Development of a bench-top extra-cleanroom for DNA amplification

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Prevention of airborne contamination has become an important factor in biotechnology; however, conventional laminar-airflow cabinets (LAF-cabinets) are no longer sufficient as a countermeasure against nano-sized airborne contaminants in the laboratory. Here we present a bench-top extra-cleanroom classified as ISO-1 that can prevent contamination from airborne nanoparticles. This bench-top extra-cleanroom consists of a novel clean-zone-creating system that is equipped with nanofibrous, nonwoven filters. In addition, the cleanroom is also equipped with an ionizer to prevent plasticware from collecting dust by electrostatic charge attraction. This combination of features allows the cleanroom to prevent DNA contamination derived from airborne nanoparticles. Our extra-cleanroom with ionizer could be useful in various areas of biotechnology that are easily affected by airborne contaminants.

Exogenous DNA contamination during DNA amplification is still a serious and vexing problem. Various countermeasures have been proposed and

implemented to reduce or eliminate DNA contamination. Laminar-airflow cabinets (LAF-cabinets) are one commonly used solution to this

problem. In addition, non-template controls (NTCs) are indispensable for confirming whether reagents, tools, and the laboratory environment are sufficiently clean for DNA amplification (1,2). The appearance of an amplicon in an NTC casts doubt regarding other amplification results, which may significantly affect the accuracy and reliability of DNA analyses.

Ever since molecular biologists discovered ϕ 29 DNA polymerase (ϕ 29 DNAP), they have used it to amplify infinitesimal amounts of genomic DNA via whole-genome amplification (WGA) (3), but this requires being vigilant for unexpected or incorrect DNA amplification (4–6). In particular, WGA is easily affected by contaminants because it is a non-specific amplification method that uses random primers (3).

We previously addressed the problem of unexpected amplification in NTCs derived from endogenous contaminating DNA in the reaction mixture (7,8). Nevertheless, we have often witnessed DNA amplification in NTCs, although all reaction mixtures were prepared in an LAF-cabinet (Supplementary Figure S1) and experimental procedures were performed under accepted guidelines for PCR (9–12). We speculated that airborne particles, such as viruses, bacteriophages, and airborne naked DNA derived from dead cells, could be a source of contamination because the HEPA filter installed in LAF-cabinets cannot absolutely prevent the passage of airborne nanoparticles (13). Indeed, it has been reported that airborne particles, including in autoclave steam, can cause false positives in PCR (14–16).

The air in the clean area produced by our LAF-cabinet, classified as ISO-5 (Supplementary Figure S2) (17), contained many airborne particles (2.0×10^4 – 3.5×10^4 particles/m³; most particles were 0.1 μ m, but a few were >0.5 μ m), as measured by a high-performance particle counter that has the ability to count 0.1 μ m particles (Lasair II 110; Particle Measuring Systems,

METHOD SUMMARY

Here we present a bench-top extra-cleanroom, classified as international standard ISO-1, that prevents contamination from airborne nanoparticles during the preparation of DNA amplification reactions.

Boulder, CO). The integrated particle numbers in the LAF-cabinet fulfilled the ISO-5 criteria; thus, the LAF-cabinet can prevent contamination by living microorganisms, spores, and pollen. However, the LAF-cabinet is unable to prevent contamination by nanoparticles, which could be a source of DNA contamination, especially for WGA.

We confirmed the origins of these contaminants by cloning and sequencing amplicons in the NTCs (Supplementary Figure S3). Based on BLASTN searches, these amplicons were derived from human and fungal chromosomal DNA, vector-like DNA, or unknown DNA sequences with no similarity to any sequences in current databases (Supplementary Tables S1 and S2). Considering that we did not extract and use these types of DNA in our laboratory at the National Food Research Institute (NFRI), these results indicate that airborne DNA could randomly contaminate the NTC tubes, even when using the same set of amplification reagents in repeated trials.

We then tested whether the source of the contamination depended on when and where the WGA reaction was done. The WGA reaction was performed in triplicate on separate days in three different laboratories (Hiroshima University (HU), NFRI, and the Isehara Research Lab (IRL) of Kanto Chemical Co., Inc) under ISO-5 conditions using the same DNA-free reagents (Supplementary Figure S4). As observed previously (18), sequencing of DNA amplified in the NTCs showed that DNA contamination under ISO-5 conditions was derived from various sources, regardless of the laboratory (Supplementary Tables S3–S5). In addition, it was confirmed that the reagents used in these experiments were DNA-free because amplicons were not found in one set of NTCs (Day 3 in IRL; Supplementary Figure S4).

The contaminants observed in the HU experiments were not living cells, such as microbes and fungi, because the LAF-cabinet of HU is routinely used for axenic culturing. Nevertheless, several of the contaminants were derived from microbes. This suggests that the DNA contamination occurring under ISO-5 conditions could considerably vary from tube to tube, day to

Figure 1. Development of a bench-top extra-cleanroom. (A) A top-view image of the particle concentrations around the novel air-cleaning system KOACH in a simulation. The arrows indicate the coherent airflows produced by the two push hoods of the KOACH system. The colors indicate the number of particles, as shown by the scale bar on the right. (B) The Table-KOACH was customized for whole-genome analysis (WGA) by the incorporation of a covering made of a PVC resin that functions as a ceiling and wall. Dedicated tools, plasticware, and stock reagents are always left in this bench-top extra-cleanroom. The blue arrows indicate the push hoods of the KOACH system. The white arrow indicates the roof of cleanroom. The yellow arrow indicates an ionizer. The red arrow indicates a handheld particle counter.

day, and lab to lab. Therefore, airborne DNA contamination would be difficult to prevent, particularly in WGA reactions.

Furthermore, two recent reports have shown that contaminating DNA can also be found in high-throughput next-generation sequencing (NGS) reads (18,19). Considering that DNA contamination could randomly occur in different tubes during preparation of the WGA reaction mixture, the DNA sequences of the NTC amplicons determined by NGS could not be used for the removal of contaminating sequences from target sequence read data of WGA samples.

Interestingly, the vector-like sequence amplified in the NTCs (preparation_2, N7, clone H06; Supplementary Table S2) is found in various DNA databases, including the mouse genome, expression sequence tags (EST), high-throughput genome sequences (HTGS), and transcript reference sequences (RefSeq_RNA), but not in databases containing the human genome and human transcripts by BLASTN (including Mouse G + T, refseq_rna, est, htgs, gss, dbsts, tsa_nt and Human G+T (Supplementary Figure S5–S11). This result suggests that clone H06 is a frequent contam-

inant in many laboratories and that some of these contaminants could be derived from airborne nano-particles. Taken together, these lines of evidence suggest that DNA amplification in NTCs of WGA is a frequent occurrence and needs to be avoided in order to ensure the validity of WGA experiments. To prevent airborne contamination, a higher level of cleanliness is therefore required for WGA.

A novel air-cleaning system called KOACH system has been developed by Koken Ltd. (Tokyo, Japan), originally intended for physics, nanotechnology, and semiconductor industry applications. The KOACH system consists of two air-supplying push-hoods facing each other, producing coherent airflows with a unidirectional vector. The two coherent airflows collide at the center and are pushed out in the vertical and horizontal directions. As a result, the intervening zone between the push-hoods is kept clean despite the absence of a ceiling and a wall (Figure 1A). Furthermore, the KOACH push-hood

uses a nanofibrous, non-woven filter (Supplementary Figure S12) (20) prepared by an electrospinning method (21) as the main filter. This nanofibrous, non-woven filter can eliminate nanoparticles >100 nm. Consequently, the KOACH system can create an extra-clean zone classified as ISO-1 (Supplementary Figure S2) (17). This level of cleanliness cannot be achieved by any conventional cleanroom system.

Since the original bench-top model (Table-KOACH; Supplementary Figure S13) was intended for use in lower-level cleanrooms (e.g., ISO-7 or ISO-8), it was unable to remove falling particles >30 μm . These particles include potential DNA contaminants, such as the spores and pollen that are present in the air of most biological laboratories; thus, we customized the Table-KOACH to prevent airborne contamination of DNA amplification reactions, especially for WGA.

A roof was attached over the clean zone so that it bridged the tops of the two push hoods and also covered

the rear of the clean zone (Figure 1B). Our concern at the time was that the attached roof might lower cleanliness by disturbing the airflow. The cleanroom classification was determined by counting airborne particles (0.1, 0.3, and 0.5 μm) at 27 different sites (Supplementary Figure S14) inside the clean zone using an airborne particle counter with the ability to count 0.1 μm particles, according to ISO 14644-1 (17). Integrated particle counts at several different points in the cleanroom fulfilled the ISO-1 criteria, and this level of cleanliness is not affected by arm movements during operation.

We compared the frequency of DNA contamination in multiple NTCs prepared in this bench-top extra-cleanroom with NTCs obtained in the LAF-cabinet using the same reaction mixtures. WGA reactions were performed as described by us previously (7,8). Ten microliters 1 $\times$ annealing buffer containing 20 μM oligonucleotide 6R5S (5'-rN_srN_srN_srN_srN_srN-3', HPLC

Figure 2. Reduction of contamination using the bench-top extra-cleanroom. Amplification products were analyzed after restriction enzyme digestion. (A) The upper panels depict the results of preparation in a laminar-airflow cabinet (LAF-cabinet) (classified as ISO-5). The lower panels represent the results of preparation in the bench-top extra-cleanroom (classified as ISO-1). Reaction mixtures were prepared using the same reagents. The numbers beneath each panel indicate the lot numbers of ϕ 29 DNA polymerase (ϕ 29 DNAP) prepared in-house (8). (B) Reaction mixtures were prepared using the same reagents used in the experiments shown in Supplementary Figure S6 under ISO-1 conditions in the presence of an ionizer. (C) Template titration assay using the same reagents used in (B); M₁: 1-kb Ladder DNA Stable Marker (Sigma-Aldrich, St Louis, MO); M₂: AccuRuler 1-kb DNA RTU ladder (Maestrogen, Las Vegas, NV); NTCs: non-template controls. The numbers above each lane indicate the input copy number of pUC19 used for positive control. Arrows indicate the 2.7-kb linear form of pUC19.

grade; Tsukuba Oligo Service, Tsukuba, Japan), 30 mM Tris-HCl (pH 7.5), 20 mM KCl, 2 mM MgCl₂, and template DNA or *UltraPURE* distilled water (μ DW; Invitrogen Life Technologies, Carlsbad, CA) was denatured for 1 min at 95°C and slowly cooled to 25°C over the course of 30 min. Subsequently, a 2 $\times$ amplification premix (10 μ L) was added, yielding a final concentration of 30 mM Tris-HCl (pH 7.5), 50 mM KCl, 14 mM MgCl₂, 20 mM (NH₄)₂SO₄, 5 mM dithiothreitol, 1 mM dNTPs, 0.002 U inorganic pyrophosphatase (Roche Diagnostics, Basel, Switzerland), and 100 ng amplifiable DNA-free ϕ 29 DNAP (made in-house or purchased from Kanto Chemical, Tokyo, Japan) (8). The reaction was performed at 30°C for 16 h, followed by incubation for 10 min at 65°C to inactivate the polymerase. The amplification products (20 μ L) were transferred into 1.5 mL centrifuge tubes and mixed with 180 μ L MilliQ water by pipetting, followed by hard mixing to lower the viscosity of the products using a microtube mixer MT-400 (Tomy, Tokyo, Japan) for 15 min at room temperature. Twenty-five microliters of the diluted product (12.5% of the original reaction product) was double digested with 20 U *Bam*HI and *Eco*RI at 37°C for 1 h in a 50 μ L volume. Then, 10 μ L of each sample (2.5% of the original reaction product) was analyzed by agarose gel electrophoresis using 1.0% TBE agarose gels, which were then visualized by staining with ethidium bromide.

We observed that amplicons from airborne nanoparticles were often present in the NTCs prepared in the LAF-cabinet, whereas no amplicons were found in any NTCs prepared in the bench-top extra-cleanroom (Figure 2A). On the other hand, with an increase in the number of amplification experiments to check reproducibility, the appearance of an amplicon in an NTC was sometimes observed in the WGA reaction in samples after preparation in the bench-top extra-cleanroom (Supplementary Figure S15). We found that plasticware and gloves carried static electricity in the portable cleanroom and noted that charged objects stored in the cleanroom collected dust once the system was turned off. Electrostatic charges

considerably influenced the level of contaminating DNA in the test tubes, interfering with subsequent reactions in the NTCs. Therefore, a roofed Table-KOACH equipped with an ionizer, which can eliminate electrostatic charge on plasticware, is necessary to maintain a high level of cleanliness and should be used to maintain a charge-free environment for WGA procedures.

We examined where an ionizer (SJ-H084A; Keyence, Osaka, Japan) should be installed in the working area to completely eliminate charges by monitoring the charge decay time when applying an initial voltage of 1000 V and measuring how rapidly the absolute values of the electric potentials decreased to 10% of the initial voltage. In the customized Table-KOACH, the charge decay time was measured at nine different sites, including the space over the bench (Supplementary Figure S16), using a charge plate monitor (Model 700A; Hugel Electronics Inc., Tokyo, Japan), with the ionizer set up either at the rear or the top of the additional hood.

When the ionizer was installed on the rear wall of the apparatus (Supplementary Figure S16, yellow bar), the applied voltage (1000 V) did not reach 100 V at positions #7 and #9 after switching on the ionizer. The average decay time at the remaining 7 positions was 5.7 ± 4.6 s [mean $\pm$ SD]. In contrast, when the ionizer was installed on the roof of the apparatus (Supplementary Figure S16, blue bar), the applied voltage decreased to 100 V at all 9 points, and the average decay time was 8.3 ± 4.1 s. These data suggest that the latter setup would be better for the effective elimination of static

charge in the working area. After setting up the ionizer in the bench-top extra-cleanroom (Figure 1B), we found that it effectively eliminated the electrostatic charge from the surfaces of objects. In addition, as a general practice, it is preferable to monitor particles in the cleanroom using a handheld particle counter (Figure 1B).

Finally, we compared the frequency of DNA contamination in multiple NTCs using this bench-top extra-cleanroom. The ISO-1 environment with an ionizer was able to suppress DNA contamination (Figure 2B), and the ionizer had little effect on the sensitivity of WGA (Figure 2C) (8). In contrast, the LAF-cabinet with an ionizer could not completely prevent contamination in NTCs (Supplementary Figure S17). The combination of an ISO-1 environment and the ionizer dramatically improved the specificity and accuracy of WGA reactions and could significantly improve practical applications in various research fields.

It has been recognized that cleanrooms are important in biological research, especially in reproductive and regenerative medicine. In fact, the cleanliness of cleanrooms significantly affects live-birth and miscarriage rates for in vitro fertilization (22). The ambient air contains many airborne nanoparticles, including bacteriophages and viruses (1.7×10^6 – 4×10^7 viruses/m³) (23). Nevertheless, the biological effects and pathogenicity of these virus-like particles have not been well studied (24). Therefore, biopharmaceuticals and stem cells should be produced under ISO-3 conditions (super-cleanroom) according to GMP guidelines (22,25). However, the super-cleanroom is an

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expensive facility that entails huge costs for both the initial purchase and for maintenance. Therefore, many molecular biologists use LAF-cabinets reluctantly, even though they require higher levels of cleanliness for reproducibility and stability.

Despite its higher cleanliness classification, the bench-top extra-cleanroom (approximately \$20,000) is much less expensive than conventional cleanroom systems. In addition, the running costs of the bench-top extra-cleanroom are the same as those of an LAF-cabinet. Therefore, we believe that the bench-top extra-cleanroom may be of great help to many biomedical researchers who otherwise would not be able to afford the construction of a super-cleanroom as a countermeasure against elusive airborne contaminants.

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Author contributions

H.T. identified the effect of nanoparticles and electrostatic charges, and conceived strategies for decontamination; T.Sa., Y.K., and T.Su. customized the Table-KOACH according to requests from H.T., Y.O., and T.K.; H.K. carried out the experiments guided by H.T.; T.H. and H.Y. carried out the experiments of WGA under ISO-5 conditions; K.Y. carried out the sequencing runs; Y.O. and T.K. obtained the necessary financial support. The paper was written primarily by H.T. and T.K., and all authors approved the final manuscript.

Competing interests

Takahiro Satoh, Yuji Kubota, and Takeo Suzuki are employed by Koken Ltd., the manufacturer of the KOACH system, the utility of which is described in this

paper. Hiroyuki Yamazaki is employed by Kanto Chemical Co. Inc., which is the supplier of the DNA-free ø29 DNA polymerase used in experiment shown in Supplementary Figure S4. The other authors declare no competing interests.

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