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Experimental transmission of chronic wasting disease agent from mule deer to cattle by the intracerebral route

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Abstract. This communication reports final observations on experimental transmission of chronic wasting disease (CWD) from mule deer to cattle by the intracerebral route. Thirteen calves were inoculated intracerebrally with brain suspension from mule deer naturally affected with CWD. Three other calves were kept as uninoculated controls. The experiment was terminated 6 years after inoculation. During that time, abnormal prion protein (PrP^{res}) was demonstrated in the central nervous system (CNS) of 5 cattle by both immunohistochemistry and Western blot. However, microscopic lesions suggestive of spongiform encephalopathy (SE) in the brains of these PrP^{res}-positive animals were subtle in 3 cases and absent in 2 cases. Analysis of the gene encoding bovine PRNP revealed homozygosity for alleles encoding 6 octapeptide repeats, serine (S) at codon 46, and S at codon 146 in all samples. Findings of this study show that although PrP^{res} amplification occurred after direct inoculation into the brain, none of the affected animals had classic histopathologic lesions of SE. Furthermore, only 38% of the inoculated cattle demonstrated amplification of PrP^{res}. Although intracerebral inoculation is an unnatural route of exposure, this experiment shows that CWD transmission in cattle could have long incubation periods (up to 5 years). This finding suggests that oral exposure of cattle to CWD agent, a more natural potential route of exposure, would require not only a much larger dose of inoculum but also may not result in amplification of PrP^{res} within CNS tissues during the normal lifespan of cattle.

Key words: Cattle; chronic wasting disease (CWD) of mule deer; prion disease.

Chronic wasting disease (CWD), a prion disease, is a neurodegenerative transmissible spongiform encephalopathy (TSE) that has been identified in captive and free-ranging cervids.[20] Chronic wasting disease has been transmitted experimentally by intracerebral inoculation of brain from mule deer into a variety of animal species, including a goat.[20] In a previous publication, preliminary findings of an experimental CWD inoculation into cattle through the intracerebral route was reported.[7] The present communication describes the final results of that study, which was terminated in the fall of 2003, 6 years after the study was initiated.

The primary objective of this study was to determine whether the CWD agent could be transmitted to cattle by intracerebral inoculation. Secondary objectives were to provide information to TSE researchers about the clinical course and lesions of CWD in this species and to determine the suitability of currently used TSE diagnostic procedures for detection of CWD in cattle.

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Table 1. Findings in 13 cattle experimentally inoculated with a mule deer isolate of the CWD agent and in 3 uninoculated control animals.

Year PI*	Ear tag No.	CWD inoculation	Survival period (mo)	Clinical course	Clinical signs	Histopathology		IHC	SAF	WB
						SE	NAD			
2	1745	i/c	23	2 mo	+	+/−	−	+	−	+
	1768	i/c	24	3 mo	+	+/−	−	+	+	+
3	1732	control	26	NA	−	−	+/−	−	−	−
	1744	i/c	28	3 days	+/−	−	−	+	+	+
4	1749	i/c	44	NA	−	−	−	−	NT	−
	1748	i/c	45	NA	−	−	+	−	NT	−
5	1741	i/c	59	NA	−	−	−	−	NT	−
	1743	i/c	59	NA	−	−	−	−	NT	−
	1746	i/c	59	7 days	+/−	+/−	+	+	NT	+
6	1765	i/c	62	1 day	−	−	−	−	NT	−
	1772	i/c	63	2 days	−	−	+	+	NT	+
	1757	i/c	72	NA	−	−	+	−	NT	−
	1760	i/c	72	NA	−	−	+	−	NT	−
	1747	i/c	72	NA	−	−	+	−	NT	−
	1730	control	72	NA	−	−	+	−	NT	−
	1731	control	72	NA	−	−	+	−	NT	−

* PI = after intracerebral inoculation; SE = spongiform encephalopathy; NAD = neuroaxonal degeneration; NT = not tested; IHC = immunohistochemistry for PrP^{res}; SAF = scrapie-associated fibrils; NA = not applicable; WB = Western blot (Prionics-Check[a]); + = lesions or antigen present; − = lesions or antigen absent; +/− = signs/lesions equivocal; and i/c = intracerebral.

Sixteen 4–6-month-old calves of mixed breed (primarily red and black Angus) were purchased from a herd of cattle outside the CWD-endemic area[14] and were assigned to inoculated (n = 13) and control (n = 3) groups. Inoculated calves were housed in a Biosafety Level 2 isolation barn at the National Animal Disease Center (NADC), Ames, Iowa. Husbandry of these animals has been described previously.[7] Personnel wore protective clothing while in the isolation facility and showered before leaving the facility.

Material for inoculation was prepared from a pool of 28 CWD-affected mule deer brains as described previously.[2,3] The pool was positive for scrapie-associated fibrils (SAF) by negative-stain electron microscopy and for PrP^{res} by Western blot (WB).[7] In addition, the inoculum produced CWD infections in mule deer fawns after oral inoculation.[7] Calves were inoculated intracerebrally with 1 ml of the CWD brain inoculum as described previously.[7] Three calves (controls) were not inoculated.

Animals were euthanized with pentobarbital, and a complete necropsy was conducted on each of the carcasses. Representative samples of lung, liver, kidney, spleen, salivary gland, thyroid gland, reticulum, rumen, omasum, abomasum, intestines (ileum, colon), adrenal gland, pancreas, urinary bladder, lymph nodes (retropharyngeal, prescapular, mesenteric, popliteal), tonsil, striated muscles (heart, tongue, masseter, diaphragm), eye, sciatic nerve, trigeminal ganglion, pituitary gland, and spinal cord (cervical, thoracic, lumbar) were immersion fixed in 10% neutral buffered formalin. The brain was cut longitudinally, and half of the brain was fixed in formalin and the remainder of the brain was frozen (−20 C). The formalin-fixed brain was cut into 2–4-mm-wide coronal sections. Sections of various anatomic sites (10–15 sections per animal) of cerebrum, cerebellum, brainstem (including the obex), and spinal cord (cervical, thoracic, and lumbar) were processed for routine histopathology, embedded in paraffin wax, and sectioned at 5-μm. The sections were stained with hematoxylin and eosin (HE), and by an immunohistochemical (IHC) method[8,13] for detection of PrP^{res}, with or without formic acid pretreatment. As described previously,[7] 3 different antiPrP primary antibodies were used, which included a rabbit polyclonal antibody and 2 monoclonal antibodies, F89/160.1.5 and F99/97.6.1,[15,16] provided by one of the authors (KIO). The latter 2 antibodies recognize PrP sequences conserved in most mammalian species in which natural TSEs have been reported.[15]

For immunodetection of PrP^{res}, a WB method[18] was performed on frozen brain (caudal medulla) using an antibody-designated 6H4.[a] The SAFs were detected in fresh brain (caudal medulla) using negative-stain electron microscopy.[19]

Genomic DNA was extracted from 3 ml of whole blood or 30 mg of spleen using a commercial kit.[b] Exon 3 of the bovine PRNP gene was amplified using approximately 0.4 μg genomic DNA in a final concentration of each of the following reagents: 2.5 μM $MgCl_2$, 200 μM dNTP stock solution, 2.5 units of *Taq* polymerase, and 0.2 μM each of forward primer (5′ ggcatatgatgctgacacc) and reverse primer (5′ tacggggctgcaggtagat). Amplifications were performed at 95 C for 5.5 minutes, followed by 30 cycles of 95 C (30 seconds), 62 C (30 seconds), and 72 C (59 seconds), followed by an extension cycle (72 C, 7 minutes). Polymerase chain reaction (PCR) products were analyzed on 1.5% agarose gels stained with ethidium bromide. After treatment of PCR products with ExoSAP[c] both DNA strands were sequenced at least once using nested forward (5′ ctggggtcaaggtggtagcc) and reverse (5′ tggtggtgactgtgtgttccttga) primers using ABI Big Dye Terminator chemistry[d] by Amplicon Express.[e]

Within 2 years after intracerebral inoculation (PI), 2 animals (Nos. 1745 and 1768; Table 1) gradually became an-

orexic and lost weight. At about the same time, 1 of these animals (No. 1768) became apprehensive and circled aimlessly in its pen. The other (No. 1745) became listless but was excited by loud noises. These behavioral changes continued with little variation until the animals were euthanized 8 and 14 weeks later. The altered behavior was subtle and most obvious to the animals' daily caretaker. Both animals were found recumbent and were euthanized 23 and 24 months PI (Table 1). At that time, a control animal (No. 1732; Table 1) was also euthanized to obtain tissues for histopathology and to test for the presence of PrP^{res}. A third inoculated animal (No. 1744; Table 1) developed lameness in 1 leg at approximately 27 months PI. Because it had overgrown hooves, its feet were trimmed under general anesthesia. Recovery was uneventful and the animal's gait appeared to improve. However, a week later, it was found recumbent and was euthanized for humane reasons.

During years 4 and 5 PI, 5 additional inoculated animals were euthanized (Table 1). Four had developed chronic leg problems as a result of being kept on concrete floors. One animal (No. 1746) became suddenly recumbent and because of a poor prognosis was euthanized a week later. This animal was found to have a fractured vertebra. Early in the sixth year PI, 2 additional animals (Nos. 1765 and 1742; Table 1) were found recumbent and were euthanized. The experiment was terminated at the end of year 6 PI. At that time, the remaining 3 inoculated and 2 control cattle, which appeared clinically normal, were euthanized (Table 1).

At necropsy, 2 animals were emaciated (Nos. 1745 and 1768; Table 1), but other gross lesions were not evident. Animal No. 1744 (Table 1), which was in a good body condition, had a large (approximately 20 cm in diameter) pulmonary abscess in 1 of the diaphragmatic lobes. The abscess contained copious, thick, greenish purulent material that was surrounded by a thick fibrous capsule. Significant lesions were not observed in other inoculated or control animals (Table 1).

Results of neurohistopathologic findings and the PrP^{res} tests are summarized in Table 1. Microscopic examination of HE-stained brain and cervical cord sections revealed isolated vacuolated neurons, a few degenerate axons, and mild astrocytosis in 3 CWD-inoculated cattle (Nos. 1745, 1768, and 1746; Table 1). However, vacuolated neurons were never seen in either the dorsal vagal or the solitary tract nuclei. Extensive neuroaxonal degeneration (NAD) was seen in the medulla oblongata in specific nuclei of most older (more than 5 years) cattle. The NAD involved nucleus Gracilis and was also seen in the 2 control animals that were euthanized at the termination of the study. One control animal (No. 1732) had moderate numbers of vacuolated neurons in the red nucleus.

The PrP^{res} was detected by IHC in the brains of 5 inoculated cattle (Nos. 1745, 1768, 1744, 1746, and 1772; Table 1). The anatomic distribution and staining pattern was similar with all 3 antibodies with or without formic acid treatment of sections. The distribution of PrP^{res} was widespread, appearing predominantly in gray matter, in some areas of white matter of the cervical spinal cord, and throughout the brain, except in cerebellar folia. The greatest amount of staining was present in the medulla oblongata and the midbrain. The staining pattern was multifocal (Fig. 1) and most of the reactivity was concentrated in or around astrocytes (Fig. 2). There was also scattered particulate or granular staining in neuropil, and small plaques (up to 40 μm in diameter) were occasionally observed. Staining in neuronal cytoplasm was uncommon, and there was no perineuronal or perivascular staining.

All other nonneuronal tissues, including the lymphoid tissues (spleen, tonsil, lymph nodes, Peyer patches) were negative. The PrP^{res} was not present in tissue sections of control animals. The PrP^{res} was also detected by WB analysis in brain material from the 5 IHC-positive animals. A distinct profile of the 3 isoforms of PrP^{res} (diglycosylated, monoglycosylated, and unglycosylated polypeptides) is shown for 3 selected positive samples in Fig. 3. Fresh, frozen, or formalin-fixed brain tissues of 4 animals revealed SAFs in 2 animals (Nos. 1744 and 1768; Table 1).

Exon 3 of the bovine PRNP gene contains either 5, 6, or 7 copies of the octapeptide repeat region, 10 noncoding changes, and 2 coding changes (S46I, S146N).[12] Codon numbering was based on the allele encoding 5 octapeptide repeats. All cattle in this study were homozygous for alleles encoding 6 octapeptide repeats, serine (S) at codon 46, and S at codon 146. Noncoding changes were identified at codons 78 (13 cattle homozygous for *cag* and 3 cattle heterozygous for *cag/caa*), 105 (9 cattle homozygous for *ccc* and 7 heterozygous for *ccc/cct*), and 184 (10 cattle homozygous for *aac*, 1 homozygous for *aat*, and 5 heterozygous cattle). No polymorphisms were observed at any other sites. No differences in PRNP were seen in cattle with or without PrP^{res}.

Cross-species transmission experiments provide valuable information for identification of potential host ranges of known TSE agents. Chronic wasting disease, similar to all other TSEs, is characterized by a long incubation period, which in deer is seldom less than 18 months.[20] In this study, 3 of the 5 cattle that were positive for PrP^{res} died or were euthanized 23–28 months PI; the other 2 animals that were positive for PrP^{res} survived longer (59 and 63 months PI).

In cervids, clinical CWD is characterized by emaciation, changes in behavior, and excessive salivation.[20] Although the latter was not observed in the 5 PrP^{res}-positive cattle described in this study, 2 animals (Nos. 1745 and 1768; Table 1) developed reduced appetite and had considerable weight loss approximately 2 years after inoculation, and their carcasses were emaciated at necropsy. These animals also showed subtle behavioral changes that were more obvious to animal caretakers who had frequent contact with the animals. The other 3 PrP^{res}-positive cattle did not show clinical signs, and whether their health problems were attributable to inoculation or husbandry could not be discerned.

Although 5 animals were positive for PrP^{res} by IHC and WB, microscopic lesions of spongiform encephalopathy in this study were either subtle ($n = 3$) or absent ($n = 2$) in these animals (Table 1). In 2 of the animals, SAFs were also detected. These findings indicate that domestic cattle are susceptible to CWD by experimental intracerebral inoculation. However, they appear to be less susceptible to CWD than to the scrapie agent as indicated by results from a previous experiment[2,3] in which 100% of cattle inoculated intracerebrally with the US scrapie agent died 14–18 months after inoculation and all were positive

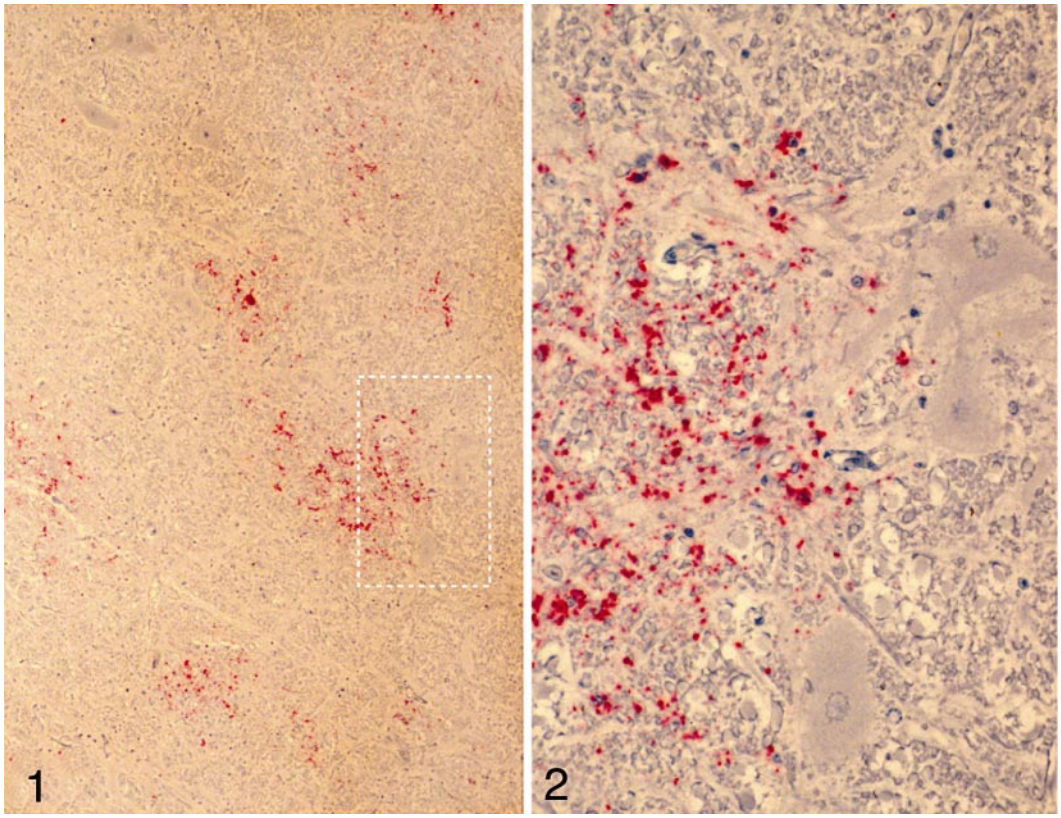

Figure 1. Brain; medulla oblongata of animal No. 1746. Multiple foci of PrPres staining (red) are present. Stained for PrP^{res} by IHC. 64×.

Figure 2. Brain; medulla oblongata of animal No. 1746. Higher magnification of area demarcated in Fig. 3 showing PrP^{res} accumulations located predominantly in or around astrocytes. Note absence of staining in or around the neurons. Stained for PrP^{res} by IHC. 512×.

for PrP^{res}. The comparatively lower susceptibility of cattle to intracerebral CWD inoculation than to intracerebral scrapie inoculation is consistent with cell-free conversion findings that PrP^{CWD} was less efficient than PrP^{Sc} in converting bovine PrP^{c} to PrP^{res} in vitro.[17]

In this experiment, the possibility that the PrP^{res} seen in tissue sections could be residual CWD material from the inoculum was ruled out because of the multifocal distribution of PrP^{res} throughout the brain (excluding cerebellar folia) and also in the cervical spinal cords of all 5 affected animals. If it were residual inoculum, the PrP^{res} would most likely have been observed as locally extensive areas of PrP^{res} in the midbrain and cerebrum (site of inoculation). Moreover, in experimental studies with sheep scrapie, it has been shown that intracerebrally inoculated brain material containing PrP^{res} is present in large enough quantity to be detected only during the first few days PI.[9]

Localization of PrP^{res} accumulation in brains of CWD-inoculated cattle was unusual because the pattern was multifocal and a primary target seemed to be astrocytes. Although PrP^{res} accumulation in astrocytes has been reported in a study of early experimental scrapie in mice,[4] this is not a prominent feature of bovine spongiform encephalopathy (BSE). The astrocytic pattern of IHC staining in CWD-inoculated cattle was also distinctly different from the PrP^{res} distribution observed in cattle inoculated intracerebrally with brain homogenate from scrapie-affected sheep.[2,3] In those animals, PrP^{res} was primarily concentrated in neuronal cytoplasm, a staining pattern that persisted even after second passage in cattle.[2] It is intriguing that 2 such different PrP^{res}-staining patterns are produced in cattle by intracerebral inoculation of TSE agents, depending on the source of inoculum (scrapie or CWD). Furthermore, these staining patterns differ from those described with naturally acquired scrapie, BSE, or CWD in their normal host species.[6] In the respective natural diseases, the IHC reactivity is described as a diffuse particulate staining of gray matter neuropil, with occasional plaques also being present in scrapie and CWD.[6] The differences in staining patterns produced by experimental BSE, scrapie, and CWD in cattle could be useful in identifying a likely exposure source(s), should new cases of prion disease arise in North American cattle.

The WB analysis of brain material from CWD-inoculated cattle revealed presence of the pathological form of the PrP, PrP^{res}, in 5 animals (Table 1; Fig. 3). As expected, all 3 isoforms (unglycosylated, monoglycosylated, and diglycosylated polypeptides) of the PrP^{res} were easily detected by WB (Fig. 3). When PrP^{res} from CWD-inoculated cattle was compared in WBs with PrP^{res} from mule deer CWD used for cattle inoculation, a lower molecular weight of the unglycosylated PrP polypeptide of the cattle-passaged CWD compared with the mule deer CWD was noted (data not shown).

Degenerative changes that were confined to the caudal medulla of both experimental and control cattle were com-

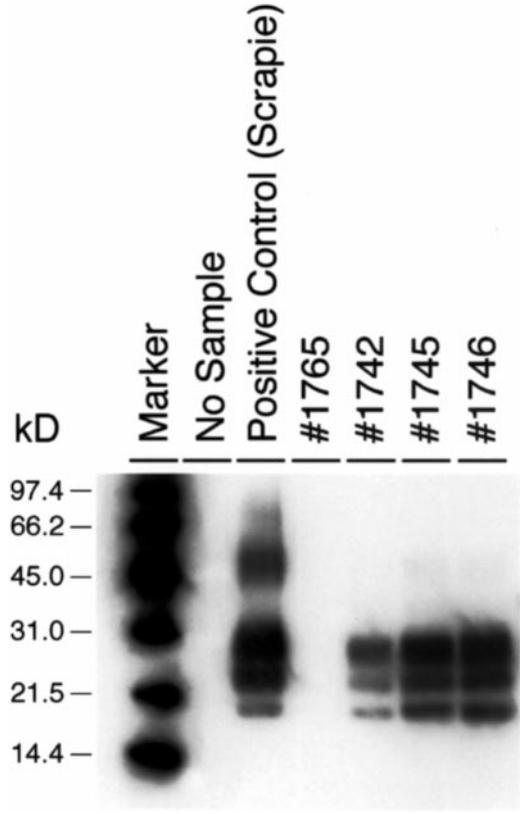

Figure 3. Western blot (with 6H4 antibody) showing distinct profile of PrP^{res} in the 3 positive animals Nos. 1742, 1745, and 1746. No signal is seen in No. ns1765, a noninoculated animal, classified as WB negative. A molecular-weight marker is shown in the first lane and a positive scrapie control is shown in the third lane.

patible with a diagnosis of NAD. Most of the affected animals were older than 5 years (Table 1) which suggests that the condition is probably a progressive one and is not observed until the animals are older. Among animals, naturally occurring NAD has been described in cats, dogs, cattle, sheep, horses, and raccoons.[5,10] Such lesions have been associated with the normal aging process, as well as degenerative diseases and experimental conditions.[11] Therefore, factors other than age (such as genetic, nutritional, and environmental) may have an influence on such degenerative axonal changes in the central nervous system of cattle.

The results in this study show that although PrP^{res} was able to amplify in only 5 of 13 (38%) cattle, the resulting PrP^{res} was easily detected by all 3 laboratory tests (IHC, WB, SAF). In addition, the IHC pattern of PrP deposition was rather unique (multifocal distribution and within small astrocytic type cells). Therefore, it appears that the diagnostic tests (IHC, WB) used at this time for confirmation of BSE cases in the United States would allow recognition of CWD in cattle, should it occur here.

Although intracerebral inoculation is an unnatural route for exposure of cattle to CWD infection, this experiment shows that CWD, similar to scrapie, has some potential for transmission to this species. Unlike scrapie in sheep, there does not appear to be any genetic predisposition in cattle for BSE, and in this study no difference was seen in the PrP gene of animals with or without PrP^{res} after CWD inoculation. The CWD isolates from other cervids (white-tailed deer and elk) may differ in their ability to amplify in cattle, and therefore, transmission studies using different CWD isolates are required. Such experiments will be initiated in the near future at the NADC (Ames, IA).

It is likely that transmission of CWD to cattle by natural-exposure routes, such as per os or by contact on range with infected cervids, would be more difficult to accomplish than the intracerebral transmissions reported here. Two experiments are currently in progress in Wyoming and Colorado, and approximately 7 years into these studies cattle orally or naturally exposed to CWD remain healthy (Williams, personal communication). On the basis of the results of this study, and on data obtained from 2 previous cross-species transmission studies (sheep-scrapie transmission to cattle by intracerebral and oral routes),[1,2] it may be concluded that under natural conditions cattle exposed to CWD would require a large dose of inoculum and also an extremely long incubation time to develop a TSE-associated disease. A second cattle passage with brain tissue from this study (infected with CWD from mule deer) may produce TSE-related disease in a larger proportion of inoculated animals in a shorter time and probably an altered clinicopathological picture of the disease. Such a study is now in progress at this Center.

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Mention of trade names or commercial products in this article is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the US Department of Agriculture.

Sources and manufacturers

a. Prionics Western, Schlieren-Zurich, Switzerland.
b. Q-BIO gene Fast DNA Kit, Carlsbad, CA.
c. USB Corp., Cleveland, OH.
d. PE-Applied Biosystems, Foster City, CA.
e. Pullman, WA.

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