Miguel J.F.: “Novel Genetics Imbalances in B-Cell Splenic Marginal Lymphomas Revealed by Comparative Genomic Hybridization and Cytogenetics”, *American Journal of Pathology*, Vol. 185, No 5, May 2005) encontrando, además, que existe una correlación entre las pérdidas de material genómico y el acortamiento del período de supervivencia. Como suele suceder con las pérdidas de material genómico implicadas en procesos tumorales, las deleciones mencionadas se presentan sólo en uno de los cromosomas, lo que parece estar relacionado con el hecho de que la pérdida del mismo fragmento en ambas copias del cromosoma hace inviables a las células.

La región 7q31-q36 en particular corresponde a una zona que parece estar, estrechamente relacionada con LEZM y en la que se encuentra a menudo deleciones en los pacientes aquejados de esta enfermedad. Se trata, sin embargo, de una zona muy amplia del genoma, en la que no se han encontrado genes candidatos (Algara P, Mateo MS, Sánchez-Beato M, *et al.*: “Deletion of the IgV(H) somatic mutations in splenic marginal zone lymphoma defines a group of unmutated cases with frequent 7q deletion and adverse clinical course”, *Blood* 2002, 99: 1299-1304; Ocio EM, Hernández JM, Mateo G, Sánchez ML, González MB, Vidriales B, Gutiérrez NC, Orfao A, San Miguel JF: “Immunophenotypic and Cytogenetic Comparison of Waldeström’s Macroglobulinemia with Splenic Marginal Zone Lymphoma”, *Clinical Lymphoma* 2005, 4: 241-245). Además, la técnica utilizada más habitualmente para realizar los estudios de los cambios producidos en los cromosomas asociados con LEZM ha sido la hibridación genómica comparada (CGH, de sus siglas en inglés *Comparative Genomic Hybridization*) (Kallioniemi Anne, Olli-P. Kallioniemi, Damiar Sudar *et al.*, “Comparative Genomic Hybridization for Molecular Cytogenetic Analysis of Solid Tumors”, *Science* 258: 818-821 (1992)), metodología que permite la determinación de ganancias y pérdidas en el número de copias entre dos muestras de ADN, mediante una hibridación competitiva de ADN normal y del ADN tumoral en estudio (cada uno de ellos marcados con un fluorocromo diferente) sobre metafases de individuos normales, evaluando después si existen zonas en las que se produzca un aumento de la señal correspondiente al fluorocromo con el que se haya marcado el ADN tumoral (lo que se considera indicativo de ganancias) o disminuciones de dicha señal, aumentando la señal correspondiente al otro fluorocromo (lo que se considera indicativo de pérdidas). A pesar de haber sido de gran utilidad en el conocimiento de las alteraciones genómicas en los procesos tumorales y en especial en los linfomas, (para cuyo conocimiento, clasificación y pronóstico ha sido de gran valor), se trata de una técnica que tiene una capacidad de resolución limitada: por un parte, los cambios que impliquen deleciones de segmentos inferiores a 7 megabases no son detectables (Bentz M, Plesch A, Stilgenbauer S, Dohner H, Lichter P: “Minimal sizes of deletions detected by comparative genomic hybridization”, *Genes Chromosomes Cancer* 1998; 21:172-175); por otra parte, la determinación de los puntos exactos del cromosoma entre los cuales se inserta la amplificación o que suponen los límites de la deleción es difícil, por lo que la amplitud del fragmento del cromosoma 7 cuya deleción se produce en la zona de 7q31-q33 ha sido difícil de precisar, ya que la región mínima delecionada es demasiado extensa como para poder ser analizada con una sonda de hibridación “*in situ*”, lo que provocó que, inicialmente, se considerara que su extensión era superior a la que realmente tiene y que sus límites podrían encontrarse en regiones más próximas al centrómero (Hernández J.M., García J.L., Gutiérrez N.C., Mollejo M., Martínez-Climent J.A., Flores T., González M.B., Piris M.A., & San Miguel J.F.: “Novel Genetics Imbalances in B-Cell Splenic Marginal Lymphomas Revealed by Comparative Genomic Hybridization and Cytogenetics”, *American Journal of Pathology*, Vol. 185, No 5, May 2005). Posteriormente, los estudios de FISH (hibridación *in situ* fluorescente, del inglés *fluorescent in situ hybridization*) combinados con los de pérdida de heterocigosidad (LOH) han situado esta alteración en la banda 7q31 (Sole F, Woessner S, Florensa L, Espinet B, Mollejo M, Martín P, Piris MA: “Frequent involvement of chromosomes 1, 3, 7 y 8 in splenic marginal B-cell lymphoma”, *Br J Haematol* 1997; 98: 446-449; Algara P, Mateo MS, Sánchez-Beato M, *et al.*: “Deletion of the IgV(H) somatic mutations in splenic marginal zone lymphoma defines a group of unmutated cases with frequent 7q deletion and adverse clinical course”, *Blood* 2002, 99: 1299-1304; Sole F, Salido M, Espinet B, García JL, Martínez-Climent JA, Granada I, Hernández JM, Benet I, Piris MA, Mollejo M, Martínez P, Vallespi T, Domingo A, Serrano S, Woessner S, Florensa L: “Splenic Marginal Zone B-Cell Lymphomas: two cytogenetics subtypes, one with gain of 3q and the other with loss of 7q”, *Haematologica* 2001, 86: 71-77; Oscier DG, Gardiner A, Mould S: “Structural abnormalities of chromosome 7q in chronic lymphoproliferative disorders”, *Cancer Genet Cytogenet* 1996, 92: 24-27; Hernández JM, Schoenmakers EF, Dal Clin P, Michaux L, Van de Ven WJ, Van de Berghe H: “Molecular delineation of the commonly deleted segment in mature B-cell lymphoid neoplasias with deletion of 7q”, *Genes Chromosomes Cancer* 1997; 18: 147-150), aunque esta región no es la única implicada dentro de este cromosoma, ya que también se han descrito casos excepcionales de LEZM con reordenamiento del gen CDK6 situado en la banda 7q21 (Corcoran MM, Mould SJ, Orchard JA, Ibbotson RE, Chapman RM, Boright AP, Platt C, Tsui LC, Scherer SW, Oscier DG:

“Disregulation of cyclin dependent kinase 6 expression in splenic marginal zone lymphoma through , chromosome 7q traslocations”, *Oncogene* 1999, 18: 6271-6277). Sin embargo, la prevalencia de reordenamientos CDK6 en los LEZM es anecdótica dado que sólo se han descrito en casos aislados de enfermos con este linfoma.

5 Las pérdidas del brazo largo del cromosoma 7 son, por lo tanto, una de las alteraciones que se han detectado hasta ahora en los estudios realizados sobre LEZM. Sin embargo, todos los estudios relativos a esta región se han centrado en un número escaso de enfermos, lo que ha impedido determinar si la frecuencia de aparición de cada una de esas alteraciones era o no significativa para ser de utilidad en el diagnóstico de LEZM y ha hecho difícil acotar la región perdida en cada caso de manera suficiente como para tener seguridad de detectarla utilizando sondas de tamaño  
10 adecuado como para ser utilizadas en las técnicas habituales que se manejan para el diagnóstico clínico. Esta situación ha motivado que, a diferencia del resto de linfomas para los que se dispone de marcadores moleculares útiles para su diagnóstico, en los LEZM se carezca de sondas que puedan usarse con fines diagnósticos.

15 Por lo tanto, aunque se tenía información sobre distintas alteraciones cromosómicas que aparecían con frecuencia asociadas a LEZM, no se había encontrado todavía ninguna alteración asociada con LEZM que fuera característica de este proceso tumoral y que, además, se encontrara con suficiente frecuencia entre la población afectada de LEZM como para garantizar el diagnóstico de una proporción significativa de pacientes mediante su detección. Además, la utilización de la hibridación genómica comparada como técnica habitual para la identificación de ganancias y pérdidas de secuencias de ADN en los tumores con bajo índice proliferativo no permite precisar la extensión de las regiones ganadas o pérdidas, lo que ha dificultado el diseño de sondas específicas, diseñadas para determinar la presencia de alteraciones asociadas con LEZM y su diferenciación de otros LNH, que pudieran integrarse en sistemas diseñados para el diagnóstico diferencial de enfermedades tumorales, como pueden ser los denominados “chips” o “arrays” de diagnóstico, conjuntos de sondas dispuestas de forma ordenada sobre un soporte físico que permiten el diagnóstico diferencial de enfermedades a partir de la señal generada tras ensayos de hibridación entre dichas sondas y el ADN  
25 del individuo en estudio.

Por ello, el grupo de los inventores decidió recurrir a una técnica diferente a la CGH, la de los arrays genómicos, también llamados CGH-arrays o arrays de CGH, que fueron usados inicialmente para identificar alteraciones en regiones específicas de cromosomas asociados con enfermedades. El array de CGH es un método para identificar alteraciones en el número de copias, pero no está diseñado para detectar traslocaciones recíprocas (Andersen CL, Gruszka-Westwood AM, Atkinson S, Matutes E, Catovsky D, Pedersen RK, Pedersen BB, Pulczynski S, Hokland P, Jacobsen E, Koch J: “Recurrent genomic imbalances in B-cell splenic marginal-zone lymphoma revealed by comparative genomic hybridization”, *Cancer Genet Cytogenet* 2005; 156: 122-128; Jonathan J. Davies, Ian M. Wilson & Wan L. Lam: “Array CGH technologies and their applications to cancer genomes”, *Chromosome Research* 13: 237-248, 2005). Se trata de una técnica que combina la tecnología de los microarrays con la CGH, en la que la hibridación se realiza reemplazando las metafases de los cromosomas por segmentos de ADN mapeados, cuyo conjunto representa todos los cromosomas o brazos de cromosomas, divididos en fragmentos clonados en cromosomas artificiales de levadura (YAC), cromosomas artificiales de bacteria (BAC), cromosomas artificiales basados en el genoma del bacteriófago P1 (PAC), cósmidos u otros vectores de clonación, y que se disponen ordenados en un soporte sólido, generalmente un portaobjetos. De esta manera, aumenta el nivel de resolución, que deja de ser cromosómico (distancias medidas en megabases) pasando en el caso de los BAC a poder ser medido en kilobases. Las ventajas de esta metodología son la mayor resolución y la posibilidad de realizar un mapeo directo del clon o clones que se encuentran involucrados en el cambio genómico.  
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45 Los análisis de alta resolución de genomas tumorales son necesarios para descubrir los genes involucrados en las enfermedades. El array de CGH tiene la capacidad de redefinir alteraciones de regiones consenso conocidas. Esto es importante, porque permite investigar de cerca las áreas pequeñas del genoma. Los análisis de alta resolución tienen una gran probabilidad de detectar nuevas alteraciones que pueden ser importantes para la enfermedad, pero que pueden no ser detectadas por técnicas de baja resolución. Sin embargo, el mayor obstáculo que limita la posibilidad del establecimiento de esta técnica para la investigación en laboratorios, es la incapacidad de manejar las cantidades enormes de datos que se generan. Por esta misma razón, los arrays de alta resolución no pueden ser utilizados todavía para uso clínico. Sin embargo, las regiones descubiertas con estos arrays sí que pueden usarse, en combinación con técnicas que puedan utilizarse de forma rutinaria en laboratorios clínicos, para diagnosticar enfermedades, como pueden ser los arrays pequeños de diagnóstico, compuesto por un número reducido de sondas que, por lo general, permite discriminar entre enfermedades relacionadas, lo que permite que el poder del array de CGH sea combinado con su fácil uso (Jonathan J. Davies, Ian M. Wilson & Wan L. Lam: “Array CGH technologies and their applications to cancer genomes”, *Chromosome Research* 13: 237-248, 2005). Sin embargo, tal como se ha comentado, en el caso de LEZM no se había definido todavía una sonda que permitiera identificar la ausencia o presencia de una alteración no asociada con ninguna otra enfermedad, pero que sí se detectara en un número significativo de pacientes de LEZM  
50 y que posibilitara el diagnóstico de esta enfermedad mediante técnicas que puedan usarse en laboratorios clínicos de forma rutinaria. La presente invención proporciona una sonda que cumple dichas características.

### Breve descripción de la invención

65 Un aspecto de la invención se refiere al uso como sonda de la secuencia correspondiente al inserto de ADN humano del BAC RP11-44M6 (SEQ ID NO:1) para el diagnóstico del linfoma esplénico de la zona marginal (LEZM). En una realización de este aspecto de la invención, el fragmento se utiliza como sonda en combinación con al menos una sonda adicional. En un caso particular de esta realización de la invención, al menos una sonda adicional corresponde a un



fragmento cuya secuencia está comprendida en la de la región 7q32-7q33. En este caso, se prefiere especialmente que al menos una sonda adicional se seleccione de entre los insertos de los clones RP11-36B6, RP11-329I5 y RP11-193I17, es decir, de entre las secuencias representadas por SEQ ID NO:2, SEQ ID NO:3 y SEQ ID NO:4, respectivamente.

5 Otro aspecto de la invención se refiere a un método de diagnóstico *in vitro* de LEZM en un ser humano que comprende las etapas de:

- a) obtener una muestra de tejido o de células separadas, procedentes de un tejido infiltrado con células de linfoma, fijadas sobre un portaobjetos y listas para llevar a cabo una hibridación *in situ* sobre ellas;
- 10 b) realizar una hibridación *in situ* sobre el portaobjetos, en presencia de ADN humano competidor, utilizando como sonda ADN correspondiente a la secuencia del inserto del BAC RP11-44M6 acoplado a una sustancia que permite su detección y utilizando opcionalmente como una segunda sonda cuya secuencia está comprendida en la región 7q32-7q33 del cromosoma 7 humano también acoplada a una sustancia que permite su detección, sustancia que es distinta de la sustancia a la que está acoplada la primera sonda y que da lugar a una señal diferenciable de la correspondiente a dicha sustancia;
- 15 c) identificar el tumor del que se tomó la muestra como linfoma esplénico de la zona marginal si en cada célula se observa una única señal correspondiente a la sustancia acoplada a la primera sonda y/o, en el caso de haberse utilizado también la segunda sonda opcional, una única señal correspondiente a la sustancia acoplada a la segunda sonda, e identificar el tumor como clasificable en otro tipo de cáncer si en cada célula se observan dos señales correspondientes a la sustancia acoplada a la primera sonda que, en el caso de haberse utilizado también la segunda sonda opcional, deberán ir acompañadas por dos señales correspondientes a la segunda sonda.
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En una realización preferida del método diagnóstico de la invención, la segunda sonda opcional se elige entre el ADN correspondiente al inserto de los clones RP11-36B6 (SEQ ID NO:2), RP11-329I5 (SEQ ID NO:3) y RP11-193I17 (SEQ ID NO:4). Independientemente de las sondas específicas elegidas, se prefiere muy especialmente que tanto la primera como la segunda sonda opcional estén marcadas con un marcador fluorescente, para que la hibridación *in situ* sea del tipo conocido como FISH (hibridación *in situ* fluorescente).

En cuanto a la muestra de partida sobre la cual se realiza la hibridación *in situ*, son realizaciones posibles del método diagnóstico de la invención tanto aquellas en las que el material fijado sobre el portaobjetos es una sección de tejido tomada de un tumor "sólido" (por ejemplo, de bazo o ganglios linfáticos), previamente incluida en parafina, como aquellas procedentes en las que el material fijado en el portaobjetos son células tratadas mediante las técnicas conocidas como "citogenética convencional", es decir, células separadas, procedentes de una suspensión celular obtenida tras el cultivo de una muestra de tejido infiltrado (sólido o líquido) extraído del paciente, suspensión compuesta por células cuya división, opcionalmente, ha sido parada en metafase (aunque son válidos también los núcleos en interfase) y que se fijan al portaobjetos mediante fijadores habituales en las técnicas citogenéticas tales como mezclas de metanol:acético o etanol.

Un aspecto más de la invención se refiere a un kit de diagnóstico que comprende una sonda que corresponde a la secuencia del inserto de ADN del cromosoma 7 humano presente en el BAC RP11-44M6 (SEQ ID NO:1) acoplada a una sustancia que permite su detección y, opcionalmente, en el mismo recipiente o en un recipiente separado, una segunda sonda, acoplada a una segunda sustancia que permite su detección y su diferenciación de la primera sustancia. Cuando está presente, se prefiere que la segunda sonda corresponda a un fragmento de la región 7q32-7q33 humana. Se prefiere especialmente que la secuencia de la segunda sonda adicional corresponda a la de un BAC que contenga un inserto correspondiente a dicha región que se selecciona entre las secuencia de los insertos de los clones RP11-36B6 (SEQ ID NO:2), RP11-329I5 (SEQ ID NO:3) y RP11-193I17 (SEQ ID NO:4).

### 50 Breve descripción de las figuras

La Figura 1 se reproduce una tabla en la que se representan los resultados obtenidos con el ADN procedente de clones de bacterias que contienen BACs con insertos correspondientes a regiones del cromosoma 7 humano en experimentos de CGH-arrays en los que dichos insertos estaban depositados de forma ordenada sobre un portaobjetos y eran hibridados con el ADN de individuos que padecían LEZM compitiendo con el ADN de un control sano. En la primera columna de la tabla aparece un código de identificación para cada uno de los clones analizados, que están ordenados de acuerdo con la posición del cromosoma 7 a la que corresponden, es decir, el primer clon sería el que correspondería a la región más próxima al extremo del brazo corto del cromosoma, mientras que el último clon correspondería a la región más próxima al extremo del brazo largo del cromosoma; los datos del clon al que corresponde dicho código de identificación (nombre del clon, código de identificación en el array, código de acceso en EMBL, región del cromosoma 7 a la que se une y los marcadores o dominios relacionados con dicha región del cromosoma) pueden encontrarse en la Tabla 1 que aparece en el Ejemplo 1. Cada fila, por tanto, representa un clon, mientras que cada columna, a partir de la segunda, corresponde a un paciente diferente. El sombreado de cada celda representa el resultado obtenido para cada clon tras la hibridación del array con el ADN del paciente correspondiente: las celdas sin ningún sombreado representan clones para los cuales no se detectaron cambios; las celdas sombreadas en tono oscuro continuo representan clones perdidos; las celdas con sombreado interrumpido por rayas verticales blancas representan clones ganados; las celdas con sombreado interrumpido por rayas inclinadas más oscuras representan clones en los que la hibridación no

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fue correcta o en los que los resultados correspondientes a cada una de las tres muestras del clon depositadas sobre el array resultaron discordantes. La Fig. 1a corresponde a los resultados obtenidos con los pacientes 1 a 34 y los clones identificados como C1 a C97 (CTB-164D18 a RP11-575G 1); la Fig. 1b corresponde a los resultados obtenidos con los pacientes 35 a 68 y los mismos clones, C1 a C97; la Fig. 1c corresponde a los resultados obtenidos con los pacientes 1 a 34 y los clones identificados como C98 a C193 (RP4-784G16 a CTB-3K23); la Fig. 1d corresponde a los resultados obtenidos con los pacientes 35 a 68 y los clones C98 a C193 (RP4-784G16 a CTB-3K23). Las celdas identificativas de los clones preferidos para ser utilizados como sondas, C120 (RP11-44M6) y C154 (RP11-36B6), C155 (RP11-329I5) y C156 (RP11-193I17) aparecen con caracteres blancos sobre fondo oscuro.

La Figura 2 corresponde a un ensayo de hibridación *in situ* realizado utilizando el método diagnóstico de la invención, sobre una muestra tomada del enfermo 21, utilizando como sondas tanto el inserto del clon RP11-4M6, marcado con el marcador "Spectrum green" (que da lugar a una señal de color verde), como el inserto del clon RP11-193I17 marcado con el marcador "Spectrum Orange" (que da lugar a una señal de color rojo/anaranjado). En ambas células se observa que sólo existe una señal de color verde por célula (puntos señalados con flechas horizontales, de color blanco), observándose dos señales de color rojo en cada una de las células (puntos señalados con flechas verticales, con relleno negro), lo que indica que existe una pérdida de material genómico en la región 7q22, no detectándose pérdida de material genómico en la región de 7q32-7q33.

### Descripción detallada de la invención

Tal como se ha comentado, en la presente invención se describe la asociación entre los LEZM y las deleciones en una región del cromosoma 7, la región 7q22.1-7q22.2, región que, como tal, no había sido asociada hasta ahora con esta enfermedad ni con ningún otro tipo de cáncer y cuyo tamaño, aproximadamente 1.600 kb, claramente inferior al tamaño mínimo detectable en ensayos de hibridación genómica comparada (7 Mb), había impedido que los estudios basados en dicha técnica de la CGH pudieran establecer asociaciones entre los LEZM y las pérdidas de fragmentos cromosómicos cuyos límites estén comprendidos en dicha región. En los experimentos que se describen más adelante en la presente memoria se demuestra que la utilización como sonda de un fragmento comprendido en dicha región, correspondiente al inserto del BAC del clon denominado RP11-44M6 (inserto que representa un fragmento de la banda 7q22.11 del genoma humano y que tiene la secuencia que se representa en SEQ ID NO:1), permite la identificación de la existencia de deleciones en la región indicada en más del 60% (63,24%) de los pacientes de LEZM incluidos en el ensayo. De esta manera, la utilización como sonda de dicho fragmento permitiría el diagnóstico de más de la mitad de los enfermos de LEZM, aportando así una solución a la cuestión pendiente de proporcionar a los médicos tanto un marcador molecular con el que diagnosticar un número significativo de enfermos de LEZM como una sonda con la que detectar la presencia o ausencia de la deleción propuesta como tal marcador molecular mediante técnicas utilizables en la práctica clínica, facilitándoles así la aplicación del correcto diagnóstico a los pacientes aquejados de linfomas.

Además, el mismo estudio permitió ratificar la asociación entre LEZM y pérdidas de material genómico en una región más distal, que anteriormente ya había sido asociada con esta enfermedad: 7q32-7q33. En el 31% de los enfermos analizados se apreciaron pérdidas en esta región detectables por al menos dos sondas contiguas. Cuando se comparan los enfermos en los que se detectan pérdidas en la región 7q22.1-7q22.2, se observa que en un 44% del total de enfermos las pérdidas en la región 7q22.1-7q22.2 se presentan de forma aislada, sin que existan pérdidas detectables en la región 7q32-7q33; esto es así en casi el 70% de los pacientes con pérdidas en dicha región. En cuanto a esta segunda región, 7q32-7q33, en el 9% de los enfermos las pérdidas en esta región se presentan de forma aislada, sin que se observen pérdidas en la región 7q22.1-7q22.2. Por lo tanto, no sólo son dos y no una sola las regiones en las que se observan deleciones asociadas a LEZM en el brazo largo del cromosoma 7 sino que, además, parece existir una cierta tendencia a que la existencia de una excluya la presencia de la otra. Sin embargo, un ensayo en el que se utilizaran como sondas ambos fragmentos permitiría detectar el 72% de los enfermos con LEZM, enfermos que, además, se habrían identificado por presentar pérdidas en el material genético lo que, como se ha comentado antes, parece tener una relación con un peor pronóstico en cuanto al período de supervivencia.

Por ello, la presente invención proporciona el uso como sonda de la secuencia correspondiente al inserto de ADN humano del BAC RP11-44M6 (SEQ ID NO:1) para el diagnóstico del linfoma esplénico de la zona marginal (LEZM). Dicha sonda puede utilizarse sola o, preferiblemente, en combinación con al menos una sonda adicional. Como sonda adicional, se tiene preferencia por aquellas cuya secuencia está comprendida en la región 7q32-7q33, especialmente por aquellas cuya secuencia se corresponda con las del inserto de un BAC seleccionado de entre RP11-36B6 (SEQ ID NO:2), RP11-329I5 (SEQ ID NO:3) y RP11-193I17 (SEQ ID NO:4).

Tal como se utiliza en la presente memoria, el término "sonda" hace referencia a una secuencia de ADN que se utiliza para detectar y/o cuantificar secuencias complementarias presentes en una muestra mediante la realización de un ensayo de hibridación y la posterior detección de la existencia o no de moléculas unidas a dicha sonda y, opcionalmente, la determinación de la cantidad de dichas moléculas unidas a la sonda. Por tanto, el concepto de "sonda" lleva implícito el marcaje de una de las secuencias de ADN con una sustancia que permite su detección; sin embargo, tal como se utiliza en la invención, no es necesariamente sobre la secuencia considerada como "sonda" sobre la cual debe efectuarse el marcaje, sino que puede ser la secuencia complementaria que se desea detectar y o cuantificar la que se una a la sustancia que permite su detección, procediendo a efectuar dicha detección y/o cuantificación tras el ensayo de hibridación. Además, dependiendo de la técnica concreta de la que forme parte el ensayo de hibridación o del procedimiento de marcaje, en el momento de realizarse la hibridación entre la sonda y la muestra de ADN que

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se desea analizar, la secuencia que se utiliza como sonda puede constituir una molécula como tal o puede utilizarse en forma de varias moléculas de ADN diferentes, la secuencia de cada una de las cuales es un fragmento de la secuencia que se desea utilizar como sonda. Así, por ejemplo, en el caso de que la sonda se marque mediante el procedimiento de “traslado de la muesca” (“*nick translation*”), la sonda marcada que se utiliza en el ensayo de hibridación no consiste en moléculas de ADN todas ellas de una misma secuencia, sino en moléculas de unos 300 pb de longitud, cuya secuencia está comprendida en la secuencia que se desea utilizar como sonda, en las que ha quedado incorporada la sustancia que se va a utilizar como marcador para la detección y/o cuantificación.

Por todo ello, están comprendidos dentro del alcance de la invención tanto el uso en el que la sonda o sondas utilizadas para el diagnóstico del LEZM se encuentren depositadas sobre un soporte sólido formando parte del array como uso en el que son células de seres humanos las que están depositadas sobre un soporte sólido y se utilizan en un ensayo de hibridación con una composición líquida que comprende la sonda o sondas utilizadas para el diagnóstico de LEZM.

Otro aspecto de la invención lo constituyen los métodos para el diagnóstico de LEZM basados en el uso como sonda de la secuencia anteriormente citada, la correspondiente al inserto del BAC RP11-44M6 (SEQ ID NO:1), sonda que se hace hibridar sobre muestras de células procedentes de tejidos infiltrados o sobre muestras de los propios tejidos y que se utiliza bien sola u, opcionalmente, junto con al menos una segunda sonda opcional. La segunda sonda opcional, preferiblemente, se elige entre aquellas cuya secuencia se corresponde con la de fragmentos comprendidos en la región 7q32-7q33 del cromosoma 7 humano. Se tiene especial preferencia para la elección de la segunda sonda opcional por aquellas sondas cuya secuencia se corresponda con las del inserto de un BAC seleccionado de entre RP11-36B6 (SEQ ID NO:2), RP11-329I5 (SEQ ID NO:3) y RP11-193I17 (SEQ ID NO:4). Para poder efectuar el método de diagnóstico de la invención, es necesario que cada una de las sondas utilizadas esté marcada con una sustancia diferente que permita su detección, de manera que pueda observarse si, en cada célula, pueden detectarse dos señales por cada una de las sondas utilizadas o sólo una.

El método diagnóstico de la invención, se basa en el hecho de que cada célula humana posee dos copias del cromosoma 7 (al igual que de cualquiera de los restantes cromosomas no sexuales) por lo que, en ausencia de deleciones en la región 7q22.1 detectables por la sonda correspondiente al inserto del BAC RP11-44M6, deberían observarse dos señales correspondientes a la sustancia acoplada a dicha sonda, una por cada uno de los cromosomas 7 de la célula. Igual argumento puede utilizarse para la sonda opcional elegida por lo que, si se utiliza adicionalmente esa segunda sonda, deberán observarse cuatro señales en cada una de las células provenientes de tumores no identificables como LEZM por el método de la invención, dos por cada una de las sondas. Puesto que las deleciones en el cromosoma 7 se producen sólo en uno de los cromosomas de la pareja, deberá observarse siempre al menos una señal por cada una de las sondas utilizadas, sirviendo dicha señal como control positivo del proceso, no siendo necesario realizar simultáneamente la hibridación con células tomadas de un individuo sano o, al menos, del que se sepa que no padece LEZM, aunque el método de la invención es compatible con la inclusión de dicho control positivo. Así, están comprendidos también dentro del alcance de la invención tanto los métodos en los que la hibridación se lleva a cabo con al menos tres sondas, siendo la tercera sonda opcional una sonda complementaria a una zona del genoma que no haya sido asociada con LEZM. Una posible realización del método de la invención en la que se incluyera un control positivo consistiría en realizar la hibridación incluyendo una tercera sonda, complementaria a la región del centrómero del cromosoma 7, como método de control numérico y de control de calidad.

En cuanto a las sustancias que permiten la detección de las sondas unidas al ADN de las células, se prefieren aquellas sustancias que den lugar a la emisión de fluorescencia, bien porque se trate de sustancias que sean ellas mismas fluoróforos *per se* (Cy3, Cy5, rodamina, fluoresceína) o bien porque la presencia de las sustancias unidas a las sondas se detecta gracias a la interacción con las mismas de moléculas que son detectables *per se* mediante la medida de la fluorescencia que emiten o porque catalizan la transformación de una sustancia en otra fácilmente medible. Este último es el caso, por ejemplo, de la biotina y la digoxigenina, que pueden ser unidas a las sondas antes de la hibridación y cuya posterior presencia puede cuantificarse, por ejemplo, gracias a anticuerpos dirigidos contra las mismas que están unidos a una enzima que cataliza la transformación de un reactivo incoloro en otra molécula distinta coloreada. Por simplicidad, se prefiere que las sustancias unidas a las sondas sean fluoróforos *per se* en las condiciones adecuadas, cada uno de los cuales emita en una longitud de onda diferente, claramente diferenciable. Así, por ejemplo, a una de las sondas podría ser “marcada” (es decir, unir a ella una sustancia que permita su detección) con rodamina, que da lugar a la emisión de luz anaranjada, mientras la otra podría ser marcada con fluoresceína o su derivado isotiocianato (FITC), que dan lugar a la emisión de luz verde. Otro par posible para obtener señales de doble color perfectamente distinguibles es el par Cy5 (rojo)/Cy3 (verde). También puede utilizarse como marcador alguna de los reactivos para marcaje fluorescente de la línea Vysis de laboratorios Abbot, como pueden ser Spectrum Green (verde), Spectrum Orange (anaranjado) o Spectrum Red (rojo). En aquellos casos en los que se utilice una tercera sonda, como puede ser, por ejemplo, aquellos en los que la tercera sonda se incluya como control positivo de la hibridación, la terna de sustancias fluorescentes utilizadas para marcar cada una de las sondas podrían ser, por ejemplo, FITC (fluorescencia verde: excitación a 494/500 nm, emisión a 517/525 nm), TexRed/Cy3.5 (fluorescencia roja: excitación a 590/581 nm, emisión a 612/596 nm) y DEAC (dietilaminocurmarina, que da lugar a fluorescencia azul turquesa: excitación a 426 nm, emisión a 480 nm).

En cuanto a la muestra de tejido o de células separadas sobre la cual se lleva a cabo la hibridación, deben proceder de un tejido infiltrado por un linfoma que se sospeche que puede ser un linfoma esplénico de la zona marginal. Tal como se comentó previamente, los tumores que se clasifican como linfoma esplénico de la zona marginal (LEZM) pueden

afectar a cualquier órgano, aunque los más comunes son el bazo y los ganglios linfáticos. Cuando se desea clasificar un tumor que afecte a uno de estos órganos, la realización del método de la invención implica que previamente se ha tomado una muestra del tumor del que se desee saber si puede o no identificarse como un linfoma esplénico de la zona marginal, muestra que se habrá tomado mediante un método adecuado, conocido por el experto en la técnica, en las condiciones más asépticas posibles. En el caso concreto de bazo y ganglios linfáticos, no es posible tomar biopsias de los mismos; es necesario proceder a la extirpación total de dichos órganos, bazo (esplenectomía) o ganglios linfáticos (linfadenectomía), cuando están afectados por tumores. El método de la invención se lleva a cabo sobre muestras tomadas de los tumores extirpados, para identificar el tipo de linfoma de que se trata y poder decidir el tratamiento adecuado que debe administrársele al paciente. En el caso de las muestras tomadas de bazo o ganglios linfáticos afectados por tumores, es posible realizar el ensayo de hibridación *in situ* sobre secciones de tejidos previamente incluidas en parafina. La inclusión en parafina de muestras de tejidos y la fijación de las mismas a portaobjetos es una metodología ampliamente conocida entre los expertos en la técnica y su uso en ensayos de hibridación *in situ* para el análisis de muestras de linfomas es también conocido, especialmente para FISH (Remstein ED, Kurtin PJ, Buno I, Bailey RJ, Proffitt J, Wyatt WA *et al.* Diagnostic utility of fluorescence *in situ* hybridization in mantle-cell lymphoma. *Br J Haematol* 2000; **110**: 856-62; Li JY, Gaillard F, Moreau A, Harousseau JL, Laboisie C, Milpied N *et al.* Detection of translocation t(11;14)(q13;q32) in mantle cell lymphoma by fluorescence *in situ* hybridization. *Am J Pathol* 1999; **154**: 1449-52). Las secciones incluidas en parafina, pueden ser hibridadas directamente siempre que sus cortes sean lo suficientemente finos como para que no exista una superposición de capas celulares. En esas condiciones serían muestras de tejidos listas para llevar a cabo una hibridación *in situ* sobre ellas.

Puede encontrarse información complementaria sobre los parámetros que influyen sobre la calidad de los ensayos FISH llevados a cabo sobre estas muestras y las posibles opciones para llevar a cabo la desparafinación, la digestión con proteasas y la desnaturalización en la página web de la Universidad de Yale, concretamente en el apartado de la guía sobre FISH dedicado a la realización de FISH sobre tejidos incluidos en parafina (<http://info.med.yale.edu/genetics/ward/tavi/fi20.html>). Aunque en dicha página se indica que el espesor de las secciones más adecuado para hibridaciones de ADN sería de 10  $\mu\text{m}$ , los inventores consideran preferible la utilización de secciones de 2-3  $\mu\text{m}$  para llevar a cabo el método de diagnóstico de la invención.

Además de proceder al análisis de los tumores de bazo o ganglios linfático partiendo de secciones de tejido fijadas sobre portaobjetos, es posible también analizarlos mediante el método diagnóstico de la invención sobre portaobjetos en los que están fijadas células separadas, procedentes de una muestra tomada del tumor, disgregada y tratada según las técnicas citogenéticas convencionales, que básicamente consisten en el cultivo de las células para que se dividan y poder para su mitosis en metafase, la toma de muestras de la suspensión celular resultante, su depósito sobre un portaobjetos y la fijación de las células a dicho soporte mediante fijadores habituales en las técnicas citogenéticas tales como mezclas de metanol:acético o etanol. Las preparaciones sobre portaobjetos obtenidas tras la aplicación de esta técnica se consideran muestras listas para llevar a cabo una hibridación *in situ* sobre ellas en lo que se refiere a muestras de células separadas. Un protocolo pormenorizado de aplicación de este tipo de técnicas puede encontrarse en el Ejemplo 2 de la presente memoria, en el que las células, cuya mitosis había sido previamente interrumpida mediante el uso de colcemida, se fijaron al portaobjetos utilizando metanol:ácido acético 3:1. También puede encontrarse información sobre esta técnica y los factores que determinan que las células fijadas sobre un portaobjetos resulten adecuadas para hibridaciones *in situ* de tipo FISH en la página web de la Universidad de Yale, concretamente en los distintos apartados que componen la “guía FISH” (<http://info.med.yale.edu/genetics/ward/tavi/FISHguide.html>). Se prefiere la utilización de portaobjetos con células que han sido tratadas mediante la técnica citogenética convencional cuando las muestras extraídas del paciente que se analicen no consistan en “tumores sólidos”, sino que se trate de “muestras líquidas” o “suspensiones de células”, como es el caso de los aspirados de médula ósea o las muestras de sangre.

No es infrecuente que los tumores ganglionares infiltren la médula ósea, de donde pueden también pasar a la sangre. Estas manifestaciones de los linfomas a nivel periférico se conocen como “linfomas leucemizados”. Debido a la existencia de este tipo de linfomas, además de partir de muestras de tumores “sólidos” (linfomas), el método diagnóstico de la invención puede llevarse a cabo partiendo de muestras de aspirados de médula ósea o de muestras de sangre tomadas al individuo. En el caso de las muestras de sangre, antes de utilizarla hay que comprobar previamente que realmente existen células de linfoma circulando. La manera más sencilla de realizar esta comprobación es tomar una muestra de sangre del enfermo, teñirla y observar si hay linfocitos de morfología anómala, generalmente de aspecto “velludo” o “piloso”, lo que es un indicio de la presencia de un linfoma en el individuo. La confirmación de que estas células proceden de un linfoma debería ser un estudio fenotípico, en el que se compruebe que los linfocitos expresan antígenos específicos tumorales, realizando, por ejemplo, un análisis de citometría de flujo en el que se compruebe cuántos linfocitos son clonales. Comprobada la existencia de linfocitos clonales en la muestra de sangre, la muestra de sangre total puede ser utilizada directamente para llevar a cabo el método de la invención, sembrándolas y cultivándolas. Otra posibilidad es centrifugar previamente la sangre total sometiéndola a un gradiente de densidad, para separar las células mononucleares antes de sembrarlas y cultivarlas, pero se prefiere no recurrir a esta segunda opción, porque los cultivos celulares deben realizarse con muestras recogidas y procesadas de la manera más aséptica posible y cada etapa de procesamiento de la muestra supone un riesgo de contaminación.

En cuanto a las muestras de aspirado de médula ósea, las mismas constituyen una especie de “tejido líquido”, similar a la sangre, por lo que su manejo es similar al de las muestras de sangre.

## Ejemplos

### Ejemplo 1

#### 5 *Estudio de alteraciones en el cromosoma 7 asociadas a LEZM*

Para localizar alteraciones cromosómicas no detectadas hasta ahora por las técnicas de CGH, se preparó un array en el que se dispusieron muestras del ADN humano contenido en los insertos de BACs y PACs correspondientes a distintos clones, de manera que se quedaran cubiertos los cromosomas 1, 3, 7, 8, 18 y 19 mediante segmentos de aproximadamente 300 kb espaciados 1 Mb. Este array se utilizó para estudiar las posibles alteraciones que pudieran presentarse en estos cromosomas en pacientes afectados de LEZM mediante la técnica de los CGH-arrays.

La ventaja de realizar experimentos de CGH-arrays utilizando arrays construidos con BACs es que, posteriormente, es posible utilizar los insertos de esos mismos BACs como sondas en estudios de confirmación tipo FISH, pues tiene en tamaño óptimo para ser visualizados mediante dicha técnica tras su marcaje con un fluoróforo.

#### 1.1. *Obtención del array*

##### - *ADN del array*

Para la fabricación del array se seleccionaron 3500 clones de la librería genómica **1 Mb clone set**, cedida por el Instituto **Wellcome Trust Sanger** (Cambridge, Inglaterra). Dicha librería genómica contiene BACs y PACs procedentes de otras distintas librerías genómicas, seleccionados de manera que abarquen la totalidad de los cromosomas humanos, mediante clones localizados a intervalos de aproximadamente 1 Mb a lo largo del genoma completo. Pueden obtenerse detalles sobre los clones en la base de datos [www.ensembl.org](http://www.ensembl.org).

Para el presente estudio se analizaron los cromosomas 1, 3, 7, 8, 18 y 19, construyendo un array con los clones de la librería genómica correspondientes a los cromosomas mencionados. En la Tabla 1 se indica información respecto a los clones correspondientes al cromosoma 7 utilizados en la construcción del array, incluyendo el nombre del clon, el código de identificación del mismo en el array, el código de acceso que permite consultar datos sobre el clon en la base de datos de EMBL (<http://www.ebi.ac.uk/embl/>), la banda cromosómica en la que se localiza el inserto del clon y los marcadores o dominios que flanquean el inserto (indicados separados por puntos suspensivos, ...) o que están contenidos en el mismo (indicados separados por líneas oblicuas, //). Se han omitido los clones que dan lugar a señales no sólo en el cromosoma 7, sino en más de un cromosoma, por considerar que esa característica los excluye de ser una herramienta válida para el diagnóstico. Los clones junto a cuyo nombre se ha añadido el superíndice "P" son de tipo PAC. Los clones preferidos para usar su inserto como sonda aparecen recuadrados con trazo más grueso.

(Tabla pasa a página siguiente)

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TABLA 1

*Clones del cromosoma 7 analizados*

	Nombre del clon (BAC/PAC)	Código de Identificación	Código de acceso en EMBL	Región del cromosoma	Marcadores o Dominio*	Código Fig. 1
5	CTB-164D18 <sup>P</sup>	PAC164D18		7p22.3	D7S2477	C1
10	RP11-449P15	bA449P15	AC073957	7p22.3	D7S2474...D7S2826	C2
	RP11-510K8	bA510K8	AC074389	7p22.3		C3
	RP4-607J2 <sup>P</sup>	dJ607J2	AC004840	7p22.3	D7S1532	C4
15	RP11-106E3	bA106E3	AC073462	7p22.2	D12S364	C5
	RP11-348A21	bA348A21	AC079231	7p22.2	D7S462...D7S2649	C6
	RP11-172O13	bA172O13	AC008167	7p22.1	D4S3184	C7
	RP1-42M2 <sup>P</sup>	dJ42M2	AC005995	7p22.1	D7S481	C8
20	RP4-810E6 <sup>P</sup>	dJ810E6	AC004895	7p22.1	D7S481...D7S3123	C9
	RP11-425P5	bA425P5	AC009412	7p22.1	D7S3123	C10
	RP4-733B9 <sup>P</sup>	dJ733B9	AC005532	7p21.3	D7s537E//D7S1659//D0S8242E//D7S1565	C11
25	RP11-505D17	bA505D17	AC006042	7p21.3	D7S2258//D20S1004	C12
	RP11-304A10	bA304A10	AC007164	7p21.3	D7S341...D7S1790	C13
	RP5-959C21 <sup>P</sup>	dJ959C21	AC004936	7p21.3	D7S1594//D7S2464	C14
30	RP11-352E12	bA352E12	AC006373	7p21.3	D7S2464...D7S1676	C15
	RP11-392P1	bA392P1	AC073098	7p21.3	D7S2464...D7S1676	C16
	RP5-855F16 <sup>P</sup>	dJ855F16	AC007029	7p21.3	D7S1846	C17
	RP11-502P9	bA502P9	AC073109	7p21.3	D7S2228	C18
35	RP5-1100A7 <sup>P</sup>	dJ1100A7	AC005228	7p21.3	D7S1597...D7S1589	C19
	RP11-195L14	bA195L14	AC007243	7p21.3	D7S1589...D7S2359	C20
	RP4-685A2 <sup>P</sup>	dJ685A2	AC004857	7p21.2	D7S790//D7S2766//D7S2837//D7S1992	C21
40	RP11-512E16	bA512E16	AC005039	7p21.2	D7S2174	C22
	RP11-547G15	bA547G15	AC006045	7p21.2	D7S1469//AFMa084wh5	C23
	RP11-196O16	bA196O16	AC006035	7p21.1	D7S1886	C24
45	RP11-403N12	bA403N12	AC098592	7p21.1	D7S2720	C25
	RP11-323K15	bA323K15	AC073205	7p21.1	AFMA046XB5	C26
	RP11-71F18	bA71F18	AC007091	7p21.1	D7S503//D7S1445//D7S2698	C27
	RP11-486P11	bA486P11	AC007001	7p21.1	D7S2551	C28
50	RP4-701O19 <sup>P</sup>	dJ701O19	AC007090	7p21.11	D7S2537//D7S713	C29
	RP11-211J15	bA211J15	AC078791	7p15.3	D7S1928	C30
	RP11-445O1	bA445O1	AC073102	7p15.3	D7S1895//D7S1868//D7S493	C31
	RP11-451F11	bA451F11	AC008176	7p15.3	D7S1632//D7S673	C32
55	RP11-343P21	bA343P21	AC079878	7p15.3	D7S1850	C33
	RP11-99O17	bA99O17	AC018706	7p15.2	D7S1534...D7S2449	C34
	RP5-1066K24 <sup>P</sup>	dJ1066K24	AC004540	7p15.2	D7S1991//D7S1977	C35
	RP11-232C20	bA232C20	AC011299	7p15.2	D7S2810...D7S1457	C36
60	RP4-781A18 <sup>P</sup>	dJ781A18	AC004885	7p15.1	D7S1808//D7S1535//D7S547//D7S516	C37
	RP4-596O9 <sup>P</sup>	dJ596O9	AC003074	7p15.1	D7S773//D7S2772	C38
65	RP11-550A18	bA550A18	AC007255	7p15.1	D7S1981//D7S2848//D7S1806//D7S2148E//D7S2403//D7S2496//D7S2496//D7S1971	C39
	RP11-111I9	bA111I9	AC009304	7p14.3	D7S2912...DXS7539	C40

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	Nombre del clon (BAC/PAC)	Código de Identificación	Código de acceso en EMBL	Región del cromosoma	Marcadores o Dominio*	Código Fig. 1
5	RP5-1143H19 <sup>P</sup>	dJ1143H19	AC004976	7p14.3	D7S2836//AFMB050YD1//D7S1538	C41
	RP11-463M14	bA463M14	AC006380	7p14.3	D9S1783...	C42
	RP11-175P8	bA175P8	AC018637	7p14.3	D7S722//D7S690	C43
10	RP11-89N17	bA89N17	AC008080	7p14.3	D7S1924//D7S2104E	C44
	RP11-179B11	bA179B11	AC009262	7p14.3	D7S656//D7S460//D7S3038	C45
	RP11-155G7	bA155G7	AC007327	7p14.2	D7S2251//D7S1422	C46
15	RP11-302L6	bA302L6	AC009530	7p14.1	D7S2206	C47
	RP11-121A8	bA121A8	AC006033	7p14.1	D7S1929...D7S2497	C48
	RP11-64I2	bA64I2	AC011290	7p14.1	D7S1609	C49
	RP5-1178G13 <sup>P</sup>	dJ1178G13	AC004988	7p14.1	D7S3094//D1S3168//D7S1472	C50
20	RP4-612F12 <sup>P</sup>	dJ612F12	AC004843	7p14.1	D7S2469...D7S2454	C51
	RP11-100C21	bA100C21	AC027269	7p14.1	D7S1768	C52
	RP5-1032B10 <sup>P</sup>	dJ1032B10	AC004455	7p13	D7S2428//D7S678	C53
25	RP11-36H20	bA36H20	AC011738	7p13	D7S1767//D7S2342	C54
	RP5-1022I14 <sup>P</sup>	dJ1022I14	AC004951	7p13	D7S1963	C55
	RP11-52M17	bA52M17	AC011894	7p13	D7S2488//D7S2697//D7S2312//D7S2329//D7S2082E//D7S2173E	C56
30	RP4-647J21 <sup>P</sup>	dJ647J21	AC004847	7p13	D7S2427...D7S621	C57
	RP11-111G20	bA111G20	AC073968	7p13	D7S2248//D7S2691	C58
	RP11-132L11	bA132L11	AC091524	7p13	D7S2379//D7S2833//D7S2146E	C59
	RP11-571N3	bA571N3	AC073115	7p13	D7S519...D7S2196	C60
35	RP11-95J15	bA95J15	AC011294	7p13	D7S2558	C61
	RP11-21H20	bA21H20	AC018998	7p12.3	D7S2506	C62
	RP4-815D20 <sup>P</sup>	dJ815D20	AC004899	7p13	D7S1415//D7S2788	C63
40	RP11-127A24	bA127A24	AC034145	7p12.3-p12.2	D7S1433//D7S1894	C64
	RP11-95E2	bA95E2	AC018705	7p12.2	D7S1606//D7S1856	C65
	RP4-718N17 <sup>P</sup>	dJ718N17	AC005999	7p12.1	D7S2361	C66
45	RP11-324M21	bA324M21	AC074263	7p12.1	D7S2451//D7S1921//AFM196xd2//AFM238WH12//D7S1761//D7S803//D7S506	C67
	RP11-449G3	bA449G3	AC019210	7p11.2	D7S2552//D7S2542	C68
	RP5-1091E12 <sup>P</sup>	dJ1091E12	AC006977	7p11.2	D7S2542...D7S793	C69
50	RP11-339F13	bA339F13	AC073324	7p11.2	D7S2935//D7S2276	C70
	RP4-725G10 <sup>P</sup>	dJ725G10	AC006970	7p11.2	D7S2765...D7S1585	C71
	RP11-10F11	bA10F11	AC095038	7p11.2	D7S1585...D7S2433	C72
	RP5-905H7 <sup>P</sup>	dJ905H7	AC006455	7p11.21	D7S1467.....	C73
55	RP11-340I6	bA340I6	AC092634	7q11.21	D7S2727	C74
	RP11-479O9	bA479O9	AC073107	7q11.21	D7S1592...D7S2549	C75
	RP4-756H11 <sup>P</sup>	dJ756H11	AC006001	7q11.21	D7S1792...D7S2368	C76
60	RP11-458F8	bA458F8	AC073335	7q11.21	D7S3250//DS2073E//D7S2304	C77
	RP4-736H5 <sup>P</sup>	dJ736H5	AC005482	7q11.22	D7S502	C78
	RP11-358M3	bA358M3	AC092637	7q11.22	D7S1639...D4S1506	C79

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	Nombre del clon (BAC/PAC)	Código de Identificación	Código de acceso en EMBL	Región del cromosoma	Marcadores o Dominio*	Código Fig. 1
5	RP11-156A14	bA156A14	AC069284	7q11.22	D7S1524//D7S1519//D7S3049//D7S1581//D7S1578//D7S1839//D7S2503	C80
	RP11-3P22	bA3P22	AC069280	7q11.22	D7S639	C81
10	RP4-715F13 <sup>P</sup>	dJ715F13	AC006317	7q11.22	D7S2500...D7S2798	C82
	RP11-409J21	bA409J21	AC073330	7q11.22	D7S1407//D7S2516//D7S1568//D7S2270E//D7S2407	C83
15	RP4-635O5 <sup>P</sup>	dJ635O5	AC004845	7q11.22	D7S1488...D7S2415	C84
	RP11-313P13	bA313P13	AC005488	7q11.23	D7S2749...D7S3287	C85
	RP11-450O3	bA450O3	AC105418	7q11.23	D7S3287...D7S2479	C86
	RP4-771P4 <sup>P</sup>	dJ771P4	AC004883	7q11.23	D7S3196...D7S2714	C87
20	RP11-107L23	bA107L23	AC079252	7q11.23	D7S715//D7S1456	C88
	RP4-799O8 <sup>P</sup>	dJ799O8	AC006972	7q11.23	D7S538E...	C89
	RP11-467H10	bA467H10	AC007000	7q11.23	...D7S2233	C90
	RP4-562A11 <sup>P</sup>	dJ562A11	AC006451	7q11.23	D7S2233	C91
25	RP11-538D15	bA538D15	AC006043	7q21.11	D7S669//D7S1885//D7S1859	C92
	RP11-343J14	bA343J14	AC073092	7q21.11	D7S1950//AFM312xb5	C93
	RP5-1057M1 <sup>P</sup>	dJ1057M1	AC004159	7q21.11	D7S1942...D7S2741	C94
30	RP5-1136A10 <sup>P</sup>	dJ1136A10	AC004972	7q21.11	D7S1797//D7S660//AFMB355XB9	C95
	RP4-560O14 <sup>P</sup>	dJ560O14	AC006145	7q21.11	D7S802//D7S2275E	C96
	RP11-575G1	bA575G1	AC008283	7q21.11	D7S1435	C97
35	RP4-784G16 <sup>P</sup>	dJ784G16	AC004886	7q21.11	D7S758//D7S2095E	C98
	RP11-242J14	bA242J14	AC073498	7q21.11	D7S1555	C99
	RP5-1093O17 <sup>P</sup>	dJ1093O17	AC004957	7q21.11	D7S1932//D7S524	C100
40	RP11-533L22	bA533L22	AC073958	7q21.11	D7S2537//D7S713//DX9848	C101
	RP4-650P9 <sup>P</sup>	dJ650P9	AC004413	7q21.11	D7S2555	C102
	RP11-212B1	bA212B1	AC079303	7q21.12	D7S2326	C103
	CTB-1137N13	bK137N13	AC005068	7q21.12	D7S2326	C104
45	CTB-60P12	bK60P12	AC002457	7q21.12	D7S2414	C105
	RP5-1078A23 <sup>P</sup>	dJ1078A23	AC006149	7q21.12- q21.13	AFMa339xa5....D7S630	C106
	RP5-998H4 <sup>P</sup>	dJ998H4	AC006350	7q21.13	D7S630...D7S2522	C107
50	RP11-263N12	bA263N12	AC006155	7q21.13	D7S2659//D7S2660	C108
	RP11-311I10	bA311I10	AC017036	7q21.13	D7S1509//AFM312yd1	C109
	RP5-1084H12 <sup>P</sup>	dJ1084H12	AC007313	7q21.2	D7S1539	C110
55	RP5-1099C19 <sup>P</sup>	dJ1099C19	AC005156	7q21.2	D7S539E....D7S2655	C111
	RP11-101N13	bA101N13	AC073886	7q21.3	D7S1489	C112
	RP11-95A10	bA95A10	AC022261	7q21.3	D13S839E....D7S2344	C113
	RP5-1145A22 <sup>P</sup>	dJ1145A22	AC007875	7q21.3	D7S618//D7S554//D1S3260	C114
60	RP11-380G21	bA380G21	AC079781	7q21.3	D7S2370...D7S1522	C115
	RP4-550A13 <sup>P</sup>	dJ550A13	AC004834	7q22.1	D7S2721	C116
	RP11-114P12	bA114P12	AC090113	7q22.1	D7S2923	C117
	RP4-604G5 <sup>P</sup>	dJ604G5	AC004522	7q22.1	D7S2320//D7S2687//D7S1619	C118
65	RP11-506M12	bA506M12	AC073842	7q22.1	D7S1619.....D7S1909	C119



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	Nombre del clon (BAC/PAC)	Código de Identificación	Código de acceso en EMBL	Región del cromosoma	Marcadores o Dominio*	Código Fig. 1
5	RP11-44M6	bA44M6	AC069281	7q22.1	D7S3095//D7S2480//D7S3098//D7S2852//D7S3103	C120
	RP5-1059M17 <sup>P</sup>	dJ1059M17	AC004953	7q22.1	D7S2245//D7S2085E	C121
10	RP11-333G13	bA333G13	AC092788	7q22.1	D7S1588//D7S2536//AF007375	C122
	RP11-401L13	bA401L13	AC007683	7q22.1	D7S1503...D7S2509	C123
	RP4-672O11 <sup>P</sup>	dJ672O11	AC006316	7q22.1	AFM211ZB8//AFMB316ZD5	C124
	RP11-148A10	bA148A10	AC007558	7q22.1	D7S1799	C125
15	RP11-22N19	bA22N19	AC007032	7q22.3	D7S1552//D7S2650	C126
	RP11-17110	bA17110	AC007031	7q22.3	D7S2650...D7S501	C127
	RP11-443I10	bA443I10	AC078853	7q31.1	D7S2456	C128
20	RP11-5N18	bA5N18	AC005487	7q31.1	D7S2648//D7S1501//AFM225XB4//D7S1608	C129
	RP11-238I13	bA238I13	AC073071	7q31.1	D7S2706//D7S1817	C130
	RP11-358A10	bA358A10	AC073326	7q31.1	D7S3152	C131
25	RP5-905M6 <sup>P</sup>	dJ905M6	AC005166	7q31.1	D7S2860//D7S3154//D7S3153//D7S2056E//D7S1763	C132
	RP11-328M22	bA328M22	AC018464	7q31.1	D7S2885...	C133
	RP11-563O5	bA563O5	AC020606	7q31.1	D7S1419...D7S2554	C134
30	RP11-45K3	bA45K3	AC092590	7q31.2	D7S2218	C135
	RP11-354H2	bA354H2	AC074021	7q31.2	D7S2054E//D7S486//D7S2886	C136
	CTB-1013N12	bK13N12		7q31.2	D7S2877//D7S1764//D7S2460//D7S2863//D7S1599//D7S1914	C137
35	RP11-374M7	bA374M7	AC106873	7q31.2	D7S2776//D7S3112//AFMB316XB9	C138
	RP5-866N18 <sup>P</sup>	dJ866N18	AC003987	7q31.2	D7S1428//D7S2097E//D7S8122//D7S3121	C139
40	RP11-126C19	bA126C19	AC040943	7q31.31	D7S1398	C140
	RP11-105B19	bA105B19	AC024259	7q31.31	D7S3116//D7S2179	C141
	RP11-376O14	bA376O14	AC006332	7q31.31	D7S2487//D7S2892	C142
	RP11-384A20	bA384A20	AC073095	7q31.32	D7S601E//D7S466	C143
45	RP11-560I19	bA560I19	AC015983	7q31.32	D7S3072//D7S3237//D7S3235//D7S3234//D7S1982	C144
	RP11-106F1	bA106F1	AC073054	7q31.32	D7S63//D7S2345//D7S727	C145
	RP5-902E20 <sup>P</sup>	dJ902E20	AC006148	7q31.33	D7S648//D7S2703	C146
50	RP11-264K23	bA264K23	AC092154	7q31.33	D7S2703...D7S1582	C147
	RP11-420H19	bA420H19	AC010099	7q31.33	D7S2471//D7S1917	C148
	RP11-21K15	bA21K15	AC013689	7q32.1	D7S1874	C149
55	RP11-224A1	bA224A1	AC009181	7q32.1	AF047018...D7S2939	C150
	RP11-128A6	bA128A6	AC018639	7q32.1	D7S2941//D7S2956//D7S1645	C151
	RP11-437L1	bA437L1	AC084865	7q32.2	D7S3063...D7S2072E	C152
60	RP5-999D10 <sup>P</sup>	dJ999D10	AC023880	7q32.2	D7S2072E//D7S2638//D7S2072E//D7S2052E	C153
	RP11-36B6	bA36B6	AC016831	7q32.3	D7S2966//D7S614//D7S1615	C154
	RP11-329I5	bA329I5	AC018642	7q32.3	D7S1575...D7S2924	C155
	RP11-193I17	bA193I17	AC009518	7q32.3	D7S2963//D7S2362	C156
65	RP11-355K3	bA355K3	AC009365	7q32.3	D7S761	C157
	RP11-239G19	bA239G19	AC009276	7q33	D7S697//D7S2792//D7S2829	C158

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Nombre del clon (BAC/PAC)	Código de Identificación	Código de acceso en EMBL	Región del cromosoma	Marcadores o Dominio*	Código Fig. 1
RP11-221G19	bA221G19	AC019014	7q33	D7S2533//D7S2330	C159
RP11-371N6	bA371N6	AC009331	7q33	D7S500	C160
RP11-88K4	bA88K4	AC020581	7q33	D7S1887//D7S2315	C161
RP11-102G17	bA102G17	AC078842	7q33	D7S2735...D7S2341	C162
RP11-269N18	bA269N18	AC020983	7q34	D7S2450//D7S3183//D7S1636	C163
RP5-886O8 <sup>P</sup>	dJ886O8	AC004914	7q34	D7S1986	C164
RP5-1173P7 <sup>P</sup>	dJ1173P7	AC006362	7q34	D7S1620//D7S1577//D7S2906// D7S1779//D7S2319	C165
RP11-237G17	bA237G17	AC099547	7q34	D7S2726//D7S1627	C166
RP5-894A10 <sup>P</sup>	dJ894A10	AC004918	7q34	D7S1627//D7S2132E//D7S810E// D7S2047E//D7S2513	C167
RP11-282G13	bA282G13	AC073079	7q34	D7S2887	C168
RP11-298A10	bA298A10	AC073264	7q34	D20S1007...D7S661	C169
RP4-721B17 <sup>P</sup>	dJ721B17	AC004864	7q35	D7S676...D7S498	C170
RP11-374N8	bA374N8	AC074384	7q35	D7S498...D7S1667	C171
RP5-819O4 <sup>P</sup>	dJ819O4	AC006007	7q35	D7S3044//D7S1798	C172
RP4-558L10 <sup>P</sup>	dJ558L10	AC005997	7q35	AFM177xd6//D7S2352	C0173
RP4-811H12 <sup>P</sup>	dJ811H12	AC004896	7q35	D7S615...D7S2692	C174
RP11-302C22	bA302C22	AC006992	7q35	D7S615...D7S1443	C175
RP5-1136G13 <sup>P</sup>	dJ1136G13	AC005229	7q36.1	D7S2442//D7S2419	C176
RP11-24N19	bA24N19	AC093743	7q36.1	D7S2640	C177
RP4-800G7 <sup>P</sup>	dJ800G7	AC004890	7q36.1	D7S2870//D7S1510//D7S2048E	C178
RP11-511P7	bA511P7	AC073111	7q36.1	D7S716//D7S2895	C179
RP4-548K24 <sup>P</sup>	dJ548K24	AC006343	7q36.1	D7S2745//D7S1826//D7S1827	C180
RP4-548D19 <sup>P</sup>	dJ548D19	AC021097	7q36.1	D7S2124E//D7S2157E// D7S642//D7S26421	C181
RP11-208G20	bA208G20	AC104692	7q36.1	D7S3200//D7S2222// AFMa153wd9	C182
RP11-312C1	bA312C1	AC072057	7q36.1-q36.2	D7S3199	C183
RP11-317C13	bA317C13	AC099341	7q36.2	D7S1521	C184
RP11-422E4	bA422E4	AC024730	7q36.2	D7S2546//D7S1544	C185
RP11-269M19	bA269M19	AC073077	7q36.2	D7S2141E//D7S2303//D7S1567// D7S2896//AFMBO58WE5	C186
RP5-1015O24 <sup>P</sup>	dJ1015O24	AC009403	7q36.3	D7S3035	C187
RP11-69O3	bA69O3	AC078834	7q36.3	D7S392//D7S550//D7S3034// D7S2392//D7S2307//D7S2398	C188
RP5-982E9 <sup>P</sup>	dJ982E9	AC005534	7q36.3	D7S3169//D7S3158//D7S3163// D7S3157//D7S559//D7S2078E// D7S2112E	C189
RP4-703P8 <sup>P</sup>	dJ703P8	AC005481	7q36.3	D7S3168...D7S2423	C190
RP11-452C13	bA452C13	AC011899	7q36.3	D7S54	C191
RP11-518I12	bA518I12	AC093856	7q36.3	D7S22//D7S1759//D7S22//D7S1 476	C192
CTB-3K23 <sup>P</sup>	PAC3K23		7q36.3	D7S749//D7S427	C193

\* Marcadores separados por líneas oblicuas (/): marcadores contenidos dentro del inserto del clon.

Marcadores separados por puntos suspensivos (...): marcadores adyacentes al inserto del clon

P: clones de tipo PAC

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Los insertos de ADN humano de los clones se aislaron tras el cultivo bacteriano, con su correspondiente antibiótico (kanamicina o cloranfenicol), a 37°C y en agitación durante toda la noche. El ADN de cada clon se preparó a partir de cultivos de 2,5 ml de cada clon utilizando el kit R.E.A.L. prep. 96 BioRobot de Qiagen, siguiendo las instrucciones del fabricante, utilizando el protocolo optimizado para BACs.

Tras la extracción, a cada ADN se le realizaron tres DOP-PCR (Fiegler H, Carr P, Douglas E.J, Burford D.C, Hunt S, Smith J, Vetrie D, Gorman P, Tomlinson I.P.M, & Carter N.P: "DNA Microarrays for Comparative Genomic Hybridization Based on DOP-PCR Amplification of BAC and PAC Clones", *Genes, Chromosomes & Cancer* 36: 361-374 (2003)) para asegurar la eficiente amplificación del ADN humano inserto en el clon y que no haya contaminación del ADN de *Escherichia coli*.

### - DOP-PCR

La amplificación del ADN inserto en cada clon se llevó a cabo siguiendo el siguiente procedimiento:

1. Tratar durante 1 hora con UV H<sub>2</sub>O miliQ, tampón TAPS, W-1 y las puntas de pipeta previamente a la preparación de las mezclas de reacción.

El tampón TAPS se preparó previamente mezclando 0,608 g de TAPS (ácido N-tris(hidroximetil)metil-3-amino-propanosulfónico) (Sigma Aldrich), 9,3 ml de H<sub>2</sub>O miliQ, 0,220 g de (NH<sub>4</sub>)SO<sub>4</sub> y 250 µl de MgCl<sub>2</sub> 1 M, ajustando el pH a 9,3 con KOH y completando con H<sub>2</sub>O miliQ hasta un volumen de 9,6 ml. Este tampón se esteriliza mediante luz UV, se divide en alícuotas de 960 µl/tubo y se almacena a -20°C. Antes de utilizarlo, han de añadirse al tubo correspondiente 31,68 µl de BSA (albúmina de suero bovino) al 5% (Sigma) y 6,72 µl de β-mercaptoetanol.

La solución de W-1 (éter de polioxietileno W-1) (Sigma Aldrich) se preparó previamente mezclando 1 g de W-1 en 100 ml de H<sub>2</sub>O.

2. Preparar la siguiente mezcla de reacción en placas de 96 pocillos:

Tampón TAPS	5,5 µl
Cebador DOP (20 µM) (Sigma Genosys)	1,5 µl
dNTP (2,5 mM cada uno) (Amersham)	4,4 µl
W-1 (1%)	2,75 µl
Taq DNA polimerasa (5 U/µl) (Promega)	0,55 µl
ADN molde (1 ng/µl)	3 µl
Volumen final	55 µl

El cebador DOP será distinto según la reacción de amplificación: DOP1 (SEQ ID NO:5), DOP2 (SEQ ID NO:6) o DOP3 (SEQ ID NO:7). Se realizan tres reacciones de amplificación distintas, cada una de ellas con un cebador diferente seleccionado entre DOP1, DOP2 y DOP3. En las secuencias correspondientes, representadas por SEQ ID NO:5, SEQ ID NO:6 o SEQ ID NO:7, respectivamente, puede observarse un fragmento de las mismas compuesto por una sucesión de "N", debido a que, en realidad, las alícuotas de cada uno de los cebadores utilizadas no contenía una única secuencia oligonucleotídica, sino una mezcla de las diferentes secuencias posibles que resultan de sustituir "N" por uno de los posibles nucleótidos A, G, C y T. Lo que permite la obtención de todas las secuencias posible para su longitud. La razón de utilizar estos cebadores degenerados es que permiten la amplificación completa del inserto de ADN genómico ya que permiten la unión del cebador en más de un lugar.

3. Amplificar siguiendo el siguiente programa, en el que las comillas simples tras un número indican minutos y las comillas dobles segundos: 3' a 94°C; 10 ciclos de 1'30" a 94°C, 2'30" a 30°C, subida hasta 72°C aumentando 0,1°C/segundo y 3' a 72°C; 30 ciclos de 1' a 94°C, 1'30" a 60°C (62°C si el cebador es DOP2), 21' a 72°C; 8' a 72°C y bajar a 12°C.

4. Mantener las placas a 4°C.

5. Comprobar que se ha producido correctamente la amplificación sometiendo a electroforesis una alícuota de 5 µl en un gel de agarosa al 2,5%. Debe observarse una mezcla de bandas diferenciadas y un fondo en forma de cola difusa (*smear*) en la zona de tamaños de 200 pb a 2 kb.

6. Guardar a -20°C hasta el momento de precipitar el ADN.

### - Precipitación de productos de PCR

1. Añadir a cada pocillo 5 µl de AcONa 3M pH 5,2 y 150 µl de EtOH 100% frío. Invertir varias veces

2. Mantener a -80°C entre 15 y 30 min

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3. Centrifugar a 3700 rpm, a 4°C, 60 min
4. Voltear para eliminar el sobrenadante
5. Añadir 150 µl de EtOH frío al 70%. Vórtex
6. Centrifugar a 3700 rpm, a 4°C, 30 min
7. Voltear para eliminar el sobrenadante
8. Mantener a 65°C en estufa hasta que los pellets estén secos
9. Resuspender en 13,4 µl de DMSO 50%
10. Mantener a 4°C durante toda la noche.
11. Comprobar en gel de agarosa
12. Guardar a -20°C

### - Obtención del array

Para obtener el array, se cultivaron los clones bacterianos elegidos, se extrajo el ADN de los mismos, se produjo su amplificación mediante DOP-PCR según se ha indicado, se precipitaron los productos de PCR y se mezclaron los productos de las tres diferentes amplificaciones (con DOP1, DOP2 o DOP3) llevadas a cabo con el ADN extraído de cada clon, de manera que cada muestra de ADN depositada en un punto del portaobjeto correspondiera a la mezcla de los tres productos de amplificación diferentes.

De cada mezcla de tres productos de amplificación correspondiente a un clon se depositó por triplicado sobre un portaobjetos de aminosilano (UltraGAPS, Corning), consiguiendo así un CGH-Array de aproximadamente 3500 sondas, cuya resolución es de 1 Mb.

En el array se han depositado aproximadamente 3500 BAC/PAC que contienen insertos del genoma humano, que al estar por triplicado corresponden en tomo a 10500 spots. Además se han utilizado como controles internos 5 BACs que corresponden a *Drosophila melanogaster*, F1y32N02, F1yP35P22, F1y39C21, F1y45C08 y F1y47P08, y un cósmido de *Schizosaccharomyces pombe*, c1782 (cuya secuencia puede consultarse en la base de datos de EMBL mediante el código AL121765). Además han sido depositados 50 spots con DMSO (control negativo) y 45 puntos que contienen una mezcla de ADN genómico de distintos individuos sanos. Con todo lo cual hay aproximadamente 10752 spots por array.

### 1.2. ADN tumorales ensayados

#### - Pacientes y ADN extraído

Se estudiaron un total de 68 pacientes diagnosticados de linfoma esplénico de la zona marginal (LEZM) de acuerdo con los criterios propuestos por la Organización Mundial de la Salud (Isaacson PG, Piris MA, Catovski D, *et al.* Splenic marginal zone lymphoma. In Jaffe ES, Harris NL, Stein H, Vardiman JW. Eds.: "Tumors of haematopoietic and lymphoid tissues. WHO classification of tumors", Lyon, Francia. IARC Press; 2001: 135-137). La mayor parte de las muestras procedían del bazo (44 casos) mientras que eran de sangre en los 24 casos restantes. Como control se usó una mezcla de ADN de varios controles sanos. Los ADNs de los pacientes y del control, empleados para el análisis de las muestras, se extrajeron mediante el protocolo estandarizado de extracción con fenol-cloroformo (Sambrook J, Fritsch EF, Maniatis T (eds): "Molecular cloning: A laboratory manual", Cold Spring Harbor, Cold Spring Harbor Laboratory, 1989).

#### - Preparación de los ADN genómicos a hibridar con el array

El ADN genómico extraído tanto de los pacientes seleccionados como del control sano se preparó para la hibridación con el array. Para ello, se sometió primero a una fragmentación mediante la digestión con la endonucleasa de restricción DpnII, incubando 2 µg del ADN correspondiente con 1,2 µl de DpnII y 3 µl del tampón correspondiente en un total de 35 µl durante 2 horas a 37°C, tras lo cual se procedió a la inactivación de la enzima manteniendo la mezcla de reacción 20 minutos a 65°C.

El tubo con el ADN digerido se mantuvo entonces 1 minuto en hielo y se purificó siguiendo el siguiente procedimiento:

1. Añadir a la mezcla de digestión:

140 µl TE 1X pH 8

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175  $\mu$ l de fenol (87,5  $\mu$ l)/cloroformo (87,5  $\mu$ l)

Vórtex unos segundos.

- 5 2. Centrifugar 5 minutos a 11.000 rpm.
3. Pasar el sobrenadante a un eppendorf nuevo y añadir:  
17.5  $\mu$ l de acetato sódico 3.0 M pH 5.2  
10 440  $\mu$ l de etanol 100% a -20°C
4. Incubar 30 minutos a -80°C
- 15 5. Centrifugar 20 minutos a 4°C. a 13.200 rpm
6. Retirar el sobrenadante con pipeta con cuidado de no eliminar el pellet.
7. Añadir 500  $\mu$ l de etanol 70%. (-20°C).
- 20 8. Centrifugar 5 minutos a 4°C a 13.200 rpm
9. Eliminar el sobrenadante con pipeta.
- 25 10. Secar al aire 5 minutos
11. Resuspender 41  $\mu$ l de agua destilada.
12. Comprobar la integridad del ADN en un gel de agarosa al 1,6%.

30 - *Marcaje y preparación de los ADNs genómicos digeridos y purificados*

Las muestras de ADN genómico (pacientes y controles) digerido y purificado se marcaron mediante la técnica de *Random-Prime* con el Kit de Marcaje Bioprimer Labelling Kit (Invitrogen). El protocolo de Marcaje de Invitrogen mediante *Random primer* está optimizado para comenzar con 2  $\mu$ g de ADN genómico. Sin embargo, en el caso de tener una menor concentración de ADN se puede eliminar la digestión del ADN o reducir el tiempo de digestión con la enzima. Por otro lado se puede aumentar el tiempo de reacción de la incorporación de nucleótidos, pudiéndose obtener una calidad optima de marcaje dejando la reacción de *Random Prime* durante toda una noche.

40 En este caso, para el marcaje se utilizaron dos fluorocromos diferentes, Cy3 para los controles y Cy5 para las muestras a testar. Brevemente, las muestras conteniendo 2  $\mu$ g de ADN genómico se mezclaron con 23,5  $\mu$ l del *random primer* presente en el kit, manteniendo la mezcla 5 minutos a 95°C, tras lo cual se pasó a hielo y se añadieron 6  $\mu$ l de CTP mix, 3,5  $\mu$ l del fluoróforo (Cy3 dCTP en el caso del control y Cy5 dCTP en el caso de las muestras de los pacientes) y 1,2  $\mu$ l de Exo-Klenow. Tras mezclar, se incubó 2 horas a 37°C, se paró la reacción con 6  $\mu$ l de tampón de  
45 parada (*Stop buffer*) y se dejó en hielo hasta el momento de purificación del ADN marcado.

La purificación se produjo siguiendo los siguientes pasos:

- 50 1. Añadir:  
67  $\mu$ l de TE 1X pH 8  
400  $\mu$ l del buffer A
- 55 2. Vortex
3. Pasar a las columnas.
4. Centrifugar 1 minutos a 10900 r.p.m. a Tª ambiente.
- 60 5. Descargar el filtrado
6. Añadir:  
65 i.600  $\mu$ l de buffer B a la columna
7. Centrifugar 1 minuto a Tª ambiente 10.900 r.p.m.

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8. Descargar y añadir 200  $\mu\text{l}$  de buffer B.
9. Centrifugar 1 minuto a  $T^{\text{a}}$  ambiente 10.900 r.p.m
- 5 10. Descargar.
11. Añadir 75  $\mu\text{l}$  de  $\text{H}_2\text{O}$  e incubar 1 minuto a  $T^{\text{a}}$  ambiente
12. Centrifugar 1 minuto a 10900 r.p.m.

10 Posteriormente se midió la incorporación de los nucleótidos marcados en el espectrofotómetro, mediante los siguientes cálculos:

- 15 - Cantidad de ADN en microgramos ( $\mu\text{g}$ ).

$$(A_{260} - A_{320}) \times 50 \times \text{Volumen sonda (en ml)} = \mu\text{g de ADN tiene que ser } > \text{ de } 2,8 \mu\text{g}$$

- 20 - Cantidad de nucleótido incorporado:

$$[\text{Cy3}]_{\text{pmoles}} = (A_{550} - A_{650})/0,15 \times (\text{volumen de sonda en } \mu\text{l})$$

$$[\text{Cy5}]_{\text{pmoles}} = (A_{650} - A_{750})/0,25 \times (\text{volumen de sonda en } \mu\text{l})$$

25 El total tiene que ser  $>$  de 100 pmoles.

- 25 - Ratios:

$$\text{Cy3} = (A_{260} \times 150.000)/(A_{550} \times 6.600)$$

$$\text{Cy5} = (A_{260} \times 250.000)/(A_{650} \times 6.600)$$

30 Estos valores deben estar en 40-80.

### 35 1.3. Hibridación del array

Para la hibridación sobre el array, se mezclaron las muestras de ADN marcado junto con ADN competidor humano Cot-1 (Life Technologies), se mezclaron con solución de hibridación y se realizó la hibridación en el aparato para el procesamiento automatizado de arrays GeneTAC (Microgen). Los detalles de proceso fueron los siguientes:

#### 40 - *Pretratamiento de los portas*

Los portaobjetos se incubaron durante 60 minutos a  $42^{\circ}\text{C}$  en agitación con una solución que contenía formamida al 25% (preparada con 25 ml de SSC 20X, 25 ml de formamida, 1 ml de SDS al 10%, 250  $\mu\text{l}$  de BSA(50 mg/ml) y 49 ml de  $\text{H}_2\text{O}$ ), se lavaron primero durante 5 minutos en SSC 0,1 X en agitación, luego se lavaron 2 veces durante 1 minuto con  $\text{H}_2\text{O}$  destilada en agitación y se centrifugaron a 2500 rpm durante 5 minutos.

#### - *Preparación de la solución de hibridación*

- 50 1. Mezclar las muestras de ADN marcadas (Cy3, Cy5) con el Cot-1:

Cy5: toda la muestra marcada: 70  $\mu\text{l}$

Cy3:  $\mu\text{l}$  según los pmoles (Se debe tener un incorporación de pmoles similar a los que se obtenga en la reacción de incorporación de Cy5).

55 Cot-1: 100  $\mu\text{l}$  de una solución de 1  $\mu\text{g}/\mu\text{l}$

2. Añadir el 10% del volumen total de la mezcla anterior de acetato sódico (3M, pH 5,2).
- 60 3. Añadir 2,5 volúmenes de etanol 100% ( $-20^{\circ}\text{C}$ ) y mezclar
4. 30 minutos a  $-80^{\circ}\text{C}$ .
5. Centrifugar 15 min. a 13.200 r.p.m. a  $4^{\circ}\text{C}$ .
- 65 6. Decantar; añadir 500  $\mu\text{l}$  de etanol al 80% frío ( $-20^{\circ}\text{C}$ ).
7. Centrifugar 5 min. a 13.200 r.p.m. a  $4^{\circ}\text{C}$ .

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8. Decantar y dejar secar.
9. Añadir 4,5  $\mu\text{l}$  de ARN de levadura (100  $\mu\text{g}/\mu\text{l}$ )(Invitrogen) y 140  $\mu\text{l}$  de tampón de hibridación (previamente caliente a 70°C): 50% formamida, 10% dextransulfano, SSC 2X, Tris 10 mM pH 7,6, SDS al 2,7% en agua.
10. Incubar 2 minutos a 70°C.
11. Incubar 1 hora a 37°C. (Empezar el pretratamiento de los portas)
12. 10 minutos a 70°C.
13. Incubar 1 hora a 37°C.
14. Centrifugar 5 minutos a 13.200 r.p.m.
15. Resuspender en 140  $\mu\text{l}$  de tampón de hibridación.

La hibridación del array con esta mezcla de hibridación tuvo lugar durante 48 horas a 42°C en el GeneTAC.

### - Lavados post-hibridación

Se llevaron a cabo primero con solución I (formamida al 50%, SSC 2X, Tween-20 0,1% v/v) y luego con solución II (PBS 1x con 0,05% v/v de Tween 20).

Una vez concluido el proceso de hibridación, el array fue escaneado por el escáner GenePix 4000B (Axon Instrument) y las imágenes fueron analizadas con el GenePix 4.0. La intensidad de la fluorescencia de cada punto fue calculada después de la sustracción del ruido de fondo.

La normalización del array se realizó eliminando los clones que el GenePix considera como no encontrados, además de los controles negativos como son: el cósmido c1782 de *S. pombe* y DMSO 50%; y haciendo que la media de todo el array sea igual a 1.

Para validar el array se realizaron una serie de hibridaciones hombre vs mujer normal y además marcando el ADN empleado como control en las hibridaciones con las muestras a testar con los distintos fluorocromos, para eliminar los clones que en estas hibridaciones se encuentran alterados porque se considerarán que no se ven afectados por la enfermedad a analizar.

Del análisis del array genómico se excluyen aquellos puntos que tienen una desviación estándar mayor o igual a 0,3, por lo que todos los clones con un valor inferior a 0,3 serán válidos para el análisis.

Para determinar que un clon está alterado se calcula el promedio de los valores de los logaritmos de los ratios de la señal correspondiente al Cy5 (ADN tumoral extraído del paciente) frente a la correspondiente al Cy3 (ADN del control) de cada triplicado del clon. Si este valor es superior a +0,3 del promedio de los logaritmos de los ratios, esto indicará que la cantidad de ADN tumoral hibridado es mayor que la cantidad de ADN control y se considera que el clon está "ganado", es decir, que en el ADN tumoral existe ganancia de la zona a la que corresponde al fragmento de ADN humano insertado en el clon correspondiente. Si este valor es inferior a -0,3 del promedio de los ratios, esto indicará que la cantidad de ADN tumoral hibridado es menor que la cantidad de ADN normal y se considera que el clon está perdido, es decir, que en el ADN tumoral hay una delección que afecta a la región del fragmento de ADN humano insertado en el clon correspondiente. Y si se encuentra entre +0,3 y -0,3 significa que el clon no está alterado, es decir que la cantidad de ADN tumoral es similar a la de ADN control. La representación gráfica se realiza con el valor del promedio de los  $\log_2$  de los ratios de cada clon frente a la posición en el genoma de dichos clones.

### 1.4. Resultados

En la Fig. 1 se muestra una representación de los datos obtenidos con cada uno de los BACs del cromosoma 7. En la misma, tal como se explicó previamente, las celdas sombreadas en tono oscuro continuo representan clones perdidos en el ADN del paciente correspondiente, las celdas con sombreado interrumpido por rayas verticales blancas representan clones ganados, mientras que las celdas con sombreado interrumpido por rayas inclinadas más oscuras representan clones en los que la hibridación no fue correcta o en los que los resultados correspondientes a cada una de las tres muestras del clon depositadas sobre el array resultaron discordantes.

En dicha Figura puede observarse como el inserto de clon denominado RP11-44M6, que tiene el código de identificación bA44M6, permite la identificación de la existencia de delecciones en la región indicada en más del 60% (63,24%) de los pacientes de LEZM incluidos en el ensayo.

Además, el mismo estudio permitió ratificar la asociación entre LEZM y pérdidas de material genómico en una región más distal, que anteriormente ya había sido asociada con esta enfermedad: 7q32-7q33. En el 31% de los

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enfermos analizados se apreciaron pérdidas en esta región que podían detectarse por la pérdida de señal de al menos dos sondas contiguas. Cuando se comparan los enfermos en los que se detectan pérdidas en la región 7q22.1-7q22.2, se observa que en un 44% del total de enfermos las pérdidas en la región 7q22.1-7q22.2 se presentan de forma aislada, sin que existan pérdidas detectables en la región 7q32-7q33; esto es así en casi el 70% de los pacientes con pérdidas en dicha región. En cuanto a esta segunda región, 7q32-7q33, en el 9% de los enfermos las pérdidas en esta región se presentan de forma aislada, sin que se observen pérdidas en la región 7q22.1-7q22.2. Por lo tanto, no sólo son dos y no una sola las regiones en las que se observan deleciones asociadas a LEZM en el brazo largo del cromosoma 7 sino que, además, parece existir una cierta tendencia a que la existencia de una excluya la presencia de la otra. Sin embargo, un ensayo en el que se utilizaran como sondas ambos fragmentos permitiría detectar el 72% de los enfermos con LEZM, enfermos que, además, se habrían identificado por presentar pérdidas en el material genético lo que, como se ha comentado antes, parece tener una relación con un peor pronóstico en cuanto al período de supervivencia.

### Ejemplo 2

#### 15 *Ensayos de hibridación in situ*

Para comprobar la validez del uso de las sondas propuestas para la detección de LEZM en ensayos de hibridación *in situ*, se realizó una segunda prueba, en este caso de hibridación *in situ* fluorescente (FISH), en la que se utilizaron células procedentes de material tumoral previamente extirpado (bazo o ganglio) o aspirado (médula ósea), células que, tras ser cultivadas, se depositaron sobre un portaobjetos y se hibridaron con ADN de los clones RP11-44M6 y RP11-193I17, marcados cada uno de ellos con un fluoróforo diferente: Spectrum Green (que da lugar a una señal de color verde), en el caso de RP11-44M6, y Spectrum Orange (que da lugar a una señal de color anaranjado/rojizo), en el caso de RP11-193I17. Los resultados obtenidos permitieron comprobar la validez del método de la invención como método para el diagnóstico de LEZM.

Las secuencias de los insertos de los clones RP11-44M6 (SEQ ID NO:1) y RP11-193I17 (SEQ ID NO:4), así como las de los clones RP11-36B6 (SEQ ID NO:2) y RP11-329I5 (SEQ ID NO:3), que se muestran en el “Listado de secuencias” son las secuencias accesibles en la base de datos de EMBL (tras la introducción del correspondiente código de acceso que aparece en la Tabla I) a fecha de 23 de Noviembre de 2006, fecha en la que la construcción del genoma humano reflejada en dicha base de datos era la construcción 88, dada a conocer en septiembre de 2006.

#### 2.1. *Marcaje de las sondas*

Se utilizó el ADN de cada uno de los clones obtenido a partir de cultivos de 2,5 ml de cada clon utilizando el kit R.E.A.L. prep. 96 BioRobot de Qiagen, siguiendo las instrucciones del fabricante, utilizando el protocolo optimizado para BACs, según se mencionó en el Ejemplo 1, que permite el aislamiento de los BACs contenidos en las bacterias.

Las sondas se marcaron mediante el procedimiento de “traslado de la muesca” (*nick translation*), utilizando Spectrum Green para marcar el ADN correspondiente al clon RP11-44M6 y Spectrum Orange para marcar el ADN correspondiente al clon RP11-193I17. Para ello se utilizó el siguiente procedimiento:

1. Agregar en un tubo “ependorf” y en frío los siguientes reactivos (las enzimas deben añadirse en último lugar):

ADN (BAC completo, RP11-44M6 o RP11-193I17)	1 $\mu$ g
10 X tampón de la reacción NT (Tris-HCl 0,5 M pH 8,0, MgCl <sub>2</sub> 50 mM, 0,5 mg/ml BSA)	5 $\mu$ L
$\beta$ -mercaptoetanol 0,1 M	5 $\mu$ L
Mezcla de dNTPs 10X (cada uno 0,5 mM salvo dTTP: 0,12 mM) (Roche)	5 $\mu$ L
Spectrum Green-dUTP (0,5 mM)/Spectrum Orange-11-dUTP (0,125 mM) (Abbot Laboratories)	2 $\mu$ L
ADNasa (3 $\mu$ g/ml)(Sigma)	2 $\mu$ L
ADN polimerasa I (10 U/ $\mu$ l)(Promega)	5 $\mu$ L
H <sub>2</sub> O bidestilada (ajustar hasta 50 $\mu$ L)	

2. Mezclar bien y centrifugar suavemente.

3. Incubar en baño a 15°C durante 30 minutos.

4. Colocar el tubo de la reacción en hielo hasta que se haya comprobado el tamaño de la sonda.

5. Comprobar la longitud de la sonda mediante la electroforesis en un gel de agarosa: cargar el gel con 7  $\mu$ L de la mezcla de la reacción (teñida con el tampón de carga) así como con el marcador de 1 Kb y dejar correr durante 30 minutos a 100 voltios. Observar el ADN bajo iluminación ultravioleta y obtener una fotografía.



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6. Si el tamaño de la sonda es el deseado, en torno a 300 pb, inactivar la ADNasa: añadir 2  $\mu\text{L}$  de EDTA 0,5 M (concentración final de 10 mM) y 1  $\mu\text{L}$  de SDS al 10% (concentración final de 0,1%) a la mezcla de la reacción y calentar durante 10 minutos a 68°C.

5 El resultado del proceso es la obtención de la sonda marcada a una concentración aproximada de 20 ng/ $\mu\text{L}$ . A continuación se puede utilizar en la hibridación o almacenar a -20°C.

### 2.2. Pacientes

10 Se analizaron un total de 12 enfermos en los que previamente se había diagnosticado LEZM de acuerdo con los mismos criterios propuestos por la Organización Mundial de la Salud a los que se hizo referencia en el Ejemplo 1.8 de ellos habían sido incluidos en el estudio de CGH-arrays descrito en el Ejemplo 1 (los pacientes referidos como los casos 4, 11, 18, 21, 22, 23, 63 y 68 en dicho Ejemplo 1) y en otros 4 no se obtuvo suficiente ADN como para realizar estudios de CGH arrays, por lo que no se habían incluidos en el Ejemplo 1.

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### 2.3. Tratamiento y desnaturalización de las preparaciones

Se parte de una suspensión de células procedentes de tumores previamente extraídos o aspirados a cada uno de los pacientes, suspensión que se obtiene tras cultivar las células de acuerdo con los procedimientos estandarizados en el laboratorio de los inventores (Hernández JM, González MB, Granada I, Gutiérrez NC, Chillón C, Ramos F, Ribera JM, González M, Feliu E, San Miguel JF: "Detection of inv(16) and t(16;16) by FISH in acute myeloid leukemia M4Eo", *Haematologica* 2000; 85: 481-485). Brevemente, se tomaron muestras de los tumores extraídos de los pacientes, que se recogieron en condiciones de máxima esterilidad y se procesaron en un tiempo inferior a 24 horas. Los tubos utilizados para la recogida y el transporte de las muestras estaban exentos de EDTA, porque inhibe el crecimiento de las células.

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#### *Procedimiento seguido*

##### 1. Preparación de la muestra:

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1.a En el caso de las muestras de aspirados de médula ósea, se realiza un recuento celular de la muestra extraída en tubos de heparina sódica, para calcular la cantidad de muestra necesaria, teniendo en cuenta que se deben cultivar aproximadamente 2 millones de células por ml de medio de cultivo.

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1.b Las muestras obtenidas de ganglio o de bazo se disgregaron con bisturí y pinzas en condiciones de máxima esterilidad, para obtener un botón celular lo más homogéneo posible, tal como ha sido descrito por Hernández *et al* (Hernández J.M., García J.L., Gutiérrez N.C., Mollejo M., Martínez-Climent J.A., Flores T., González M.B., Piris M.A., & San Miguel J.F. Novel Genetics Imbalances in B-Cell Splenic Marginal Lymphomas Revealed by Comparative Genomic Hybridization and Cytogenetics. *American Journal of Pathology*, Vol. 185, No 5, May 2005).

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2. Incubar las células en 10 mL de medio de cultivo (RPMI 1640, Invitrogen) en frascos estériles en una estufa de CO<sub>2</sub> al 5% a 37°C durante 24 horas sin estimulante.

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3. Trascorrido el tiempo de incubación, interrumpir el ciclo celular con 0,1 mL de Colcemid (Sigma) (concentración final: 0,1  $\mu\text{g}/\text{mL}$ ) durante 50 minutos a 37°C.

4. Transferir el producto del cultivo a un tubo cónico y centrifugar a 1200 r.p.m. durante 10 minutos. Decantar el sobrenadante y resuspender el sedimento obtenido.

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5. Añadir suavemente la solución hipotónica (KCl 0,0075 M) precalentada a 37°C e incubar durante 20 minutos en estufa a 37°C.

6. Centrifugar a 1200 r.p.m. durante 10 minutos. Decantar el sobrenadante.

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7. Añadir el fijador Carnoy (metanol:ácido acético 3:1) gota a gota. Centrifugar.

8. Decantar, resuspender el sedimento nuevamente con fijador y volver a centrifugar al menos otras dos veces, para obtener un botón celular limpio. Conservar a -20°C.

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### 2.4. Tratamiento y desnaturalización de las sondas

1. Colocar en un tubo "eppendorf" 100 ng de sonda y 20  $\mu\text{L}$  de ADN Cot1 (enriquecido con ADN altamente repetitivo) que actuará como competidor evitando que la sonda hibride en las regiones cromosómicas que poseen secuencias altamente repetitivas (regiones pericentroméricas).

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2. Precipitar la sonda añadiendo 1,5  $\mu\text{L}$  de acetato sódico 3 M y 50  $\mu\text{L}$  de etanol al 100% a -20°C. Mezclar bien e incubar a -80°C durante 30 minutos.

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3. Centrifugar a 12.000 r.p.m. durante 10 minutos a 4°C.

4. Decantar el sobrenadante. Añadir 100 µL de etanol al 80% y centrifugar a 12.000 r.p.m. durante 10 minutos. Eliminar el sobrenadante y dejar secar.

4. Resuspender el ADN con 7 µL del tampón de hibridación y agitar durante 30 minutos a temperatura ambiente.

5. Desnaturalizar el ADN a 75°C 5 minutos, incubar en hielo otros 5 minutos y realizar el preanillado de la sonda en baño a 37°C durante 45 minutos.

6. Agregar 7 µL de la sonda desnaturalizada y preanillada sobre el portaobjetos.

7. Cubrir con un cubreobjetos y sellar con pegamento.

### 2.5. Hibridación y lavados

Para proceder a la hibridación, las preparaciones se introdujeron en una cámara húmeda y se incubaron con tampón de hibridación (formamida al 60%, dextranulfato al 2%, SSC 0,01X) durante 24 horas a 37°C.

Terminada la hibridación, se eliminó suavemente el pegamento y los cubreobjetos de las preparaciones. En primer lugar, se introdujeron en un recipiente que contenía formamida al 60%/SSC 2X pH 7,0 a 42°C, realizando cuatro lavados de 5 minutos cada uno. A continuación se realizaron tres lavados de 5 minutos en una solución de SSC 0,1X a 60°C. Por último, se realizó un lavado de 5 minutos a temperatura ambiente en 4T (SSC 4X, Tween-20 0,05% v/v).

### 2.6. Contratinción

La contratinción de las células, adicional a la realizada al poner en contacto las preparaciones con las sondas previamente marcadas con los fluoróforos, se realizó con DAPI (4,6-diamidino-2-fenilindol), introduciendo el portaobjetos en un recipiente que contenía una solución de DAPI, durante 2 minutos en agitación y a temperatura ambiente.

A continuación, se agregaron 20 µl de “VECTASHIELD” (Laboratorios Vector) para mantener la fluorescencia, y se cubrió con un cubreobjetos. Las preparaciones se mantuvieron a 4°C y protegidas de la luz hasta el momento de realizar la adquisición y análisis digital de las imágenes.

### 2.7. Adquisición y análisis de las imágenes

Los portaobjetos se observaron en un microscopio epifluorescente con rueda de filtros (DAPI, FTC, TRITC). Las imágenes se procesaron con el sistema de análisis de imagen CYTOVISION (Applied Imaging).

### 2.8. Resultados

Tal como se explicó previamente, debido a que la región cromosómica con la que hibrida cada una de las sondas está duplicada en cada una de las células, si dicha zona no está delecionada, debe observarse en la célula dos señales con el color correspondiente al fluoróforo con el que se haya marcado dicha sonda. Por tanto, en las células procedentes de pacientes que presenten tumores no clasificables como linfoma esplénico de la zona marginal deben apreciarse 4 señales, dos correspondientes a la sonda RP11-44M6 (que en este caso serán de color verde), y otras dos correspondientes a la sonda RP11-193I17 (que en este caso serán de color anaranjado/rojizo). Las muestras se clasificarán como procedentes de un linfoma esplénico de la zona marginal (LEZM) cuando se observe una única señal del color correspondiente a al menos una de las sondas, es decir, según los fluoróforos utilizados en este caso específico, se diagnosticará que el tumor es un linfoma esplénico de la zona marginal tanto si la célula presenta una única señal de verde (lo que indica pérdida de la zona con la que hibrida el inserto del clon RP11-44M6 en uno de los cromosomas) y dos señales de color anaranjado/rojizo, como si la célula presenta una única señal de color anaranjado/rojizo (lo que indica pérdida de la zona con la que hibrida el inserto del clon RP11-193I17) y dos señales de color verde, o también si la célula presenta una única señal de color verde y una única señal de color anaranjado/rojizo (lo que indica pérdida tanto de la zona con la que hibrida el inserto del clon RP11-44M6 como pérdida de la zona con la que hibrida el inserto del clon RP11-193I17).

En los 4 enfermos en los que no se realizaron estudios de CGH arrays se observó pérdida del clon RP11-44M6 en 3 de ellos, de los cuales en 1 se observaba también pérdida del clon RP11-193I17. De los 8 casos analizados también por CGH arrays se observó que en los enfermos número 4, 11 y 63 había pérdida de ambos clones, mientras que en los casos números 18, 21, 22 y 23 se observaba sólo pérdida del clon RP11-44M6. El caso 68 no presentaba pérdida de ninguno de los dos clones analizados por hibridación “*in situ*”. Los hallazgos encontrados en la FISH confirmaron los observados en el ensayo de CGH-array del Ejemplo 1 en los enfermos analizados.

La Figura 2 corresponde a un ensayo de hibridación *in situ* realizado utilizando el método diagnóstico de la invención, sobre una muestra tomada del enfermo 21, utilizando como sondas tanto el inserto del clon RP11-4M6, marcado con el marcador “Spectrum green” (que da lugar a una señal de color verde), como el inserto del clon RP11-193I17 marcado con el marcador “Spectrum Orange” (que da lugar a una señal de color rojo/anaranjado). En ambas células se

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observa que sólo existe una señal de color verde por célula (puntos señalados con flechas horizontales, de color blanco), observándose dos señales de color rojo en cada una de las células (puntos señalados con flechas verticales, con relleno negro), lo que indica que existe una pérdida de material genómico en la región 7q22, no detectándose pérdida de material genómico en la región de 7q32-7q33, resultado que confirma los obtenidos para este mismo paciente en el experimento del CGH-array descrito en el Ejemplo 1.

Por tanto, el sistema propuesto es válido para el diagnóstico de LEZM mediante la utilización de las sondas propuestas marcadas con alguna sustancia que permita su detección, preferentemente fluoróforos diferentes, cada uno de los cuales emite el longitudes de onda diferentes, que dan lugar a la observación de colores diferentes.

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REIVINDICACIONES

- 5 1. Uso de la secuencia representada por SEQ ID NO:1 como sonda para el diagnóstico del linfoma esplénico de la zona marginal (LEZM) en seres humanos.
2. Uso según la reivindicación 1, en el que la sonda de SEQ ID NO:1 se utiliza para el diagnóstico del linfoma esplénico de la zona marginal en seres humanos en combinación con al menos una sonda adicional.
- 10 3. Uso según la reivindicación 2, en el que la secuencia de al menos una sonda adicional está comprendida en la región 7q32-7q33 del cromosoma 7 humano.
- 15 4. Uso según la reivindicación 3, en el que la secuencia de al menos una sonda adicional está comprendida en un fragmento de la región 7q32-7q33 del cromosoma 7 humano que se selecciona entre las secuencias representadas por SEQ ID NO:2, SEQ ID NO:3 y SEQ ID NO:4.
- 20 5. Uso según una cualquiera de las reivindicaciones 1 a 4, en el que la sonda o sondas utilizadas para el diagnóstico del linfoma esplénico de la zona marginal se encuentran depositadas sobre un soporte sólido formando parte de un array.
- 25 6. Uso según una cualquiera de las reivindicaciones 1 a 4, en el que la sonda o sondas utilizadas para el diagnóstico del linfoma esplénico de la zona marginal se utilizan formando parte de una composición líquida que se utiliza en un ensayo de hibridación con el genoma de células de seres humanos depositadas sobre un soporte sólido.
- 30 7. Un método de diagnóstico *in vitro* de LEZM en un ser humano que comprende las etapas de:
- a) obtener una muestra de tejido o de células separadas, procedentes de un tejido infiltrado con células de linfoma, fijadas sobre un portaobjetos y listas para llevar a cabo una hibridación *in situ* sobre ellas;
  - 35 b) realizar una hibridación *in situ* sobre el portaobjetos, en presencia de ADN humano competidor, utilizando como sonda ADN correspondiente a la secuencia del inserto del BAC RP11-44M6 (SEQ ID NO:1) acoplado a una sustancia que permite su detección y utilizando opcionalmente como una segunda sonda cuya secuencia está comprendida en la región 7q32-7q33 del cromosoma 7 humano también acoplada a una sustancia que permite su detección, sustancia que es distinta de la sustancia a la que está acoplada la primera sonda y que da lugar a una señal diferenciable de la correspondiente a dicha sustancia;
  - 40 c) identificar el tumor del que se tomó la muestra como linfoma esplénico de la zona marginal si en cada célula se observa una única señal correspondiente a la sustancia acoplada a la primera sonda y/o, en el caso de haberse utilizado también la segunda sonda opcional, una única señal correspondiente a la sustancia acoplada a la segunda sonda, e identificar el tumor como clasificable en otro tipo de cáncer si en cada célula se observan dos señales correspondientes a la sustancia acoplada a la primera sonda que, en el caso de haberse utilizado también la segunda sonda opcional, deberán ir acompañadas por dos señales correspondientes a la segunda sonda.
- 45 8. Método según la reivindicación 7, en el que la segunda sonda opcional se selecciona entre los insertos de los BACs RP11-36B6 (SEQ ID NO:2), RP11-329I5 (SEQ ID NO:3) y RP11-193I17 (SEQ ID NO:4).
- 50 9. Método según la reivindicación 8, en el que la segunda sonda opcional corresponde al inserto del BAC RP11-193I17 (SEQ ID NO:4).
- 55 10. Método según una cualquiera de las reivindicaciones 7 a 9, en el que tanto la sustancia acoplada a la primera sonda como la sustancia acoplada a la segunda sonda opcional dan lugar a la emisión de fluorescencia.
11. Método según la reivindicación 10, en el que tanto la sustancia acoplada a la primera sonda como la sustancia acoplada a la segunda sonda son fluoróforos unidos covalentemente a la sonda correspondiente.
12. Método según la reivindicación 11, en el que el fluoróforo unido covalentemente a una de las sondas es Cy3 y el fluoróforo unido covalentemente a la otra sonda es Cy5.
- 60 13. Método según la reivindicación 11, en el que el fluoróforo unido covalentemente a una de las sondas es rodamina y el fluoróforo unido covalentemente a la otra sonda es fluoresceína.
14. Método según la reivindicación 11, en el que los fluoróforos unidos a cada una de las sondas se seleccionan del grupo de Spectrum Green, Spectrum Orange y Spectrum Red.
- 65 15. Método según la reivindicación 14, en el que el fluoróforo unido covalentemente a una de las sondas es Spectrum Green y el fluoróforo unido covalentemente a la otra sonda es Spectrum Orange.

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16. Método según una cualquiera de las reivindicaciones 7 a 15, en el que la muestra fijada sobre un portaobjetos sobre la cual se lleva a cabo la hibridación *in situ* es una sección de tejido tomada de un tumor de bazo o de ganglios linfáticos.

5 17. Método según la reivindicación 16, en el que la sección de tejido tiene un espesor de 2-3  $\mu\text{m}$ .

18. Método según una cualquiera de las reivindicaciones 7 a 15, en el que la muestra fijada sobre un portaobjetos sobre la cual se lleva a cabo la hibridación *in situ* se compone de células separadas procedentes de una muestra de tejido infiltrado extraída previamente del paciente y que han sido sometida a los procesos característicos de la citogenética convencional.

19. Método según la reivindicación 18, en el que las células proceden de un tumor de bazo, un tumor de ganglios linfáticos, un aspirado de médula ósea o de una muestra de sangre total.

15 20. Método según una cualquiera de las reivindicaciones 7 a 19, en el que se incluye al menos una tercera sonda opcional, complementaria a una región no asociada con LEZM.

21. Método según la reivindicación 21, en el que la tercer sonda opcional es complementaria a la región del centrómero del cromosoma 7.

22. Un kit de diagnóstico que comprende una primera sonda que corresponde a la secuencia del inserto del BAC RP11-44M6 (SEQ ID NO:1) acoplada a una sustancia que permite su detección y, opcionalmente, en el mismo recipiente o en un recipiente separado, una segunda sonda, acoplada a una segunda sustancia que permite su detección y su diferenciación de la primera sustancia, sonda que corresponde a la secuencia de un fragmento de la región 7q32-7q33.

23. Kit de diagnóstico según la reivindicación 22, en el que la sonda que corresponde a la secuencia de un fragmento de la región 7q32-7q33 se selecciona entre los insertos de los BACs RP11-36B6 (SEQ ID NO:2), RP11-329I5 (SEQ ID NO:3) y RP11-193I17 (SEQ ID NO:4).

24. Kit de diagnóstico según la reivindicación 23, en el que la segunda sonda opcional corresponde al inserto del BAC RP11-193I17 (SEQ ID NO:4).

25. Kit de diagnóstico según una cualquiera de las reivindicaciones 22 a 24, en el que tanto la sustancia acoplada a la primera sonda como la sustancia acoplada a la segunda sonda opcional son fluoróforos.

26. Kit de diagnóstico según la reivindicación 25, en el que la primera sonda está unida covalentemente al fluoróforo Spectrum Green y la segunda sonda está unida covalentemente al fluoróforo Spectrum Orange.

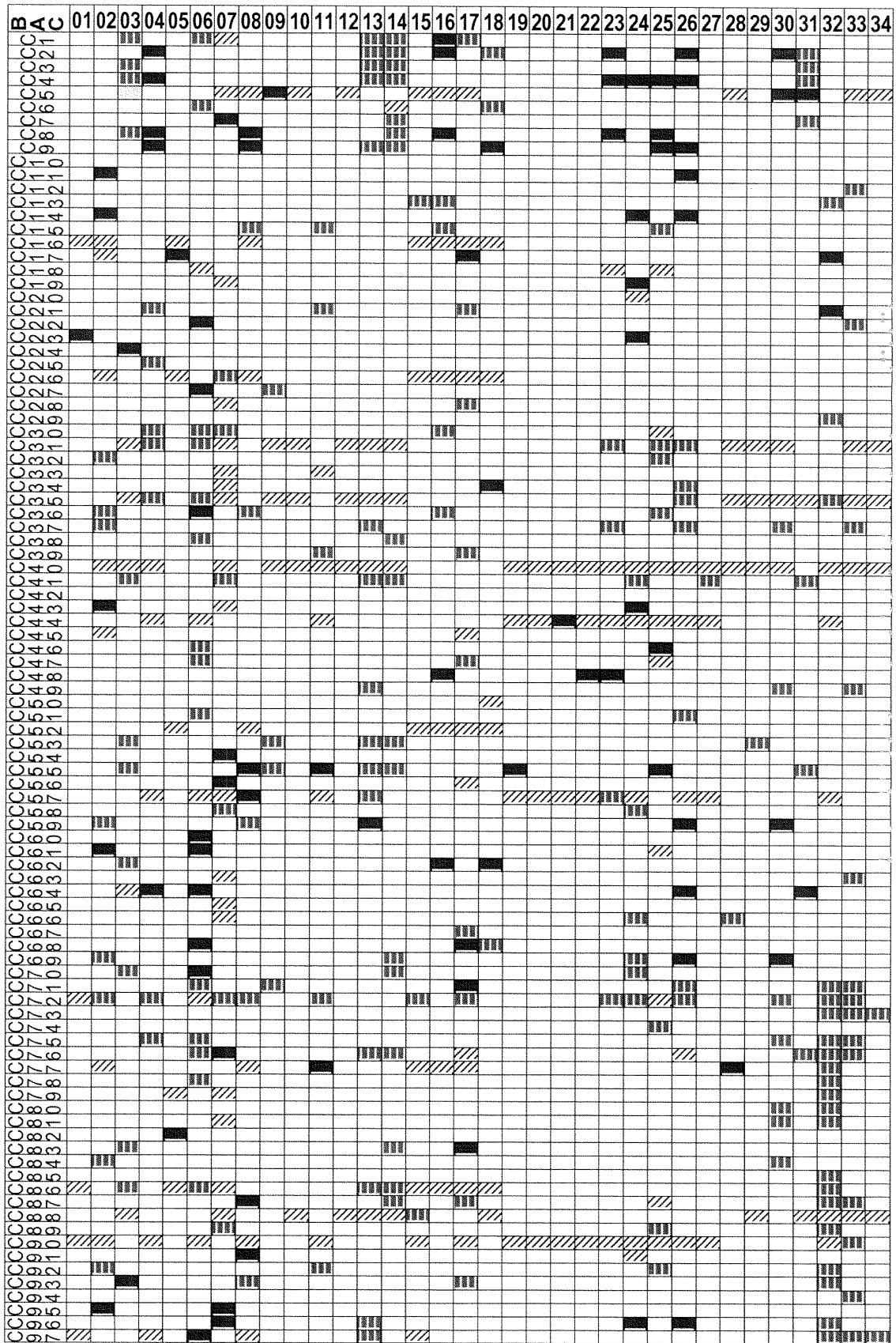


Fig. 1a

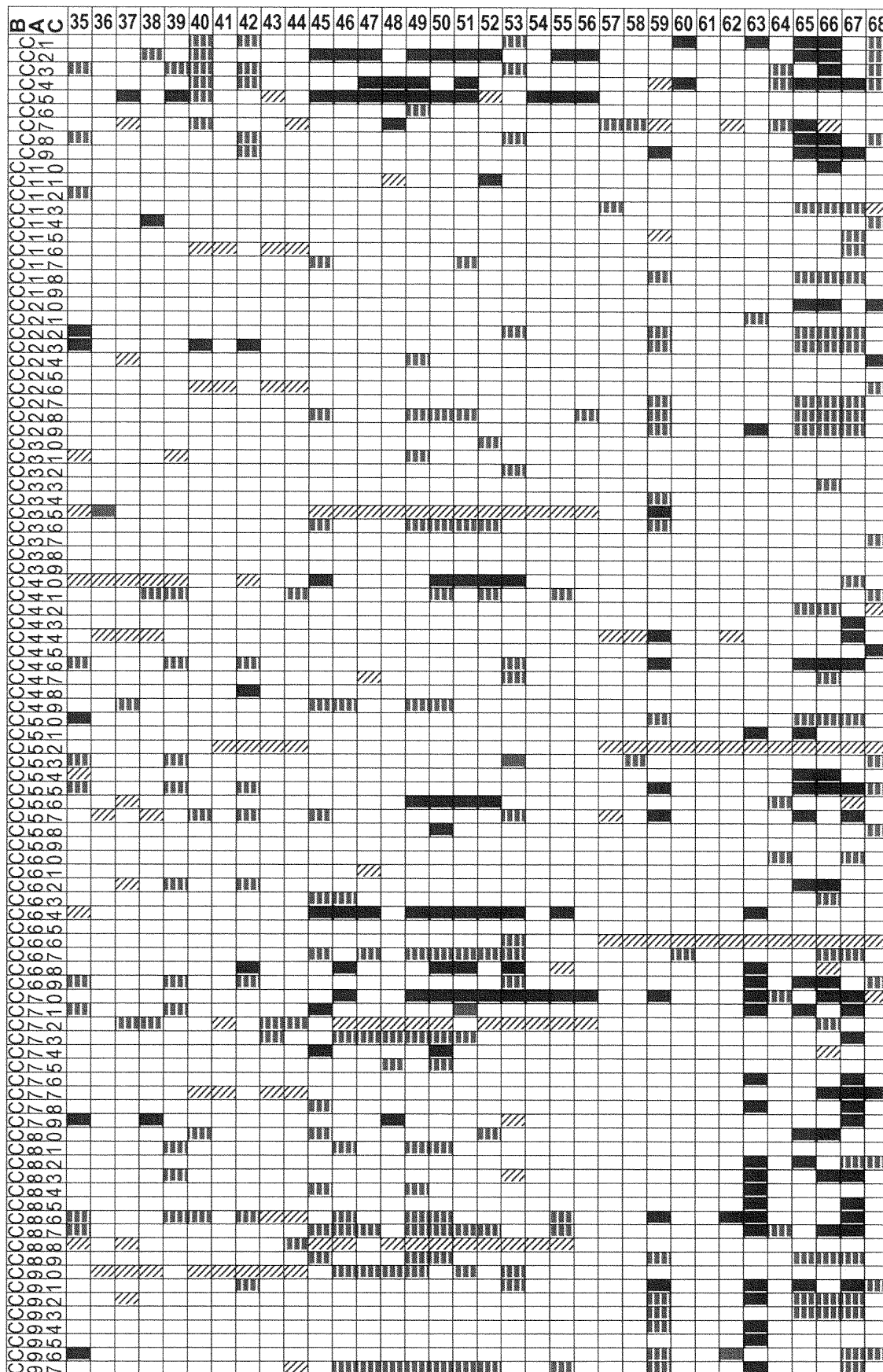


Fig. 1b







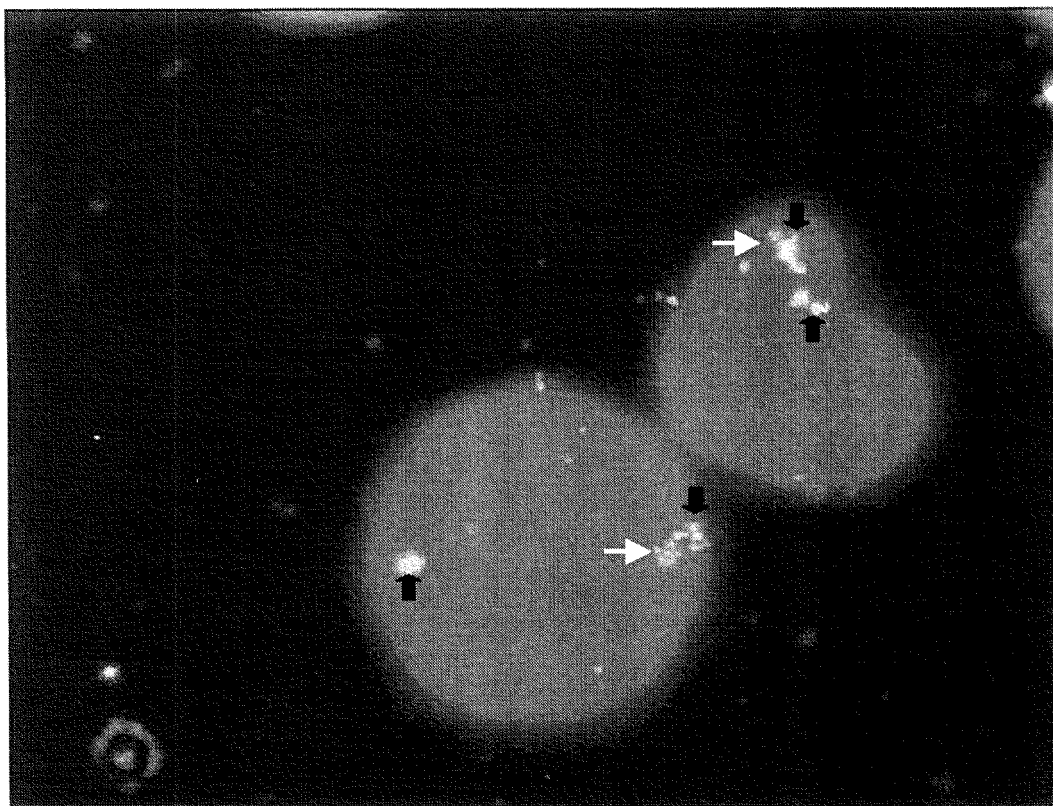


Figura 2

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## LISTA DE SECUENCIAS

<110> FUNDACIÓN DE INVESTIGACIÓN DEL CÁNCER

5 <120> USO DE SONDAS PARA EL DIAGNÓSTICO DEL LINFOMA ESPLÉNICO DE LA ZONA MARGINAL

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10 <160> 7

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25 <222> (1)..(113687)

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OFICINA ESPAÑOLA DE  
PATENTES Y MARCAS

ESPAÑA

① ES 2 284 408

② Nº de solicitud: 200700241

③ Fecha de presentación de la solicitud: 30.01.2007

④ Fecha de prioridad:

## INFORME SOBRE EL ESTADO DE LA TÉCNICA

⑤ Int. Cl.: C12Q 1/68 (2006.01)

### DOCUMENTOS RELEVANTES

Categoría	Documentos citados	Reivindicaciones afectadas
X	ROBLEDO C. et al. "Chromosomal imbalances detected by CGH-array in B-cell splenic marginal zone lymphomas (SMZL)". EUROPEAN JOURNAL OF MEDICAL GENETICS. Octubre 2005. Vol. 48, Nº 4, páginas 520-521.	1,2,5,6, 10-21
Y		3,4,7-9, 22-26
Y	ANDERSEN CLAUS L. et al. "A narrow deletion of 7q is common to HCL, and SMZL, but not CLL". Eur. J. Haematol. Junio 2004. Vol. 72, Nº 6, páginas 390-402.	3,4,7-9, 22-26
A	ANDERSEN CLAUS L. et al. "Recurrent genomic imbalances in B-cell splenic marginal-zone lymphoma revealed by comparative genomic hybridization". Cancer Genetics and Cytogenetics. Enero 2005. Vol. 156, Nº 2, páginas 122-128.	1-26
A	VIAGGI S. et al. "Uncommon cytogenetic findings in a case of splenic marginal zone lymphoma with aggressive clinical course". Cancer Genet Cytogenet. Enero 2004. Vol. 148, Nº 2, páginas 133-136.	1-26
A	HERNANDEZ JM. et al. "Novel genomic imbalances in B-cell splenic marginal zone lymphomas revealed by comparative genomic hybridization and cytogenetics". Am. J. Pathol. Mayo 2001, Vol. 158, Nº 5, páginas 1843-1850.	1-26
A	BASE DE DATOS EMBL_HUM, "En línea". 20 Mayo 2005 (20.05.2000). HAAKENSEN W. "The sequence of Homo Sapiens BAC clone RP11-44M6". Número de Acceso: AC069281. Esta secuencia tiene una identidad del 100% respecto a SEQ ID Nº 1, con solapamiento sobre 18687 nt.	1-26

#### Categoría de los documentos citados

X: de particular relevancia

Y: de particular relevancia combinado con otro/s de la misma categoría

A: refleja el estado de la técnica

O: referido a divulgación no escrita

P: publicado entre la fecha de prioridad y la de presentación de la solicitud

E: documento anterior, pero publicado después de la fecha de presentación de la solicitud

#### El presente informe ha sido realizado

para todas las reivindicaciones

para las reivindicaciones nº:

<b>Fecha de realización del informe</b> 07.05.2007	<b>Examinador</b> J. Manso Tomico	<b>Página</b> 1/1
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