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(54) Título: MÉTODO E KIT PARA QUANTIFICAÇÃO DE MATERIAL DE ORIGEM BOVINA E BUBALINA EM PRODUTOS DE ORIGEM ANIMAL

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(57) Resumo: MÉTODO E KIT PARA QUANTIFICAÇÃO DE MATERIAL DE ORIGEM BOVINA E BUBALINA EM PRODUTOS DE ORIGEM ANIMAL A presente invenção descreve método ekit para quantificação de material de origem bovina ebubalina em produtos alimentícios lácteos ou cárneos edemais produtos de origem animal através de um conjunto de iniciadores/sonda específicos. Para aquantificação de DNA, utiliza-se osistema TaqMan de PCR em Tempo Real como padrão, porém ametodologia também se adequa ao sistema SYBR Green. A presente invenção descreve ainda um novo método para normalização da quantidade total de DNA presente em determinada amostra ea contrução de curvas de calibração de acordo com otipo de material aser analisado.

REIVINDICAÇÕES

1- **MÉTODO PARA QUANTIFICAÇÃO DE MATERIAL DE ORIGEM BOVINA E BUBALINA EM PRODUTOS DE ORIGEM ANIMAL PELA TÉCNICA DE PCR EM TEMPO REAL,** caracterizado por utilizar iniciadores específicos capazes de amplificar moléculas de DNA bubalino e bovino, representados pelas SEQ ID N^{os} 1, 2, 4 e 5.

2- **MÉTODO PARA QUANTIFICAÇÃO DE MATERIAL DE ORIGEM BOVINA E BUBALINA EM PRODUTOS DE ORIGEM ANIMAL PELA TÉCNICA DE PCR EM TEMPO REAL,** de acordo com a reivindicação 1, caracterizado por utilizar, preferencialmente, o sistema TaqMan, com as sondas representadas pelas SEQ ID N^{os} 3 e 6.

3- **MÉTODO PARA QUANTIFICAÇÃO DE MATERIAL DE ORIGEM BOVINA E BUBALINA EM PRODUTOS DE ORIGEM ANIMAL PELA TÉCNICA DE PCR EM TEMPO REAL,** de acordo com as reivindicações 1 e 2, caracterizado pelas sondas serem marcadas com o fluoróforo FAM, não limitante.

4- **MÉTODO PARA QUANTIFICAÇÃO DE MATERIAL DE ORIGEM BOVINA E BUBALINA EM PRODUTOS DE ORIGEM ANIMAL PELA TÉCNICA DE PCR EM TEMPO REAL,** de acordo com a reivindicação 1, caracterizado por utilizar, opcionalmente, o sistema SYBR Green.

5- **MÉTODO PARA QUANTIFICAÇÃO DE MATERIAL DE ORIGEM BOVINA E BUBALINA EM PRODUTOS DE ORIGEM ANIMAL PELA TÉCNICA DE PCR EM TEMPO REAL,** de acordo com as reivindicações 1 a 4, caracterizado pela quantidade total de DNA presente em uma determinada amostra ser calculada através da soma das quantificações específicas obtidas com os iniciadores específicos para amplificação de DNA bovino e bubalino, representados pelas SEQ ID N^{os} 1, 2, 4 e 5, ou iniciadores/sonda, representados pelas SEQ ID N^{os} 1, 2, 3, 4, 5 e 6, utilizando uma curva de calibração de referência.

6- **KIT PARA QUANTIFICAÇÃO DE MATERIAL DE ORIGEM BOVINA E BUBALINA EM PRODUTOS DE ORIGEM ANIMAL PELA TÉCNICA DE PCR EM TEMPO REAL,** caracterizado por compreender iniciadores

específicos para amplificação de DNA bovino e bubalino, representados pelas SEQ ID N^{os} 1, 2, 4 e 5, para utilização nos sistemas TaqMan e SYBR Green, sondas para utilização no sistema TaqMan, representadas pelas SEQ ID N^{os} 3 e 6, e um software que permite o cálculo da quantidade de material bovino ou bubalino presente nas amostras testadas, levando em consideração os desvios computados na curva de calibração de referência.

Espacenet

Bibliographic data: CN104894263 (A) — 2015-09-09

Multiple-species-component real-time fluorescence PCR (polymerase chain reaction) combined detection method

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Abstract of CN104894263 (A)

The invention discloses a real-time fluorescence PCR (polymerase chain reaction) combined detection method for simultaneously determining multiple animal-derived components in a meat product. The method can design species-specific primers according to the mitochondrion gene polymorphism difference among the animal species and simultaneously identify more than 10 species components in one PCR reaction system. The CT value is inversely proportional to the logarithm of the initial DNA (deoxyribonucleic acid) concentration, and thus, the difference of 7 above on the CT value indicates that the template DNA content difference is more than a hundred times, thereby eliminating the false positive result caused by contamination according to such judgment standard. The method can complete all animal-derived component identification on any unknown animal-derived component meat product or multiple-animal-derived-component mixed meat product within the identification rage by a single test, and is suitable for detection and identification of raw and cooked meat products and other meat products processed by various processing methods. The method does not need to perform electrophoresis, thereby shortening the analysis time and reducing the product pollution.

Notice

DESCRIPTION CN104894263A

10 Combined detection method of real-time fluorescent PCR for multi-species components

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12 **多物种成分实时荧光PCR联合检测方法**

[0001]

18 field of invention

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20 **发明领域**

[0002]

26 The invention relates to a real-time fluorescent PCR detection method for meat components in meat products, in particular to a real-time fluorescent PCR combined detection method for simultaneously measuring multiple animal source components in meat products, and belongs to the technical field of food safety detection applications.

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31 **本发明涉及肉制品中肉类成分的实时荧光PCR检测方法，特别是涉及一种同时测定肉制品中多种动物源成分的实时荧光PCR联合检测方法，属于食品安全检测应用技术领域。**

[0003]

38 Background technique

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40 背景技术

[0004]

46 Meat adulteration and counterfeiting is a common phenomenon. Some unscrupulous traders sell low-priced meat instead of high-priced meat, which damages the interests of consumers and causes unfair competition; With the globalization of trade, consumers are more eager to know the exact composition of food, so it is necessary to establish a fast and accurate identification method for meat products.

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51 肉制品掺 假造假 是常见的现象,某些不法商贩以低价肉类代替高价肉类出售,损害了消费者的利益,造成了不正当竞争；随着疯牛病和禽流感等疾病的流行和食品贸易的全球化,消费者更迫切地要求知晓食品的确切成分,因此必须建立快速、准确的肉制品品种鉴定方法。

[0005]

59 At present, the identification methods of meat products mainly include protein identification (isoelectric focusing electrophoresis, enzyme-linked immunosorbent assay, chromatography, etc.) and molecular identification, among which the molecular identification method based on genetic differences between species is a research hotspot The main reasons are: ① DNA has stronger heat resistance than protein, and small fragments of DNA can still be extracted from high-temperature-treated foods, while most proteins will denature, which cannot meet the needs of identification; ② Molecular identification methods do not depend on tissue and cell types; ③ Different gene fragments have different evolutionary efficiencies, and different target genes can be selected for research according to the purpose of the experiment; ④ DNA has higher interspecies polymorphism than protein, which is conducive to species identification.

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69 目前，肉制品品种鉴定方法主要有蛋白质鉴定(等电聚焦电泳、酶联免疫吸附试验、色谱等)和分子学鉴定两类,其中以物种间基因差异为基础的分子学鉴定方法是研究的热点,主要原因是：①DNA比蛋白质的耐热性强,高温处理过的食品中仍能提取出小片段的DNA,而多数蛋白质会发生变性,不能满足鉴定需要；②分子学鉴定方法不依赖于组织和细胞的类型；③不同的基因片段进化效率不同,可以根据实验目的选择不同的目的基因进行研究；④DNA比蛋白质具有更高的种间多态性,有利于品种鉴定。

[0006]

80 The main molecular detection methods currently used for the identification of meat products and their advantages and disadvantages:

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83 目前用于肉制品鉴定的主要分子学检测方法及其优缺点：

[0007]

89 1.

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91 1.

94 PCR-FINS: This method is based on the sequence differences of specific gene fragments to identify species and consists of 4 steps: ① Establish a method for isolating DNA from a wide range of biological samples, including processed foods (canned, semi-processed, pressed, (salted, smoked); ②Use universal primers to amplify specific DNA fragments; ③Sequence the amplified DNA fragments; ④Compare the nucleic acid sequence with the submitted database to directly identify the species or find the database The species most closely related to it.

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101 PCR-FINS：该方法是基于特定基因片段的序列差异来鉴定物种,由4个步骤组成：①建立从广泛的生物样品中分离DNA的方法,包括处理过的食品(罐头、半加工、压制、盐渍、熏制)；②应用通用引物扩增出特定的DNA片段；③对扩增的DNA片段进行测序；④将这个核酸序列与被提交数据库进行序列比对,从而直接鉴定出物种或者找到数据库中与其亲缘关系最接近的物种。

107 Advantages: It can directly determine the species source of unknown ingredients in food.

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109 优点：可以直接判断食品中未知成分的种属来源。

112 Disadvantages: Due to the use of universal primers, multiple components in the mixture are usually amplified at the same time, which will cause confusion in the sequencing results, so it is not suitable for the identification of mixtures.

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116 缺点：由于使用了通用引物,混合物中通常有多种成分被同时扩增,会造成测序结果的杂乱,因此不适用 于混合物的鉴定。

[0008]

123 2.

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125 2.

128 PCR-RFLP: also known as cleavage amplified polymorphic sequence analysis, its basic steps include: PCR amplification of specific gene fragments, restriction endonuclease digestion, electrophoresis of digestion products, comparison of enzyme digestion maps and species identification .

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132 PCR-RFLP：又称为切割扩增多态性序列分析，其基本步骤包括：PCR扩增特定基因片段、限制性内切酶酶切、酶切产物的电泳、酶切 图 谱的比较和物种鉴定。

136 Advantages: simple, fast, cheap, and more suitable for routine testing.

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138 优点：简单、快速、便宜，更适用于常规检测。

141 Disadvantages: It is susceptible to intraspecific polymorphism. If the restriction site is mutated, the restriction map will be changed, resulting in wrong identification results.

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144 缺点：易受种内多态性的影响，如果酶切位点发生了突变，就会改变酶切 图 谱，导致出现 错误的鉴定结果。

[0009]

151 3.

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153 3.

156 Species-specific PCR: Species-specific PCR is based on the design of specific primers based on the differences in gene sequences between species, so that the primers can only amplify a fragment of a specific length in a specific species, and there is no corresponding fragment in any other species According to the presence or absence of amplified fragments, species identification can be realized.

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161 物种特异性PCR：物种特异性PCR是根据物种之间基因序列的差异设计特异性引物,使该引物只能在特定的物种中扩增出特定长度的片段,在其它任何物种中都没有相应片段的出现,根据扩增片段的有无实现物种鉴定。

166 Advantages: The source of species can be directly determined through electrophoresis results, without subsequent sequencing or enzyme digestion treatment, simpler and faster, and can be used for routine detection of large samples. Disadvantages: PCR products are prone to aerosol contamination.

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170 优点：可以通过电泳结果直接判定物种来源,不需要后续的测序或酶切处理,更简单、快速,可用于大样本的常规检测。 缺点：PCR产物容易发生气溶胶污染。

[0010]

177 4.

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179 4.

182 Fluorescent real-time quantitative PCR: This technology uses fluorescent energy transfer technology on the basis of conventional PCR, adding fluorescent labeled probes or fluorescent dyes, and detecting PCR products with the help of fluorescent signals. According to the different fluorescent labels, it can be divided into two categories: one is the detection of specific sequences, quantified by fluorescently labeled specific hybridization probes, such as TaqMan. The other is the detection of non-specific sequences, using DNA binding dyes, such as SYBR green, which increases the fluorescence signal after binding to the small groove of double-stranded DNA, and the binding process has nothing to do with the sequence.

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190 荧光实时定量PCR:该技术是在常规PCR基础上运用荧光能量传递技术,加入荧光标记探针或荧光染料,借助于荧光信号来检测PCR产物。 根据荧光标记的不同,可以分为两类：一类是对特异性序列的检测,用荧光标记的特异性杂交探针定量,如TaqMan。 另一类是对非特异性序列的检测,应用DNA结合染料,如SYBR　green,它与双链DNA的小槽结合后荧光信号增强,结合过程与序列无关。

[0011]

199 The advantages of fluorescent real-time quantitative PCR are: ① Quantitative detection can be realized.

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201 荧光实时定量PCR的优势在于:①可实现定量检测。

204 ② Its fluorescence data can be directly detected by real-time PCR instrument or fluorescence detector, without electrophoresis, which shortens the analysis time and reduces product contamination. ③Applicable

to the amplification of small fragments, which is especially important in the detection of processed foods, especially foods with severe DNA degradation.

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209 ②其荧光数据可以通过实时PCR仪器或荧光检测器直接检测,不需要进行电泳,缩短了分析时间，减少产物污染。③适用于小片段的扩增,这在检测处理过的食品尤其是DNA降解严重的食品时尤为重要。

[0012]

217 All of the above methods can be applied to the identification of a single species, but the cost of PCR detection for a single species is high, and there may be other species in meat products. A method in which multiple pairs of primers are added to the reaction system to simultaneously amplify multiple DNA fragments.

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221 以上方法都可以应用于单一物种的鉴定，但单一物种的PCR检测成本高，而肉品中可能存在其它品种，单重PCR已经满足不了现有的要求，进而出现了多重PCR技术，即在同一反应体系中加入多对引物同时扩增多条目的DNA片段的方法。

226 It has been reported that multiplex PCR technology is used to detect 18 common animal species. However, according to the size of the PCR products, capillary electrophoresis is used to separate the fragments to determine the species. The subsequent processing is cumbersome, and multiplex PCR is also difficult There are some disadvantages, such as the low amplification efficiency caused by the existence of multiple pairs of primers in the system, and the inconsistent amplification efficiency between different templates, etc., which limit the commercial application of this technology. Therefore, it has always been a problem to be solved to establish a rapid, sensitive and specific detection method for the detection of unknown raw meat varieties, and the combined detection method is expected to solve this problem.

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235 已有报道有利用多重PCR技术对18种常见动物种类进行检测的技术，但要根据其PCR产物大小不同，用毛细管电泳对其片段进行分离后判断物种的种类，后续处理繁琐，且多重PCR也存在一些不足，如体系 中存在多对引物而导致的扩增效率较低，不同模板之间的扩增效率不一致等，这些都限制了此项技术的商业化应用。因此，建立一种能快速、灵敏、特异的检测未知生肉品种的检测方法一直是有待解决的难题，而联合检测方法将有望解决这一难题。

[0013]

245 The so-called joint detection means that all possible results can be obtained in one detection using the same reaction system and reaction conditions.

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248 所谓联合检测，即使用相同的反应体系和反应条件，一次检测可以得到所有可能的结果。

251 This method is different from the multiplex PCR technique, each reaction is carried out in a different reaction tube, avoiding mutual interference between primers. The difficulty of joint detection is that the designed primers should be highly specific to species, not have cross-reaction, and should be optimized to the same PCR reaction conditions. At present, the use of joint detection to identify the species of meat products has been reported using Taqman probes to determine the components of beef, pork, mutton, chicken, turkey, and ostrich meat in the mixture, and it has also been reported that the SYBR Green method was used to successfully identify mixed meat species. Red deer, European deer, roe deer, chamois and Pyrenees. However, there are still some problems in the above detection: the probe method has high requirements for probe design, and because the detection sensitivity is too high, the requirements for experimental operation are high, and some tiny pollution may also be detected. The dye method has high requirements for the design of primers, which is prone to non-specific amplification, and false positives cannot be ruled out only by the interpretation of melting curves.

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264 此方法不同于多重PCR技术，每个反应是在不同的反应管中进行，避免了引物之间的相互干扰。联合检测的难点在于设计的引物要物种特异性强，不能有交叉反应，且要能优化到同一PCR反应条件。 目前报道的利用联合检测鉴定肉制品种属的有应用Taqman探针对混合物中牛肉、猪肉、羊肉、鸡肉、火鸡肉、鸵鸟肉成分进行了测定，也有报道用SYBR　Green法成功鉴别混合肉种的马鹿、欧洲鹿、狍、岩羚羊和比利牛斯山羊。 但以上检测也存在一些问题：探针法对探针设计要求较高，且因检测灵敏度太高,对实验操作的要求较高，一些微小的污染也可能被检测出来。染料法对引物的设计要求较高，容易有非特异性扩增，且仅以溶解曲线判读结果不能排除假阳性。

[0014]

277 In view of the above problems, the present invention proposes a real-time fluorescent PCR combined detection method for simultaneous determination of multiple animal-derived components in meat products: by designing specific primers for multiple species, multiple species can be detected simultaneously in one PCR reaction system. It is especially suitable for the detection of mixed components in meat products.

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282 针对以上问题，本发明提出一种同时测定肉制品中多种动物源成分的实时荧光PCR联合检测方法：通过对多个物种分别设计特异性引物，可以在一个PCR反应体系内同时对多个物种的特异性片段进行扩增，特别适用于目前肉制品中混合成分的检出。

287 Fluorescent signals are detected by adding fluorescent dyes, without the need to design specific probes, which saves costs. Finally, negative and positive results are determined by comparing the amplified CT value with the internal reference to eliminate false positive results caused by contamination. The characteristics of this

method: 1. By designing specific primers for multiple species, the specific fragments of multiple species can be amplified simultaneously in one PCR reaction system without interference to the fluorescent signal, especially suitable for meat and identification of mixed ingredients in meat products.

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294 通过加入荧光染料来检测荧光信号，不用设计特异性探针，节约成本，最后通过扩增的CT值与内参的对照来确定阴阳性，排除因沾染造成的假阳性结果。 该方法的特点：1、通过对多个物种分别设计特异性引物，可以在一个PCR反应体系内同时对多个物种的特异性片段进行扩增，且对荧光信号无干扰，特别适用于肉类及肉制品中混合成分的鉴定。

2、

303 2. The reaction tube has been integrated, and only need to add template to perform PCR detection when using, which is easy to operate.

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306 反应管已集成化，使用时仅需加入模板即可进行PCR检测，操作简便。

3、

312 3. Determine the negative and positive results by comparing with the internal reference to eliminate false positive results caused by contamination.

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315 通过与内参对照判定阴阳性，排除因沾染造成的假阳性结果。

4、

321 4. The types of meat products identified and the number of test samples can be flexibly combined according to needs.

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324 可以根据需要灵活组合鉴定的肉制品种类和检测样本的份数。

[0015]

330 Contents of the invention

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332 发明内容

[0016]

338 The technical problem to be solved by the present invention is to overcome the deficiencies of the prior art, to provide a real-time fluorescent PCR combined detection method for simultaneously measuring multiple animal-derived components in meat products, to design specific primers for multiple species, and to Specific fragments of multiple species are simultaneously amplified in the reaction system.

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343 本发明所要解决的技术问题是克服现有技术的不足，提供一种同时测定肉制品中多种动物源成分的实时荧光PCR联合检测方法，针对多个物种分别 设计特异性引物，并在一个PCR反应体系内同时对多个物种的特异性片段进行扩增。

348 Combined with the use of fluorescent dyes, negative and positive can be directly interpreted.

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350 结合荧光染料的使用，可以直接判读阴阳性。

353 The method is low in cost, time-saving and efficient, and can identify multiple components at the same time.

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355 该方法成本低、省时、高效，可对多种成分同时鉴别。

[0017]

361 For solving above technical problem, the present invention adopts following technical scheme: 1.

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363 为解决以上技术问题，本发明采用如下技术方案：1.

366 A real-time fluorescent PCR combined detection method for simultaneous determination of multi-species components in products. Species-specific primers are designed according to the differences in genetic polymorphisms between species on mitochondrial DNA, and the reaction conditions are optimized to the same system, that is, it can be used in one In the PCR system, multiple species components are identified at the same time, the fluorescent signal is detected by adding fluorescent dyes, and finally the negative and positive are determined by comparing the amplified CT value with the internal reference; this method simultaneously detects more than 10 species of animals in meat and meat products source components were tested.

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375 一种同时测定产品中多物种成分的实时荧光PCR联合检测方法，根据线粒体DNA上物种间基因多态性的差异设计各物种特异性的引物，并将反应条件优化到同一体系，即可以在一个PCR体系中同时对多种物种成分进行鉴定，通过加入荧光染料来检测荧光信号，最后用扩增的CT值与内参的对照来确定阴阳性；该方法同时对肉及肉制品中10种以上动物源性成分进行检测。

[0018]

384 The sequence of more than 10 species-specific primers and one internal reference primer included in the real-time fluorescent PCR method.

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387 所述实时荧光PCR法包含的10个以上物种的特异性引物和1个内参引物的序列。

[0019]

393 In the real-time fluorescent PCR detection method, the detection of multiple animal source components is completed once in a PCR reaction system and under the same reaction conditions.

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396 所述实时荧光PCR检测方法对多种动物源成分的检测是在一个PCR反应体系中、同一个反应条件下一次完成的。

[0020]

403 The fluorescent dye used in the real-time fluorescent PCR method is SYBR Green, but not limited to SYBR Green, and can also be fluorescent dyes such as Eva Green and LC Green, and the specificity of amplification is analyzed by reading the melting curve.

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407 所述实时荧光PCR法的所用荧光染料SYBR Green，但不限于SYBR Green，也可以是Eva Green、LC Green等荧光染料，并通过对溶解曲线的判读分析扩增的特异性。

[0021]

414 The method for interpreting the results of the real-time fluorescent PCR method: use the amplified CT value and the comparison of the internal reference to determine negative and positive; the direct interpretation

without CT value is negative; the amplified CT value and the internal reference primer amplified CT value For comparison, the interpretation of greater than or equal to 7 is negative, and the interpretation of less than 7 is positive.

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420 所述实时荧光PCR法的结果的判读方法：用扩增的CT值与内参的对照来确定阴阳性；无CT值的直接判读为阴性；有扩增CT值的与内参引物扩增CT值进行比较，大于等于7的判读为阴性，小于7的判读为阳性。

[0022]

428 The real-time fluorescent PCR method is applied to the detection of any species in more than 10 species.

---

430 所述实时荧光PCR法应用于10个以上物种中的任一物种检测。

[0023]

436 The real-time fluorescent PCR method is applied to the detection of any mixed species among more than 10 species.

---

439 所述实时荧光PCR法应用于10个以上物种中的任意混合物种的检测。

[0024]

445 Advantage of the present invention and beneficial effect are:

---

447 本发明的优点和有益效果为：

[0025]

453 1. By designing specific primers for multiple species, the specific fragments of multiple species can be amplified simultaneously in one PCR reaction system without interference to the fluorescent signal, especially suitable for mixing in meat and meat products Identification of ingredients.

---

457 1、通过对多个物种分别设计特异性引物，可以在一个PCR反应体系内同时对多个物种的特异性片

段进行扩增，且对荧光信号无干扰，特别适用于肉类及肉制品中混合成分的鉴定。

[0026]

464 2. The reaction tube has been integrated, and only need to add template to perform PCR detection when using, which is easy to operate.

---

467 2、反应管已集成化，使用时仅需加入模板即可进行PCR检测，操作简便。

[0027]

473 3. Determine the negative and positive results by comparing with the internal reference to eliminate false positive results caused by contamination.

---

476 3、通过与内参对照判定阴阳性，排除因沾染造成的假阳性结果。

[0028]

482 4. The types of meat products identified and the number of test samples can be flexibly combined according to needs.

---

485 4、可以根据需要灵活组合鉴定的肉制品种类和检测样本的份数。

[0029]

491 Detailed ways

---

493 具体实施方式

[0030]

499 The following will clearly and completely describe the technical solutions in the embodiments of the present

invention. Obviously, the described embodiments are only some of the embodiments of the present invention, rather than all the embodiments.

---

503 下面将对本发明实施例中的技术方案进行清楚、完整地描述，显然，所描述的实施例仅是本发明的一部分实施例，而不是全部的实施例。

507 Based on the embodiments of the present invention, all other embodiments obtained by persons of ordinary skill in the art without making creative efforts belong to the protection scope of the present invention.

---

510 基于本发明中的实施例，本领域普通技术人员在没有做出创造性劳动前提下所获得的所有其它实施例，都属于本发明保护的范围。

[0031]

517 A real-time fluorescent PCR combined detection method for simultaneous determination of multiple animal-derived components in meat products. The identification method is: design specific primers for each species according to the differences in polymorphisms between animal species in mitochondrial genes, and optimize the reaction conditions To the same system, more than 10 species components can be identified in a PCR system at the same time, the fluorescent signal is detected by adding the fluorescent dye SYBR, and finally the negative and positive are determined by comparing the amplified CT value with the internal reference.

---

524 一种同时测定肉制品中多种动物源成分的实时荧光PCR联合检测方法，其鉴定方法为：根据线粒体基因上动物种间多态性的差异设计各物种特异性的引物，并将反应条件优化到同一体系，即可以在一个PCR体系中同时对10个以上的物种成分进行鉴定，通过加入荧光染料SYBR来检测荧光信号，最后用扩增的CT值与内参的对照来确定阴阳性。

530 The RT-PCR method can be used for single meat products or multiple animal components of various animal-derived components (pigs, cattle, sheep, dogs, rabbits, cats, chickens, ducks, geese, mice, etc.) Mixed meat products are tested and identified.

---

534 该RT-PCR方法可以同时对肉及肉制品中多种动物源性成分(猪、牛、羊、狗、兔、猫、鸡、鸭、鹅、鼠等)的单一肉制品或多种动物的混合肉制品进行检测鉴定。

[0032]

541 Further, the sequence of the species-specific primer and one internal reference primer of the real-time

fluorescent PCR method.

---

544 进一步的，所述实时荧光PCR法的物种特异性引物和1个内参引物的序列。

[0033]

550

553

[0034]

560

563

[0035]

570 Further, the PCR reaction system and conditions of the real-time fluorescent PCR method.

---

572 进一步的，所述实时荧光PCR法的PCR反应体系和条件。

[0036]

578 PCR system:

---

580 PCR体系：

[0037]

586

589

[0038]

596 PCR conditions:

---

598 PCR条件：

[0039]

604

607

[0040]

614

617

[0041]

624 The fluorescent dye used in the real-time fluorescent PCR method is SYBR, and the specificity of amplification is analyzed by reading the melting curve.

---

627 所述实时荧光PCR法的所用的荧光染料SYBR，并通过对溶解曲线的判读分析扩增的特异性。

630 The method for interpreting the results of the real-time fluorescent PCR method: use the amplified CT value to compare with the internal reference to determine negative or positive.

---

633 所述实时荧光PCR法的结果的判读方法：用扩增的CT值与内参的对照来确定阴阳性。

636 The direct interpretation without CT value was negative; the amplification CT value was compared with the internal reference primer amplification CT value, and the interpretation of greater than or equal to 7 was negative, and the interpretation of less than 7 was positive.

---

640 无CT值的直接判读为阴性；有扩增CT值的与内参引物扩增CT值进行比较，大于等于7的判读为阴性，小于7的判读为阳性。

644 The CT value is inversely proportional to the logarithm of the initial DNA concentration, so a difference of more than 7 in the CT value indicates a difference of more than 100 times in the template DNA content, and this criterion can exclude false positive results caused by contamination.

---

648 CT值与起始DNA浓度的对数成反比，因此CT值相差7以 上表 示模板DNA含量相差百倍以上，以此判断标准可以排除因沾染造成的假阳性结果。

652 The real-time fluorescent PCR method is applicable to the detection of any species in multiple species.

---

654 所述实时荧光PCR法的应用于多个物种中的任一物种检测。

657 The real-time fluorescent PCR method is applicable to the detection of any mixed species among multiple species.

---

660 所述实时荧光PCR法的应用于多个物种中的任意混合物种的检测。

[0042]

666 Example 1

---

668 **实施例1**

[0043]

674 Single Component Detection:

---

676 **单一成分检测：**

[0044]

682 **1.**

---

684 **1.**

687 Material:

---

689 **材料：**

[0046]

695 [0042]

---

697 [0042]

[0047]

703 2.

---

705 2.

708 Genomic DNA extraction: Use the OMEGA tissue DNA extraction kit to extract the genomic DNA of each of the above materials.

---

711 基因组DNA提取：用OMEGA组织DNA提取试剂盒提取以上各个材料的基因组DNA。

[0048]

717 3.

---

719 3.

722 PCR amplification reaction:

---

724 PCR扩增反应：

[0049]

730 Prepare the PCR reaction solution according to the following components, and finally add the sample DNA.

---

732 按下列组分分别配制PCR反应液，最后加入样本DNA。

[0050]

738

741

[0051]

748 Carry out PCR reaction according to the following conditions:

---

750 按下列条件进行PCR反应：

[0052]

756

759

[0053]

766 4.

---

768 4.

771 Result analysis:

---

773 **结果分析：**

[0054]

779 Check whether the amplification curve and melting curve are normal, and whether the baseline and threshold need to be adjusted manually.

---

782 **检查扩增曲线和溶解曲线是否正常，是否需要手动调整基线和阈值。**

785 After the curve is adjusted, read the CT value of the amplification curve.

---

787 **待调整好曲线后读取扩增曲线的CT值。**

[0055]

793 5.

---

795 5.

798 Result judgment:

---

800 **结果判定：**

[0056]

806

809

[0057]

816

819

[0058]

826 The results are judged as follows: 1-pig 2-cow 3-sheep 4-chicken 5-duck 6-goose 7-dog 8-rabbit 9-cat 10-rat

---

828 **结果判定如下：1-猪 2-牛 3-羊 4-鸡 5-鸭 6-鹅 7-狗 8-兔 9-猫 10-鼠**

[0059]

835 Example 2

---

837 实施例2

[0060]

843 Mixture detection:

---

845 混合成分检测：

[0061]

851 1.

---

853 1.

856 Material:

---

858 材料：

[0063]

864 2.

---

866 2.

869 Genomic DNA extraction: Use the OMEGA tissue DNA extraction kit to extract the genomic DNA of each of the above materials.

---

872 基因组DNA提取：用OMEGA组织DNA提取试剂盒提取以上各个材料的基因组DNA。

[0064]

878 3.

---

880 3.

883 PCR amplification reaction:

---

885 PCR扩增反应：

[0065]

891 Prepare the PCR reaction solution according to the following components, and finally add the sample DNA.

---

893 按下列组分分别配制PCR反应液，最后加入样本DNA。

[0066]

899

902

[0067]

909

912

[0068]

919 Carry out PCR reaction according to the following conditions:

---

921 按下列条件进行PCR反应：

[0069]

927

930

[0070]

937 4.

---

939 4.

942 Result analysis: Check whether the amplification curve and melting curve are normal, and whether the baseline and threshold need to be adjusted manually.

---

945 结果分析：检查扩增曲线和溶解曲线是否正常，是否需要手动调整基线和阈值。

948 After the curve is adjusted, read the CT value of the amplification curve.

---

950 待调整好曲线后读取扩增曲线的CT值。

[0071]

956 5.

---

958 5.

961 Result judgment:

---

963 结果判定:

[0072]

969

972

[0073]

979 The results are judged as follows: 1-mixed ingredients of pigs and cattle 2-mixed ingredients of cattle and sheep 3-mixed ingredients of pigs and sheep 4-mixed ingredients of pigs and chickens.

---

982 结果判定如下：1-猪、牛混合成分　2-牛、羊混合成分　3-猪、羊混合成分4-猪、鸡混合成分。

[0074]

988 It will be apparent to those skilled in the art that the invention is not limited to the details of the above-described exemplary embodiments, but that the invention can be embodied in other specific forms without departing from the spirit or essential characteristics of the invention.

---

992 对于本领域技术人员而言，显然本发明不限于上述示范性实施例的细节，而且在不背离本发明的精神或基本特征的情况下，能够以其他的具体形式实现本发明。

996 Therefore, no matter from all points of view, the inventive examples should be regarded as exemplary and non-restrictive, and the scope of the present invention is defined by the appended claims rather than the above description, so it is intended that the scope of the invention will fall within the scope of the claims. All changes within the meaning and range of equivalents of the elements are embraced in the present invention.

1001 因此，无论从哪一点来看，均应将发明例看作是示范性的，而且是非限制性的，本发明的范围由所附权利要求而不是上述说明限定，因此旨在将落在权利要求的等同要件的含义和范围内的所有变化囊括在本发明内。

Notice

CLAIMS CN104894263A

1.

13 A real-time fluorescent PCR combined detection method for simultaneous determination of multi-species components in a product, characterized in that: Species-specific primers are designed according to the differences in genetic polymorphisms between species on mitochondrial DNA, and the reaction conditions are optimized to the same system, That is to say, multiple species components can be identified in a PCR system at the same time, the fluorescent signal is detected by adding fluorescent dyes, and finally the negative and positive are determined by comparing the amplified CT value with the internal reference; More than 10 kinds of animal-derived ingredients were tested.

---

21 一种同时测定产品中多物种成分的实时荧光PCR联合检测方法，其特征在于：根据线粒体DNA上物种间基因多态性的差异设计各物种特异性的引物，并将反应条件优化到同一体系，即可以在一个PCR体系中同时对多种物种成分进行鉴定，通过加入荧光染料来检测荧光信号，最后用扩增的CT值与内参的对照来确定阴阳性；该方法同时对肉及肉制品中10种以上动物源性成分进行检测。

2.

30 A real-time fluorescent PCR combined detection method for simultaneous determination of multiple animal-derived components in meat products according to claim 1, characterized in that: the real-time fluorescent PCR method includes more than 10 species-specific primers and one Sequences of internal reference primers.

---

34 根据权利要求1所述的一种同时测定肉制品中多种动物源成分的实时荧光PCR联合检测方法，其特征在于：所述实时荧光PCR法包含的10个以上物种的特异性引物和1个内参引物的序列。

3.

41 A real-time fluorescent PCR combined detection method for simultaneous determination of multiple animal-derived components in meat products according to claim 1 or 2, characterized in that: the real-time fluorescent PCR detection method detects multiple animal-derived components in In a PCR reaction system, it is completed once under the same reaction conditions.

---

46 根据权利要求1或2所述的一种同时测定肉制品中多种动物源成分的实时荧光PCR联合检测方法，其特征在于：所述实时荧光PCR检测方法对多种动物源成分的检测是在一个PCR反应体系中、同一个反应条件下一次完成的。

4.

54 A real-time fluorescent PCR combined detection method for simultaneous determination of multiple animal-derived components in meat products according to claim 3, characterized in that: the fluorescent dye used in the real-time fluorescent PCR method is SYBR Green, but not limited to SYBR Green, It can also be fluorescent dyes such as Eva Green and LC Green, and the specificity of amplification can be analyzed by reading the melting curve.

---

60 根据权利要求3所述的一种同时测定肉制品中多种动物源成分的实时荧光PCR联合检测方法，其特征在于：所述实时荧光PCR法的所用荧光染料SYBR Green，但不限于SYBR Green，也可以是Eva Green、LC Green等荧光染料，并通过对溶解曲线的判读分析扩增的特异性。

5.

68 According to claim 1 or 4, a real-time fluorescent PCR combined detection method for simultaneous determination of multiple animal-derived components in meat products is characterized in that: the method for interpreting the results of the real-time fluorescent PCR method: using amplified CT Values were compared with internal reference to determine negative and positive; no CT value was directly interpreted as negative; those with amplified CT value were compared with the amplified CT value of internal reference primers, and those greater than or equal to 7 were interpreted as negative, and those less than 7 were interpreted as positive.

---

76 根据权利要求1或4所述的一种同时测定肉制品中多种动物源成分的实时荧光PCR联合检测方法，其特征在于：所述实时荧光PCR法的结果的判读方法：用扩增的CT值与内参的对照来确定阴阳性；无CT值的直接判读为阴性；有扩增CT值的与内参引物扩增CT值进行比较，大于等于7的判读为阴性，小于7的判读为阳性。

6.

85 A real-time fluorescent PCR combined detection method for simultaneous determination of multiple animal-derived components in meat products according to claim 5, characterized in that: the real-time fluorescent PCR method is applied to any one of more than 10 species detection.

---

89 根据权利要求5所述的一种同时测定肉制品中多种动物源成分的实时荧光PCR联合检测方法，其特征在于：所述实时荧光PCR法应用于10个以上物种中的任一物种检测。

7.

96 A real-time fluorescent PCR combined detection method for simultaneous determination of multiple animal-derived components in meat products according to claim 6, characterized in that: the real-time fluorescent PCR method is applied to the detection of any mixed species among more than 10 species .

---

100 根据权利要求6所述的一种同时测定肉制品中多种动物源成分的实时荧光PCR联合检测方法，其特征在于：所述实时荧光PCR法应用于10个以上物种中的任意混合物种的检测。

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(54)发明名称

多物种成分实时荧光PCR联合检测方法

(57)摘要

本发明公开一种同时测定肉制品中多种动物源成分的实时荧光PCR联合检测方法，该方法根据线粒体基因上动物种间多态性的差异设计各物种特异性的引物，即可实现在一个PCR反应体系中同时对10个以上的物种成分进行鉴定。CT值与起始DNA浓度的对数成反比，因此其CT值相差7以上表示模板DNA含量相差百倍以上，以此判断标准可以排除因沾染造成的假阳性结果。本发明可以对鉴定范围内的任何一种未知动物源成分的肉制品，或多种动物源成分的混合肉制品一次试验完成全部动物源成分鉴定，适用于生、熟及各种加工方法制备的肉制品的检测鉴定。该方法不需要进行电泳，从而缩短了分析时间，减少了产物污染。

CN 104894263 A

1. 一种同时测定产品中多物种成分的实时荧光 PCR 联合检测方法，其特征在于：根据线粒体 DNA 上物种间基因多态性的差异设计各物种特异性的引物，并将反应条件优化到同一体系，即可以在一个 PCR 体系中同时对多种物种成分进行鉴定，通过加入荧光染料来检测荧光信号，最后用扩增的 CT 值与内参的对照来确定阴阳性；该方法同时对肉及肉制品中 10 种以上动物源性成分进行检测。

2. 根据权利要求 1 所述的一种同时测定肉制品中多种动物源成分的实时荧光 PCR 联合检测方法，其特征在于：所述实时荧光 PCR 法包含的 10 个以上物种的特异性引物和 1 个内参引物的序列。

3. 根据权利要求 1 或 2 所述的一种同时测定肉制品中多种动物源成分的实时荧光 PCR 联合检测方法，其特征在于：所述实时荧光 PCR 检测方法对多种动物源成分的检测是在一个 PCR 反应体系中、同一个反应条件下一次完成的。

4. 根据权利要求 3 所述的一种同时测定肉制品中多种动物源成分的实时荧光 PCR 联合检测方法，其特征在于：所述实时荧光 PCR 法的所用荧光染料 SYBR Green，但不限于 SYBR Green，也可以是 Eva Green、LC Green 等荧光染料，并通过对溶解曲线的判读分析扩增的特异性。

5. 根据权利要求 1 或 4 所述的一种同时测定肉制品中多种动物源成分的实时荧光 PCR 联合检测方法，其特征在于：所述实时荧光 PCR 法的结果的判读方法：用扩增的 CT 值与内参的对照来确定阴阳性；无 CT 值的直接判读为阴性；有扩增 CT 值的与内参引物扩增 CT 值进行比较，大于等于 7 的判读为阴性，小于 7 的判读为阳性。

6. 根据权利要求 5 所述的一种同时测定肉制品中多种动物源成分的实时荧光 PCR 联合检测方法，其特征在于：所述实时荧光 PCR 法应用于 10 个以上物种中的任一物种检测。

7. 根据权利要求 6 所述的一种同时测定肉制品中多种动物源成分的实时荧光 PCR 联合检测方法，其特征在于：所述实时荧光 PCR 法应用于 10 个以上物种中的任意混合物种的检测。

多物种成分实时荧光 PCR 联合检测方法

发明领域

[0001] 本发明涉及肉制品中肉类成分的实时荧光 PCR 检测方法，特别是涉及一种同时测定肉制品中多种动物源成分的实时荧光 PCR 联合检测方法，属于食品安全检测应用技术领域。

背景技术

[0002] 肉制品掺假造假是常见的现象，某些不法商贩以低价肉类代替高价肉类出售，损害了消费者的利益，造成了不正当竞争；随着疯牛病和禽流感等疾病的流行和食品贸易的全球化，消费者更迫切地要求知晓食品的确切成分，因此必须建立快速、准确的肉制品品种鉴定方法。

[0003] 目前，肉制品品种鉴定方法主要有蛋白质鉴定（等电聚焦电泳、酶联免疫吸附试验、色谱等）和分子学鉴定两类，其中以物种间基因差异为基础的分子学鉴定方法是研究的热点，主要原因是：① DNA 比蛋白质的耐热性强，高温处理过的食品中仍能提取出小片段的 DNA，而多数蛋白质会发生变性，不能满足鉴定需要；②分子学鉴定方法不依赖于组织和细胞的类型；③不同的基因片段进化效率不同，可以根据实验目的选择不同的目的基因进行研究；④ DNA 比蛋白质具有更高的种间多态性，有利于品种鉴定。

[0004] 目前用于肉制品鉴定的主要分子学检测方法及其优缺点：

[0005] 1. PCR-FINS：该方法是基于特定基因片段的序列差异来鉴定物种，由 4 个步骤组成：①建立从广泛的生物样品中分离 DNA 的方法，包括处理过的食品（罐头、半加工、压制、盐渍、熏制）；②应用通用引物扩增出特定的 DNA 片段；③对扩增的 DNA 片段进行测序；④将这个核酸序列与被提交数据库进行序列比对，从而直接鉴定出物种或者找到数据库中与其亲缘关系最接近的物种。优点：可以直接判断食品中未知成分的种属来源。缺点：由于使用了通用引物，混合物中通常有多种成分被同时扩增，会造成测序结果的杂乱，因此不适用 于混合物的鉴定。

[0006] 2. PCR-RFLP：又称为切割扩增多态性序列分析，其基本步骤包括：PCR 扩增特定基因片段、限制性内切酶酶切、酶切产物的电泳、酶切图谱的比较和物种鉴定。优点：简单、快速、便宜，更适用于常规检测。缺点：易受种内多态性的影响，如果酶切位点发生了突变，就会改变酶切图谱，导致出现错误的鉴定结果。

[0007] 3. 物种特异性 PCR：物种特异性 PCR 是根据物种之间基因序列的差异设计特异性引物，使该引物只能在特定的物种中扩增出特定长度的片段，在其它任何物种中都没有相应片段的出现，根据扩增片段的有无实现物种鉴定。优点：可以通过电泳结果直接判定物种来源，不需要后续的测序或酶切处理，更简单、快速，可用于大样本的常规检测。缺点：PCR 产物容易发生气溶胶污染。

[0008] 4. 荧光实时定量 PCR：该技术是在常规 PCR 基础上运用荧光能量传递技术，加入荧光标记探针或荧光染料，借助于荧光信号来检测 PCR 产物。根据荧光标记的不同，可以分为两类：一类是对特异性序列的检测，用荧光标记的特异性杂交探针定量，如 TaqMan。

另一类是对非特异性序列的检测，应用 DNA 结合染料，如 SYBR green，它与双链 DNA 的小槽结合后荧光信号增强，结合过程与序列无关。

[0009] 荧光实时定量 PCR 的优势在于：①可实现定量检测。②其荧光数据可以通过实时 PCR 仪器或荧光检测器直接检测，不需要进行电泳，缩短了分析时间，减少产物污染。③适用于小片段的扩增，这在检测处理过的食品尤其是 DNA 降解严重的食品时尤为重要。

[0010] 以上方法都可以应用于单一物种的鉴定，但单一物种的 PCR 检测成本高，而肉品中可能存在其它品种，单重 PCR 已经满足不了现有的要求，进而出现了多重 PCR 技术，即在同一反应体系中加入多对引物同时扩增多条目的 DNA 片段的方法。已有报道有利用多重 PCR 技术对 18 种常见动物种类进行检测的技术，但要根据其 PCR 产物大小不同，用毛细管电泳对其片段进行分离后判断物种的种类，后续处理繁琐，且多重 PCR 也存在一些不足，如体系 中存在多对引物而导致的扩增效率较低，不同模板之间的扩增效率不一致等，这些都限制了此项技术的商业化应用。因此，建立一种能快速、灵敏、特异的检测未知生肉品种的检测方法一直是有待解决的难题，而联合检测方法将有望解决这一难题。

[0011] 所谓联合检测，即使用相同的反应体系和反应条件，一次检测可以得到所有可能的结果。此方法不同于多重 PCR 技术，每个反应是在不同的反应管中进行，避免了引物之间的相互干扰。联合检测的难点在于设计的引物要物种特异性强，不能有交叉反应，且要能优化到同一 PCR 反应条件。目前报道的利用联合检测鉴定肉制品种属的有应用 Taqman 探针对混合物中牛肉、猪肉、羊肉、鸡肉、火鸡肉、鸵鸟肉成分进行了测定，也有报道用 SYBR Green 法成功鉴别混合肉种的马鹿、欧洲鹿、狍、岩羚羊和比利牛斯山羊。但以上检测也存在一些问题：探针法对探针设计要求较高，且因检测灵敏度太高，对实验操作的要求较高，一些微小的污染也可能被检测出来。染料法对引物的设计要求较高，容易有非特异性扩增，且仅以溶解曲线判读结果不能排除假阳性。

[0012] 针对以上问题，本发明提出一种同时测定肉制品中多种动物源成分的实时荧光 PCR 联合检测方法：通过对多个物种分别设计特异性引物，可以在一个 PCR 反应体系内同时对多个物种的特异性片段进行扩增，特别适用于目前肉制品中混合成分的检出。通过加入荧光染料来检测荧光信号，不用设计特异性探针，节约成本，最后通过扩增的 CT 值与内参的对照来确定阴阳性，排除因沾染造成的假阳性结果。该方法的特点：1、通过对多个物种分别设计特异性引物，可以在一个 PCR 反应体系内同时对多个物种的特异性片段进行扩增，且对荧光信号无干扰，特别适用于肉类及肉制品中混合成分的鉴定。2、反应管已集成化，使用时仅需加入模板即可进行 PCR 检测，操作简便。3、通过与内参对照判定阴阳性，排除因沾染造成的假阳性结果。4、可以根据需要灵活组合鉴定的肉制品种类和检测样本的份数。

发明内容

[0013] 本发明所要解决的技术问题是克服现有技术的不足，提供一种同时测定肉制品中多种动物源成分的实时荧光 PCR 联合检测方法，针对多个物种分别 设计特异性引物，并在一个 PCR 反应体系内同时对多个物种的特异性片段进行扩增。结合荧光染料的使用，可以直接判读阴阳性。该方法成本低、省时、高效，可对多种成分同时鉴别。

[0014] 为解决以上技术问题，本发明采用如下技术方案：1. 一种同时测定产品中多物种成分的实时荧光 PCR 联合检测方法，根据线粒体 DNA 上物种间基因多态性的差异设计各物

种特异性的引物，并将反应条件优化到同一体系，即可以在一个 PCR 体系中同时对多种物种成分进行鉴定，通过加入荧光染料来检测荧光信号，最后用扩增的 CT 值与内参的对照来确定阴阳性；该方法同时对肉及肉制品中 10 种以上动物源性成分进行检测。

[0015] 所述实时荧光 PCR 法包含的 10 个以上物种的特异性引物和 1 个内参引物的序列。

[0016] 所述实时荧光 PCR 检测方法对多种动物源成分的检测是在一个 PCR 反应体系中、同一个反应条件下一次完成的。

[0017] 所述实时荧光 PCR 法的所用荧光染料 SYBR Green，但不限于 SYBR Green，也可以是 Eva Green、LC Green 等荧光染料，并通过对溶解曲线的判读分析扩增的特异性。

[0018] 所述实时荧光 PCR 法的结果的判读方法：用扩增的 CT 值与内参的对照来确定阴阳性；无 CT 值的直接判读为阴性；有扩增 CT 值的与内参引物扩增 CT 值进行比较，大于等于 7 的判读为阴性，小于 7 的判读为阳性。

[0019] 所述实时荧光 PCR 法应用于 10 个以上物种中的任一物种检测。

[0020] 所述实时荧光 PCR 法应用于 10 个以上物种中的任意混合物种的检测。

[0021] 本发明的优点和有益效果为：

[0022] 1、通过对多个物种分别设计特异性引物，可以在一个 PCR 反应体系内同时对多个物种的特异性片段进行扩增，且对荧光信号无干扰，特别适用于肉类及肉制品中混合成分的鉴定。

[0023] 2、反应管已集成化，使用时仅需加入模板即可进行 PCR 检测，操作简便。

[0024] 3、通过与内参对照判定阴阳性，排除因沾染造成的假阳性结果。

[0025] 4、可以根据需要灵活组合鉴定的肉制品种类和检测样本的份数。

具体实施方式

[0026] 下面将对本发明实施例中的技术方案进行清楚、完整地描述，显然，所描述的实施例仅是本发明的一部分实施例，而不是全部的实施例。基于本发明中的实施例，本领域普通技术人员在没有做出创造性劳动前提下所获得的所有其它实施例，都属于本发明保护的范围。

[0027] 一种同时测定肉制品中多种动物源成分的实时荧光 PCR 联合检测方法，其鉴定方法为：根据线粒体基因上动物种间多态性的差异设计各物种特异性的引物，并将反应条件优化到同一体系，即可以在一个 PCR 体系中同时对 10 个以上的物种成分进行鉴定，通过加入荧光染料 SYBR 来检测荧光信号，最后用扩增的 CT 值与内参的对照来确定阴阳性。该 RT-PCR 方法可以同时对肉及肉制品中多种动物源性成分（猪、牛、羊、狗、兔、猫、鸡、鸭、鹅、鼠等）的单一肉制品或多种动物的混合肉制品进行检测鉴定。

[0028] 进一步的，所述实时荧光 PCR 法的物种特异性引物和 1 个内参引物的序列。

[0029]

引物	序列	目的基因	大小
猪上	5-atgaaacattggagtagtcc-3	猪线粒体 CYTB 基因	149bp
猪下	5-ctacgaggtctgttccgata-3		
牛上	5-gtaatgccaattataattgg-3	牛线粒体 COXI 基因	113bp
牛下	5-atgctagaagtagtaggaagg-3		
羊上	5-ggttgaggccggagcaggaac-3	羊线粒体 COXI 基因	144bp
羊下	5-ataaaattaatggctcctag-3		
狗上	5-gacacacgagcgtactttac-3	狗线粒体 COX1 基因	172bp

[0030]

狗下	5-caatacctgttaacccgcct-3		
兔上	5-taatcgtcaccgcacatgcc-3	兔线粒体 COXI 基因	117bp
兔下	5-ggaaggctatgtcaggagcc-3		
鸡上	5-aagacgagaagaccctgtgg-3	鸡线粒体 16SrRNA 基因	249bp
鸡下	5-gattgcgctgttatccctgg-3		
鸭上	5-gcctcaggcgtaactgtcac-3	鸭线粒体 COXIII 基因	161bp
鸭下	5-acgctgtcggcgattgag-3		
猫上	5-caggtgtctcctcaatcttggg-3	猫线粒体 COXI 基因	148bp
猫下	5-aagactggaagtgatagaag-3		
鹅上	5-tcgttaccgctcacgcctttg-3	鹅线粒体 COXI 基因	221bp
鹅下	5-agggtagacagttcagcctg-3		
鼠上	5-agaacccttcggatatatag-3	鼠线粒体 COXI 基因	140bp
鼠下	5-taattatagtggcagatgtaaag-3		
内参上	5-tctgccctatcaactttcgatggta-3	真核生物 18SrRNA 基因	142bp
内参下	5-aatttgcgcgcctgctgccttcctt-3		

[0031] 进一步的，所述实时荧光 PCR 法的 PCR 反应体系和条件。

[0032] PCR 体系：

[0033]

2×SYBR Premix ExTaq II	10ul
模板（50ng 左右）	1ul
引物 Mix（20uM each）	1ul
H_2O	8ul
	20ul

[0034] PCR 条件：

[0035]

95℃ 5min

[0036]

95℃ 15s
60℃ 40s
72℃ 45s
} 35 个循环

95℃ 15s
60℃ 15s
95℃ 30s
} 溶解曲线

[0037] 所述实时荧光 PCR 法的所用的荧光染料 SYBR，并通过对溶解曲线的判读分析扩增的特异性。所述实时荧光 PCR 法的结果的判读方法：用扩增的 CT 值与内参的对照来确定阴阳性。无 CT 值的直接判读为阴性；有扩增 CT 值的与内参引物扩增 CT 值进行比较，大于等于 7 的判读为阴性，小于 7 的判读为阳性。CT 值与起始 DNA 浓度的对数成反比，因此 CT 值相差 7 以上表示模板 DNA 含量相差百倍以上，以此判断标准可以排除因沾染造成的假阳性结果。所述实时荧光 PCR 法的应用于多个物种中的任一物种检测。所述实时荧光 PCR 法的应用于多个物种中的任意混合物种的检测。

[0038] 实施例 1

[0039] 单一成分检测：

[0040] 1. 材料：

[0041]

编号	样品	成分
1	肉加工品	猪
2	肉加工品	牛
3	肉加工品	羊
4	肉加工品	鸡

5	肉加工品	鸭
6	肉加工品	鹅
7	组织	狗
8	组织	兔

[0042]

9	组织	猫
10	组织	鼠

[0043] 2. 基因组 DNA 提取：用 OMEGA 组织 DNA 提取试剂盒提取以上各个材料的基因组 DNA。

[0044] 3. PCR 扩增反应：

[0045] 按下列组分分别配制 PCR 反应液，最后加入样本 DNA。

[0046]

2×SYBR Premix ExTaq II	10ul
样本 DNA	1ul
引物 Mix（20uM each）	1ul
dH_2O	8ul
	20ul

[0047] 按下列条件进行 PCR 反应：

[0048]

95℃	5min	
95℃	15s	35 个循环
60℃	40s	
72℃	45s	
95℃	15s	溶解曲线
60℃	15s	
95℃	30s	

[0049] 4. 结果分析：

[0050] 检查扩增曲线和溶解曲线是否正常，是否需要手动调整基线和阈值。待调整好曲线后读取扩增曲线的 CT 值。

[0051] 5. 结果判定：

[0052]

样本 \ 引物	猪	牛	羊	鸡	鸭	鹅	狗	兔	猫	鼠	内参
1	12.27	—	30.14	29.42	32.5	—	32.54	33.31	—	34.95	16.05

[0053]

2	32.31	13.22	—	31.31	32.85	—	30.68	—	—	33.09	15.66
3	27.8	29.37	17.43	30.78	31.27	—	33.76	34.23	—	31.2	16.14
4	—	—	—	15.32	—	—	33.4	33.94	34.5	—	16.71
5	30.29	—	31.66	—	16.28	34.12	29.1	32	—	—	16.45
6	32.34	—	32.02	33.41	30.43	14.21	35	—	34.65	32.39	14.91
7	31.21	—	—	32.62	33.6	—	19.2	34.9	—	32.16	18.6
8	34.15	33.25	32.21	34.5	34.65	—	—	15.78	—	—	18.71
9	—	—	30.18	—	—	32.92	33.76	—	14.65	—	15.64
10	30.87	32.08	—	—	—	—	33.63	34.2	—	13.84	14.54

[0054] 结果判定如下：1- 猪 2- 牛 3- 羊 4- 鸡 5- 鸭 6- 鹅 7- 狗 8- 兔 9- 猫 10- 鼠

[0055] 实施例 2

[0056] 混合成分检测：

[0057] 1. 材料：

[0058]

编号	样品	成分
1	肉骨粉 1	猪、牛源混合成分
2	肉骨粉 2	牛、羊源混合成分
3	肉骨粉 3	猪、羊源混合成分
4	火腿肠	猪、鸡源混合成分

[0059] 2. 基因组 DNA 提取：用 OMEGA 组织 DNA 提取试剂盒提取以上各个材料的基因组 DNA。

[0060] 3. PCR 扩增反应：

[0061] 按下列组分分别配制 PCR 反应液，最后加入样本 DNA。

[0062]

2×SYBR Premix ExTaq II	10ul
样本 DNA	1ul
引物 Mix（20uM each）	1ul
dH_2O	8ul
20ul	

[0063]

[0064] 按下列条件进行 PCR 反应：

[0065]

95℃ 5min

95℃ 15s
60℃ 40s
72℃ 45s
} 35 个循环

95℃ 15s
60℃ 15s
95℃ 30s
} 溶解曲线

[0066] 4. 结果分析：检查扩增曲线和溶解曲线是否正常，是否需要手动调整基线和阈值。待调整好曲线后读取扩增曲线的 CT 值。

[0067] 5. 结果判定：

[0068]

引物 \ 样本	1	2	3	4
猪	12.47	27.15	12.78	18.4
牛	11.87	13.22	32.97	30.82
羊	28.37	14.28	13.28	29.35
狗	—	—	33.94	—
兔	—	31.67	34.02	—
鸡	32.62	33.09	—	18.44
鸭	29.92	33.12	32.67	—
猫	—	—	—	—
鹅	—	—	—	—
鼠	33.56	33.09	31.07	34.95
内参	16.21	13.92	15.3	15.61

[0069] 结果判定如下：1- 猪、牛混合成分 2- 牛、羊混合成分 3- 猪、羊混合成分 4- 猪、鸡混合成分。

[0070] 对于本领域技术人员而言，显然本发明不限于上述示范性实施例的细节，而且在

不背离本发明的精神或基本特征的情况下，能够以其他的具体形式实现本发明。因此，无论从哪一点来看，均应将发明例看作是示范性的，而且是非限制性的，本发明的范围由所附权利要求而不是上述说明限定，因此旨在将落在权利要求的等同要件的含义和范围内的所有变化囊括在本发明内。

Alinhamentos

```
CLUSTAL O(1.2.4) multiple sequence alignment


BosF            CGGAGTAATCCTTCTGCTCACAGT    24
SEQ1-BRPI       CGGAGTAATCCTTCTGCTCACAGT    24
                ************************


#
#
#  Percent Identity  Matrix - created by Clustal2.1
#
#

     1: BosF         100.00  100.00
     2: SEQ1-BRPI    100.00  100.00



CLUSTAL O(1.2.4) multiple sequence alignment


BosR            GGATTGCTGATAAGAGGTTGGTG     23
SEQ2-BRPI       GGATTGCTGATAAGAGGTTGGTG     23
                ***********************


#
#
#  Percent Identity  Matrix - created by Clustal2.1
#
#

     1: BosR         100.00  100.00
     2: SEQ2-BRPI    100.00  100.00

CLUSTAL O(1.2.4) multiple sequence alignment


Bub2F           TCAGCCCAAAGAAAAATAAACCA     23
SEQ4-BRPI       TCAGCCCAAAGAAAAATAAACCA     23
                ***********************


#
#
#  Percent Identity  Matrix - created by Clustal2.1
#
#

     1: Bub2F        100.00  100.00
     2: SEQ4-BRPI    100.00  100.00

CLUSTAL O(1.2.4) multiple sequence alignment


Bub2R           GTCACCCCAACCGAAACTGT        20
SEQ5-BRPI       GTCACCCCAACCGAAACTGT        20
                ********************


#
#
#  Percent Identity  Matrix - created by Clustal2.1
#
#

     1: Bub2R        100.00  100.00
     2: SEQ5-BRPI    100.00  100.00
```