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ScienceDirect
Food Control 18 (2007) 1137–1142
FOOD CONTROL
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Detection of *Listeria monocytogenes* using a commercial PCR kit and different DNA extraction methods

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Received 10 April 2005; received in revised form 12 June 2006; accepted 13 June 2006

Abstract

The aim of our work was to evaluate a new commercial test kit for the detection of *Listeria monocytogenes* by PCR, using different DNA extraction methods. Food samples (pork sausage and "mozzarella" cheese) were spiked with known concentrations of *L. monocytogenes* and culture-enriched for 24 h. DNA extracted using three commercial kits and two standard methods, was amplified in species-specific PCR employing a *L. monocytogenes* PCR Detection Kit (Diatheva). The PCR-based method proved to be a reliable means of detecting the pathogen in food samples independently from the extraction procedure used, even for a contamination cell number of 1 cfu/g before culture enrichment. The molecular assay, showing perfect agreement with standard microbiological tests and a considerably shortened analysis time, provides a sensitive and rapid alternative for applications in the testing of foods for microbiological contamination, and highlights the potential of PCR technology in routine food control.
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Keywords: *Listeria monocytogenes*; PCR detection kit; Magnetic DNA extraction

1. Introduction

Listeria monocytogenes is an important food pathogen when considering public health. In fact it causes severe illnesses associated with the contamination of various categories of foods, particularly cheeses and dairy products (Rudolf & Scherer, 2001), beef and pork (Heredia, Garcia, Rojas, & Salazar, 2001). The mortality rate of listeriosis is very high, approximately 30% (Griffiths, 1989), and for this reason the FDA maintains a policy of zero-tolerance for *L. monocytogenes* (Anon, 2003). With this concern, the ability to detect this pathogen at low levels is considered essential because most of the foods susceptible to contamination are ready-to-eat products, which are not cooked or otherwise processed before consumption. These considerations underline the benefit of sensitive *L. monocytogenes* monitoring to ensure the microbiological quality of foods and to reduce risks for public health. Current microbiological culture methods rely on growth in culture media, followed by isolation, and biochemical and serological identification. However, the detection of this pathogen in food by these standard culture methods is made difficult by the sporadic or low levels of contamination (<100 cfu/g), by the presence of a high level of background microflora and competitor organisms that could mask the presence of *L. monocytogenes*, and by interference due to food matrix components (Norton et al., 2001). Moreover, these methods are laborious and time consuming, requiring a minimum of five days to recognize *Listeria* spp. and about 10 days to identify *L. monocytogenes* by confirmatory tests (Anon, 1996), while immediate action should be taken in

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doi:10.1016/j.foodcont.2006.06.012

case of contamination since it is of fundamental importance to ensure the safety of food products, especially in the case of those food matrices having short shelf-lives, such as meat or dairy products.

In the past years, advancements in biotechnology have resulted in the development of rapid methods that reduce analysis time and offer great sensitivity and specificity in the detection of pathogens. Among these, PCR has been increasingly used for the rapid, sensitive and specific detection of foodborne pathogens (Olsen et al., 1995). However, the successful application of PCR assays to food samples has been hindered by the lack of a rapid and efficient method for the preparation of PCR-amplifiable DNA (Lantz, Hahn Hagcrdal, & Radstrom, 1994). The presence of PCR inhibitors in food samples (Bickley, Short, McDowell, & Parkes, 1996; Rossen, Norskov, Holmstrom, & Rasmussen, 1992) represents the main limitation in this kind of assays due to the production of false negative results. For this reason, the application of PCR-based methods is closely linked to the selection of suitable methods for DNA extraction.

This study evaluates different procedures for the extraction of *L. monocytogenes* DNA from enriched food samples, followed by detection using the new commercial assay "*Listeria monocytogenes* PCR Detection Kit" (Diatheva, Italy). Additionally, the equivalence of the molecular method and the reference culture method is investigated.

2. Materials and methods

2.1. Samples and media

Food samples of pork raw sausage and "mozzarella" cheese were obtained from a local supermarket.

For bacterial growth and samples enrichment the following media were used: Listeria Enrichment Broth Base supplemented with Listeria Selective Enrichment Supplement (LEB); Fraser Broth Base supplemented with Fraser Selective Supplement (FB); Listeria Selective Agar Base (Oxford) supplemented with Listeria Selective Supplement (Oxford), all reconstituted and supplemented according to producer's recommendations. All culture media were purchased from Oxoid (Basingstoke, UK).

Chemicals were obtained from Sigma-Aldrich (St. Louis, Missouri, USA).

2.2. Inoculum and samples preparation

The bacterial species used in this study is a collection strain of *Listeria monocytogenes* (ATCC 9525). *L. monocytogenes* was growth in LEB at 37 °C with shaking at 200 rpm, to an optical density of 0.065 (at 600 nm), corresponding to about 10^8 cfu/ml. The bacterial concentration of the contaminating culture was determined by plating on Oxford medium and incubation at 37 °C for 24–48 h. Serial 10-fold dilutions in 0.9% NaCl were made, and 1 ml of each was used for contamination of food samples prepared as follows: twenty-five grams each of sausage and "mozzarella" cheese were homogenized separately in 225 ml of FB or LEB, using a Stomacher 400 homogenizer (Seward, Worthington, UK). Subsequently, food homogenates were inoculated with the described bacterial dilutions. The contamination rate of food samples ranged from 10^3 to 1 cfu/g. A culture-negative control, consisting of an uncontaminated food sample in culture medium, was also included for each food matrix.

Food homogenates were incubated at 30 °C for 24 h. Aliquots of 1 ml of the enriched samples were collected and sterile gauze-filtered, then centrifuged at 5000*g* for 10 min at 4 °C. Bacterial pellets were subjected to DNA extraction.

2.3. Nucleic acid isolation

In this section are outlined methods used for DNA isolation from enriched food samples. Brief descriptions are reported for DNA extraction methods carried out with commercial kits, while full detail is provided for lab-based procedures.

DNeasy Tissue Kit (Qiagen, Hilden, Germany). The extraction was performed following the manufacturer's protocol for Gram-positive bacteria. Cellular lysis was carried out by enzymatic treatment with lysozyme, RNase A and proteinase K in detergents and guanidium salts-containing buffers. After buffering condition adjustment, DNA was selectively bound to a silica-gel membrane. Contaminants were removed by two washing steps and finally DNA was eluted in water.

Puregene Yeast and Gram Positive Bacteria Kit (Gentra Systems, Minneapolis, Minnesota, USA). As described by the manufacturer, bacteria were lysed with an anionic detergent and a lytic enzyme solution in presence of a DNA stabilizer. Complete cell disruption was also ensured by a heating step at 80 °C. RNA was removed by a 40 min RNase A digestion while proteins were salt precipitated and then discarded. Bacterial DNA was recovered by precipitation with isopropanol and dissolved in a buffered solution.

Listeria monocytogenes DNA Isolation Kit: Milk (Diatheva, Fano, Italy). The kit was designed for a magnetic based-DNA isolation using paramagnetic nanoparticles. Bacterial cells were lysed and enzymatically digested with lysozyme, RNase A and proteinase K, according to manufacturer's instructions. The lysates were then mixed with the magnetic beads in presence of the specific binding buffer to allow DNA adsorbtion. DNA bound to magnetic particles was then washed, eluted in water and finally concentrated by ethanol precipitation.

Phenol–chloroform extraction. The organic extraction was performed according to the standard method by Sambrook and Russel (2001). Briefly, pelletted cells were resuspended in 500 μl of lysis solution (8 M urea, 0.3 M NaCl, 10 mM Tris–HCl) with the addition of 500 μl 10% SDS. After 20 min at 37 °C, DNA was extracted with 2 volumes

of phenol, mixed 10 min and centrifuged at 3500*g* for 10 min. An equal volume of chloroform:isoamyl alcohol was added to the aqueous phase, samples were mixed as above and centrifuged at 12000*g* for 5 min. Nucleic acids in the aqueous phase were precipitated with 2.5 volumes of ethanol and 1/10 volume of 3 M sodium acetate pH 5.2, incubating at −20 °C for 1 h. After centrifugation, DNA pellets were washed with 70% ethanol and dissolved in 150 µl of water.

Boiling method (Bansal, McDonell, Smith, Arnold, & Ibrahim, 1996). Bacterial pellets were washed once with 1 ml phosphate buffered saline (PBS), pH 7.4, resuspended in a same volume of cold water and incubated in a boiling water bath for 10 min. The clear supernatants obtained after a 5 min centrifugation at 12000*g* were used for PCR reaction.

2.4. Quality and yield of extracted nucleic acids

To investigate the quality of the extracted nucleic acids, sample absorbances at 260 and 280 nm were measured (Pharma Spec UV-1700 spectrophotometer; Shimadzu, Kyoto, Japan) and the A_{260}/A_{280} ratios were calculated. The spectrophotometrical analysis also permitted to evaluate the yield of the differently extracted DNAs (Sambrook & Russel, 2001).

2.5. DNA amplification and electrophoretic analysis

*L. monocytogene*s DNA amplification was carried out according to the "*Listeria monocytogenes* PCR Detection Kit" (Diatheva) manual. Ten microliters of each extracted sample were used in the PCR reaction. Additionally, for every amplification round, a reagent blank and a positive control (supplied in the kit) were included. Besides reagents and enzyme for detection of *L. monocytogenes* target sequence, the PCR mix also contained an internal control to identify reaction inhibition.

Twenty-five of 50 µl amplified mixture were electrophoresed through a 2% agarose (w/v) gel, stained with ethidium bromide in presence of a low molecular weight DNA standard (ΦX174 DNA/*Hae*III, Roche, Basel, Switzerland). Amplicons were visualized and analyzed on a Gel Doc 2000 apparatus using the Quantity One Quantitation Software (Bio-Rad, Hercules, California, USA).

An amplicon of 172 bp with or without the presence of a 112 bp DNA fragment indicated a positive result. Negative samples free of PCR inhibitors were identified by a 112 bp gel-band alone.

2.6. Detection limits and reproducibility of the *L. monocytogenes* PCR Detection Kit

The sensitivity of the PCR kit was assessed by amplifying dilutions in NaCl 0.9% of a *L. monocytogenes* culture prepared as described in Section 2.2. Bacterial concentration ranged from 1×10^5 to 2×10^2 cfu/ml, corresponding to 10^3 to 2 cfu per PCR reaction. Each dilution was tested in quadruplicate to assess the reproducibility of the results. Amplification and gel analysis were carried out as described in previous paragraph.

2.7. *Listeria monocytogenes* detection in food samples by reference method

Standard culture protocols, according to the official Italian regulatory agency "Istituto Superiore di Sanità Nazionale" (Anon, 1996), were used as reference methods for *L. monocytogenes* detection in artificially contaminated food samples. Briefly, samples were homogenized in enrichment media (LEB for cheese samples and FB for sausages) and contaminated as described in 2.2 section. Enrichment cultures were incubated at 30°C for 48 h. Subsequently, 0.1 ml were streaked onto Oxford plates, and incubated at 37 °C for 48 h. Presumptive *Listeria* colonies were subjected to biochemical (API Listeria, bioMérieux, Marcy l'Etoile, France) and serological (Listeria Rapid Test, Oxoid) confirmation tests.

3. Results

3.1. Quality and yield of extracted DNAs

Since both the purity and quality of extracted DNAs constitute pre-requisites in PCR-based detection assays, we evaluated the A_{260}/A_{280} ratios of purified samples from both food enrichments used in this study contaminated with a starting bacterial concentration of 10^3 cfu/g (Table 1).

The results indicated that the quality of DNA extracted from sausage enrichment cultures was invariably higher than that isolated from "mozzarella" cheese. The best A_{260}/A_{280} ratio values were shown for extractions using the Qiagen kit, phenol–chloroform and boiling methods, while the lowest ratio was obtained for Gentra extracted samples. Nevertheless, the purity of DNA recovered was still more than sufficient for subsequent application and isolated DNA functioned satisfactorily in PCR amplification. Finally, the data indicated that non commercial methods yielded the highest amount of DNA, followed by Diatheva and Qiagen, with similar results (Table 1).

3.2. Detection limits and reproducibility of the *L. monocytogenes* PCR Detection Kit

The PCR kit employed in this work was tested for sensitivity and reproducibility, to evaluate its capability to reveal low levels of contamination. As shown in Fig. 1, the detection endpoint occurred at 2 cfu per PCR reaction, and this result was confirmed in all four replicates with the same level of contamination, showing a high degree of sensitivity and good reproducibility.

Table 1
Detection protocols comparison

	Culture method	Phenol–chloroform extraction	Boiling method	Qiagen	Diatheva	Gentra
Time required	10 d	3 h	1 h	2 h	3.5 h	3.5 h
Relative cost[a]	++	+	+	+++	+++	++
DNA yield (μg)						
"Mozzarella" cheese	–	34.1	23.1	4.7	7.6	1.3
Sausage	–	24.9	19.1	5.3	2.3	1.9
DNA purity (A_{260}/A_{280})						
"Mozzarella" cheese	–	1.63	1.51	1.45	1.18	1.01
Sausage	–	1.77	1.64	1.64	1.36	1.25

[a] Relative costs of the used method in €: +++ from 250 to 150; ++ from 150 to 80; + from 80 to 20.

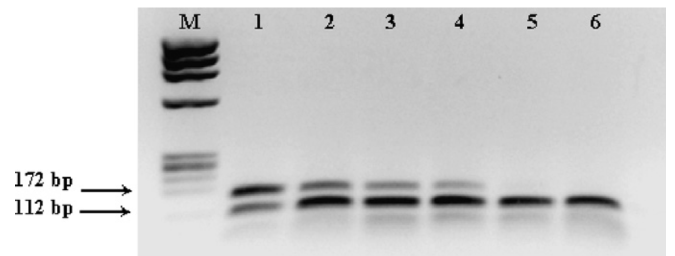


Fig. 1. Sensitivity of *L. monocytogenes* PCR Detection Kit with *L. monocytogenes* DNA. Lanes: M, ΦX174 DNA/*Bsu*RI (*Hae*III) marker (MBI Fermentas); lanes 1–4, amplification of 50, 10, 4 and 2 cfu/PCR; lanes 5–6, PCR negative controls (no DNA).

3.3. PCR detection

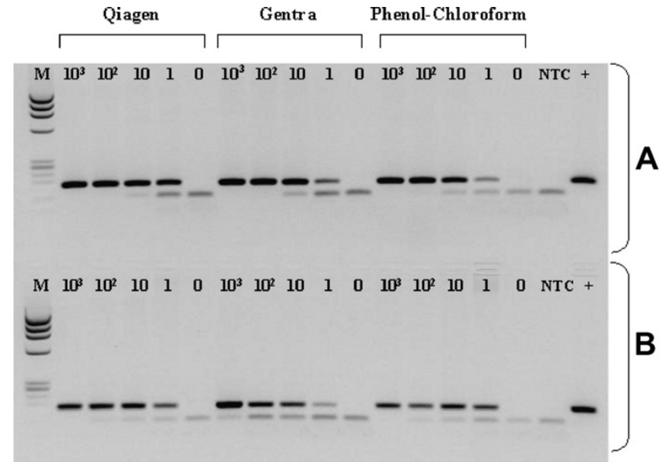
To determine the accuracy and versatility of the *L. monocytogenes* PCR Detection Kit, the assay was evaluated using DNA extracted from two types of food enrichments using five different procedures.

Fig. 2 summarizes the results obtained after amplification of the extracted nucleic acids. The *L. monocytogenes* specific 172 bp amplification product was visualized in all contaminated samples independent of the DNA purification method employed. In particular, samples obtained from sausage enrichments showed marginally better amplification, with a stronger intensity in gel electrophoresis analysis.

Culture and PCR negative controls showed only the 112 bp band, corresponding to the internal control amplification. In every case, no inhibitory effects on PCR were noticed, meaning that contaminants of food origin were not present in the differently extracted DNAs at a concentration that could affect amplification success. However, samples obtained by the boiling method produced weaker signals in agarose gel analysis.

3.4. Standard culture method

The microbiological procedure used for the detection of *L. monocytogenes* in sausage and "mozzarella" cheese, performed according to official protocols of the Italian regulatory agency (Anon, 1996), allowed to correctly identify the presence of *L. monocytogenes* in all the artificially contaminated samples, from 10^3 to 1 cfu/g.



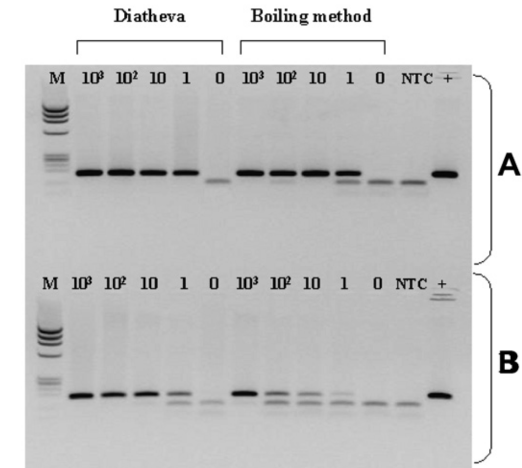


Fig. 2. Application of *L. monocytogenes* PCR Detection Kit to post-enrichment food samples after DNA extraction with the five different methods indicated. M, molecular weight marker ΦX174 DNA/*Hae*III, (Roche); 10^3 to 0, starting bacterial concentration per g of food product; NTC, PCR negative control (no DNA); +, PCR positive control. (A) Sausage, and (B) "mozzarella" cheese.

4. Discussion

Conventional techniques for the detection of bacteria involve intensive labour and time consuming cultural procedures including enrichment in selective media, agar isolation, biochemical and serological identification. Moreover, these methods may lead to false-negative results if the

sample contains the target species in a high background of a mixed bacterial population. For example, several researchers have observed that *L. innocua* can outcompete *L. monocytogenes* if the two species are cultivated together in commonly used enrichment media, including LEB (MacDonald & Sutherland, 1994; Petran & Swanson, 1993). Consequently, DNA amplification technology has proven to be beneficial when working with foodborne pathogens because of its high specificity, sensitivity and rapidity. The main limitation associated with PCR application to food-contaminating microorganisms concerns the presence of inhibitory substances that are coextracted with DNA and may be present in the sample, causing a failure in the amplification reaction which leads to false negative results. Therefore, quality and purity of extracted nucleic acids are primary requirements for a PCR-based detection assay and the selection of a proper extraction method is determinant for a successful and valid PCR analysis. Mainly due to the high cost of labour and time constraints in processing numerous samples, commercial kits have been developed and are becoming widely used for pathogen DNA detection. Some of these have been specifically developed for food analysis, particularly for *L. monocytogenes*.

In this study we report the application of the commercial assay "*Listeria monocytogenes* PCR Detection Kit" using DNA differently extracted by different methods from two artificially contaminated foodstuffs: pork sausage, that is a matrix having a high protein content, and "mozzarella" cheese, containing fat and calcium ions, well known to be PCR inhibitors (Bickley et al., 1996). DNA isolation was carried out by either commercially available kits or conventional phenol–chloroform and lysis by boiling methods. The three manufacturers did not supply complete information regarding the ingredients contained in their commercial kits due to the proprietary nature of kit components. However, purity and yield of the extracted nucleic acids were compared, resulting in higher DNA content for conventional extraction methods and relatively low A_{260}/A_{280} ratios for the majority of DNA samples. This result indicates contamination with other species, which could most probably be identified as proteins, having no inhibitory effects on DNA polymerase. Moreover, the PCR Kit correctly identified all contaminated samples, even at the lowest contamination ratio (1 cfu/g before culture enrichment). However, different substances of food origin, such as calcium ions (Bickley et al., 1996), polysaccharides or fats (Rossen et al., 1992) that commonly cause amplification inhibition are not detectable in the A_{280} reading. This consideration could explain results obtained for samples extracted using the boiling method suggested by Bansal et al. (1996), showing faint signals in gel analysis, particularly in the case of "mozzarella" samples, but with an A_{260}/A_{280} ratio comparable to that of DNA obtained by other procedures. Thus, although lysis by boiling appears the most convenient extraction protocol, particular care should be taken when food contamination with very low bacterial count is suspected.

The magnetically driven extraction (Diatheva), even if specifically recommended for DNA isolation directly from milk, proved to be suitable for enriched food products as well. Nevertheless, although these extraction procedures worked well for raw pork sausage and "mozzarella" cheese, they cannot extrapolate results when applied to other foods known to contain PCR inhibitors, such as certain fruits (e.g., rasberries).

The magnetic isolation was slightly more time consuming and laborious than the column system (Qiagen), but the limitation could be circumvented by automation of the entire procedure, offering several advantages to the end user, especially when large sample numbers are processed. Moreover, it is safer than using phenol–chloroform, avoiding the use and manipulation of harmful organic solvents.

The molecular assay also revealed results which were equivalent to those obtained with standard methods used in microbiology, however the time required for analysis was reduced from 7-10 to two working days. The application of the amplification assay after cultural enrichment offers some advantages, increasing the amount of target sequences, diluting non-target DNA and compounds interfering with the PCR, and ensuring that a positive result is obtained from viable cells.

In conclusion, the PCR Kit tested showed very high sensitivity and robustness, requiring relatively little experience in bio-molecular techniques. The presence of the internal control, capable of revealing every possible PCR failure caused by substances of food origin, provides complete certainty of negative results. It can be proposed in the routine investigation of foods for a rapid preliminary screening of negative samples, furnishing presumptive positive results that should be confirmed by official reference methods.

Acknowledgement

We would like to thank Diatheva srl for providing reagents and equipment for this study.

References

Anon. (1996). Rapporti ISTISAN ISSN 1123-3117, 96/35.

Anon. (2003). FDA/Center for Food Safety and Applied Nutrition, USDA/Food Safety and Inspection Service, Centers for Disease Control and Prevention, Quantitative assessment of relative risk to public health from foodborne *Listeria monocytogenes* among selected categories of ready-to-eat foods, September 2003.

Bansal, N. S., McDonell, F. H., Smith, A., Arnold, G., & Ibrahim, G. F. (1996). Multiplex PCR assay for the routine detection of *Listeria* in food. *International Journal of Food Microbiology, 33*, 293–300.

Bickley, J., Short, J. K., McDowell, D. G., & Parkes, H. C. (1996). Polymerase chain reaction (PCR) detection of *Listeria monocytogenes* in diluted milk and reversal of PCR inhibition caused by calcium ions. *Letters in Applied Microbiology, 22*, 153–158.

Griffiths, M. W. (1989). *Listeria monocytogenes*: its importance in the dairy industry. *Journal of the Science of Food and Agriculture, 47*, 133–158.

Heredia, N., Garcia, S., Rojas, G., & Salazar, L. (2001). Microbiological condition of ground meat retailed in Monterrey, Mexico. *Journal of Food Protection, 64*, 1249–1251.

Lantz, P.-G., Hahn Hagcrdal, B., & Radstrom, P. (1994). Sample preparation methods in PCR-based detection of food pathogens. *Trends in Food Science and Technology, 5*, 384–389.

MacDonald, F., & Sutherland, A. D. (1994). Important differences between the generation times of *Listeria monocytogenes* and *Listeria innocua* in two *Listeria* enrichment broths. *Journal of Dairy Research, 61*, 433–436.

Norton, D.-M., McCamey, M. A., Gall, K. L., Scarlett, J. M., Boor, K. J., & Wiedmann, M. (2001). Molecular studies on the ecology of *Listeria monocytogenes* in the smoked fish processing industry. *Applied and Environmental Microbiology, 67*(1), 198–205.

Olsen, J. E., Aabo, S., Hill, W., Notermans, S., Wernars, K., Granum, P. E., et al. (1995). Probes and polymerase chain reaction for detection of food-borne bacterial pathogens. *International Journal of Food Microbiology, 28*, 1–78.

Petran, R. L., & Swanson, K. M. J. (1993). Simultaneous growth of *Listeria monocytogenes* and *L. innocua*. *Journal of Food Protection, 56*, 616–618.

Rossen, L., Norskov, P., Holmstrom, K., & Rasmussen, O. F. (1992). Inhibition of PCR by components of food samples, microbial diagnostic assays and DNA extraction solutions. *International Journal of Food Microbiology, 17*, 37–45.

Rudolf, M., & Scherer, S. (2001). High incidence of *Listeria monocytogenes* in European red smear cheese. *International Journal of Food Microbiology, 63*, 91–98.

Sambrook, J., & Russel, D. W. (2001). *Molecular cloning: A laboratory manual* (3rd ed.). Cold Spring Harbor, NY, USA: Cold Spring Harbor Press.