A community-based model of rapid autopsy in end-stage cancer patients

To the Editor:

Systematic genomic studies, including the Cancer Genome Atlas (TCGA)[1] and the International Cancer Genome Consortium (ICGC)[2], have provided an unprecedented catalog of driver mutations in human cancer. However, these studies use mainly primary, pre-treatment tumor material obtained at surgery with curative intent. There is an urgent need to identify and characterize resistance mechanisms to understand how cancers can evade even the best medical efforts and kill patients; therefore, access to end-stage disease is important. Solid cancers show considerable spatial[3], temporal[4,5] and genomic heterogeneity at diagnosis. Selective pressure and mutagenic impact of treatment[6] drives intra-patient evolution of cancer cell populations[4,7]. Understanding acquired resistance requires access to paired pre- and post-treatment samples[4,7]; however, curative surgery is typically confined to patients with locoregional disease, and opportunities for tumor sampling in advanced disseminated disease are limited. Here, we describe Cancer Tissue Collection After Death (CASCADE), an autopsy program that overcomes logistical challenges to enable collection of samples at end stage for research in melanoma and breast, ovarian and prostate cancers.

For the CASCADE study, we aimed to recruit cancer patients close to the end of life, including those outside the minority of patients who die in hospitals. To preserve tissue integrity, autopsies must commence within a few hours of death, requiring access to around-the-clock services. Intervention in the emotionally charged end-of-life environment must be managed in an ethical manner and to a high standard. Finally, we aimed for the study to be highly cost-effective. We believe our approach to meeting these challenges is applicable to researchers in other large urban centers.

Here we summarize the main steps in CASCADE's operating protocol and our experiences from the initial 3 years and 30 autopsies performed (**Fig. 1**). Information about institutional review board approvals (including a detailed patient information-and-consent form), the autopsy procedure and certain laboratory processes is given in **Supplementary Methods** and **Supplementary Figure 1**. Recruitment of participants was led by the clinicians. Such discussions require careful consideration, in timing and in language, and were initiated only if there was a perception that tissue donation would be acceptable to the patients and their families. Factors suggesting acceptability include the emotional stability of the participant and family members and their clarity about and acceptance of the terminal nature of the disease. On occasion, participants prompted discussion by asking about organ or body donation. Consent discussions typically involved oncologists and/or palliative care physicians employed at recruiting hospitals who had established a care relationship with the participant and their family during the patient's cancer journey. Frequently, the study was introduced at one meeting and discussed over several subsequent clinic visits, allowing patients and their families time to consider participation. We view the involvement of family members in the consent process as essential to support the participant and facilitate decision-making. Involvement of family members also ensures that they are fully aware of the autopsy process and helps to clarify funeral arrangements for the study team. After obtaining consent, study investigators collated clinical information, including that related to past and current treatment and diagnostic procedures such as imaging, on an ongoing basis. Between September 2012 and August 2015, 40 patients were approached, and 37 (92.5%) expressed interest in participating. Of those 32 patients (80%) consented; the other 5 had rapid clinical deterioration precluding

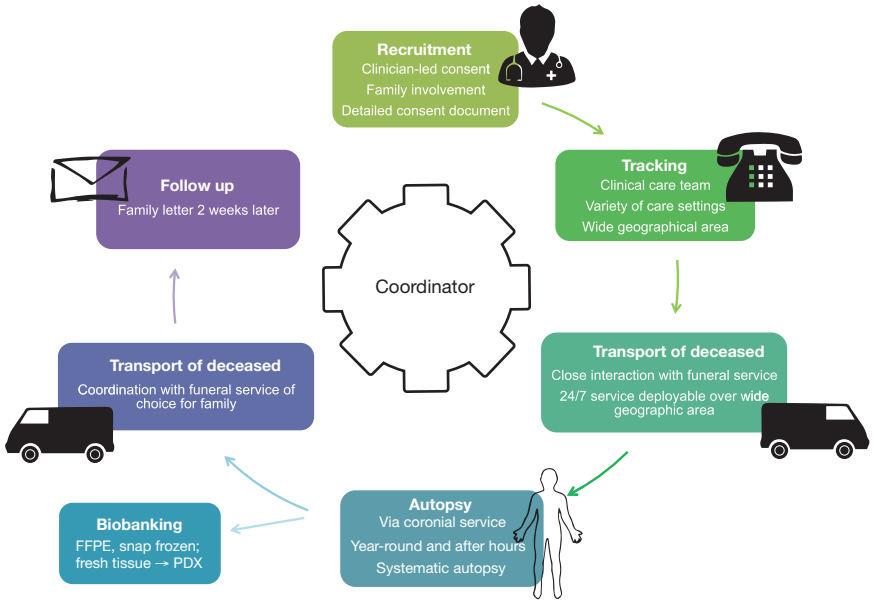

Figure 1 Schematic of the steps involved in CASCADE and essential success elements for the protocol.

participation. The 3 patients who declined did so owing to concerns about the impact of participation on family members or for religious reasons. To date, 30 of the 32 participants have died.

Most participants were not hospital inpatients at the time of their recruitment and subsequently moved in and out of various care settings as their disease progressed. To minimize intrusion, a program coordinator followed participants' movements via palliative care providers—both hospital based and in the community—rather than contacting the participant or family members directly. A 24-h phone number was provided to the participants' care teams to facilitate communication with the study investigators. After-hours contact with care teams was shared among senior research investigators. The frequency of contact with the care team typically increases as participants' health deteriorates, and the care teams are encouraged to alert the investigators when death is imminent. The median time between consent and death to date is 30 d (**Table 1**). No participants or relatives have withdrawn from the study after consent. Typically, the on-call investigator was notified within minutes of a participant's death (median 10 min; **Table 1**). Nine patients (30%) died in hospitals, 13 (43.3%) in palliative care facilities and 8 (26.7%) at home. Half of the patients died >20 km from the study center (**Table 1** and **Supplementary Fig. 2**).

Acquisition of a participant's death certificate is coordinated by the CASCADE investigator on call at the time of death. The timing of release of the participant to CASCADE by the family is monitored by the care team and communicated to the project coordinator. Participants have died in various care settings in or near metropolitan Melbourne, a city of more than 4 million people. We collaborate with a funeral service, Tobin Brothers, that has sufficient resources to transport the deceased across a wide area and after hours. Once the family is ready to release the deceased, Tobin Brothers is notified and transports the participant to the Victorian Institute of Forensic Medicine (VIFM), where a whole-body computed tomography (CT) scan of the body is performed.The CT scan is part of a routine assessment at VIFM to look for objects that could be hazardous during autopsy, but it is also useful to CASCADE investigators for assessing the brain for metastases. VIFM operates 24 h per day and 365 d per year under the auspices of the Department of Justice of the Australian State of Victoria and performs approximately 2,500 autopsies per year; pathology and mortuary technical staff perform full diagnostic autopsies.

After external examination of the deceased, an incision was made from the shoulders to the sternum and down to the pubic bone. Organs were removed from the body *en bloc*, weighed and reviewed systematically for macroscopic evidence of disease. Large organs were sectioned every 1–2 cm to allow thorough examination. If the clinical summary, prior imaging or post-mortem CT showed an indication of brain metastases, and the participant had given consent, the brain was exposed. Two attending researchers collected metastatic tissue throughout the autopsy, guiding both sampling and photography to aid annotation. A median of 16 sites per participant were sampled, depending on the extent of disease (range 4–27 sites; **Table 1**). Sites of metastatic disease were reviewed macroscopically to avoid the collection of necrotic tissue, and adjacent normal tissue was occasionally collected as well. Sampling instruments were rinsed between sites to avoid cross-contamination. Date of collection, time of processing and the location of all biological fractions were recorded on a standard form. Tissue samples were snap frozen in liquid nitrogen and collected in neutral buffered formalin for routine clinical histopathology processing in formalin-fixed paraffin-embedded (FFPE) blocks before being transported to the Peter MacCallum Cancer Centre for centralized biobanking. To allow xenografting into immunocompromised mice, tissue was collected into RPMI 1640 or Hank's balanced salt solution and kept on ice. Patient-derived xenografts (PDXs) were transplanted into nonobese diabetic severe combined immunodeficient (NOD–SCID) or NOD–SCID–IL-2Rγ (NSG) mice within a few hours of collection as described previously[8–12]. FFPE tissue blocks were processed immediately, and tissues were reviewed by anatomical pathologists at the Peter MacCallum Cancer Centre. The post-mortem CT, diagnostic autopsy report and histopathological findings are made available to researchers. To date, the median time between death and the start of autopsy is 5.5 h (range 3–12 h; **Table 1**). Although proximity of the deceased to the VIFM was an important factor in the time to autopsy, it was not the primary determinant (**Supplementary Fig. 2**). Twenty-three of the 30 deaths occurred outside of business hours and were enabled by 24-h on-call processes.

After autopsy, the research coordinator contacts the funeral service nominated by the family to arrange transfer of the deceased. Two weeks after the death of the participant, family members are sent letters of appreciation from the study investigators and are offered an opportunity to meet with the clinical team if they had outstanding questions. To date, none have done so.

Because our study aims to enable genomic and functional studies, complete histories of pre-mortem treatment were collated from participant records. DNA and RNA have been isolated from CASCADE tissue samples for massively parallel sequencing, including for a recent study providing insights into disease resistance in epithelial ovarian cancer[7]. Double-stranded DNA isolated from needle-macrodissected snap-frozen tumor tissue has an average A_{260}/A_{280} ratio of 1.99 (range 1.86–2.13; **Supplementary Table 1**) and is suitable for whole-genome sequencing. RNA was partially degraded (from needle-macrodissected snap-frozen tissue average RNA integrity number 5.17 (range N/A–7.4; **Supplementary Table 1**) but was of sufficient quality to validate mutations identified through massively parallel DNA sequencing[7]. RNA quality was consistent with previously published data on post-mortem tissue quality[13].

CASCADE samples have also been used for immunohistochemical studies. We stained FFPE sections with markers commonly used in melanoma (S100), ovarian cancer (p53) or breast cancer (estrogen receptor and HER2) (**Fig. 2**). Staining was comparable to that seen in freshly frozen surgical samples from other patients, and we did not observe evidence of decreased staining associated with time to

Table 1 CASCADE study participants

Number of autopsies	Time enrolled (d)		Place of death			Time between death and notification (min)		Time between death and autopsy (h)		Distance from study center (km)		Number of metastatic sites sampled	
	Median	Range	Home	Hospice	Hospital	Median	Range	Median	Range	Median	Range	Median	Range
30	30	<1–385	8 (26.7%)	13 (43.3%)	9 (30.0%)	10	3–180	5.5	3–12	18.75	4.5–177	16	4–27

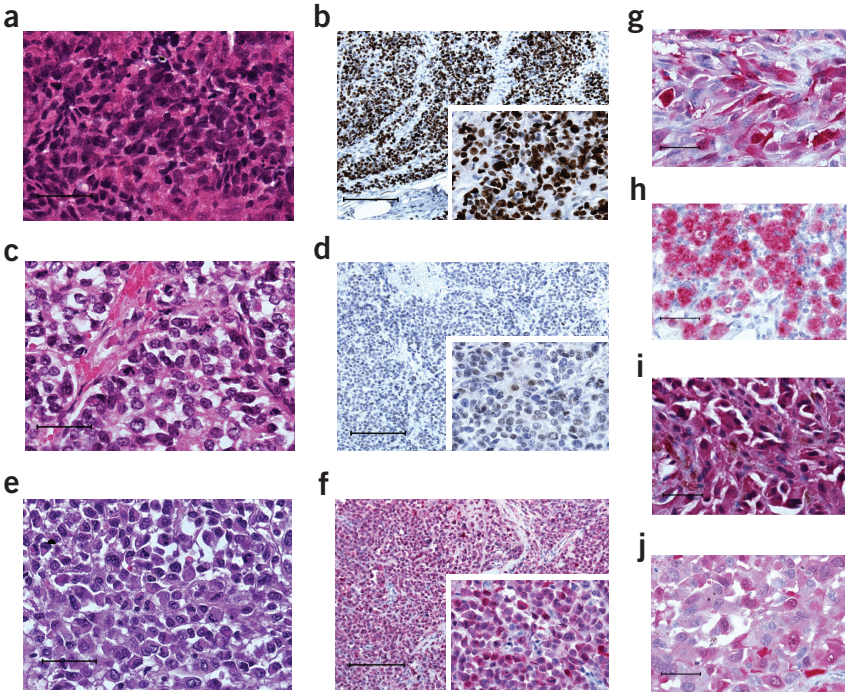

Figure 2 Tumor-type-specific immunohistochemistry of post-mortem CASCADE samples. (**a**) Hematoxylin and eosin (H&E) staining of metastatic serous ovarian cancer sampled at 6 h after death (scale bar, 50 μm). (**b**) p53 staining, indicative of high-grade serous ovarian carcinoma (scale bar, 200 μm). (**c**) H&E staining of metastatic breast cancer autopsied at 5.5 h after death (scale bar, 50 μm). (**d**) Retained estrogen receptor expression, as per primary tumor phenotype (scale bar, 200 μm). (**e**) H&E staining of metastatic melanoma, sampled at 7 h after death (scale bar, 50 μm). (**f**) S100 expression (3-amino-9-ethylcarbazole (AEC) chromagen; scale bar, 200 μm). Inset panels in (**b**), (**d**) and (**f**) are representative high power images of the corresponding tissue sections. (**g**–**j**) S100 expression in additional metastatic melanomas, sampled at 3 h (**g**), 3.5 h (**h**), 5 h (**i**) and 9.5 h (**j**) after the death of the patient (scale bars, 50 μm).

autopsy in this small series (**Fig. 2g**–**j**). We also generated PDXs by transferring tissue fragments or flow cytometry–sorted cells into immunocompromised mice. Using a threshold of 300 d after engraftment, we have produced transplantable PDXs for 13 of 17 cases (76.5%; **Supplementary Fig. 3** and **Supplementary Methods**).

The end of life is often an emotionally charged time, and consideration of involvement in research can further complicate a sensitive situation for patients and their families. Despite this, we have developed a process that meets stringent ethical criteria, allows collection of tumor samples in a timely and efficient manner and respects the end-of-life wishes of participants and their families. There are several essential elements to the program (**Fig. 1**). Careful selection of patients minimizes the possibility of causing distress and allows the discussion to take place across multiple clinical appointments, providing opportunities for patients and families to identify concerns and address specific questions. We are not aware of any emotional adverse events arising for participants or family members as a result of the program. The overall acceptance rate has thus far been high, and this has probably been aided by the close relationships formed between the participants and their doctors. Although potentially upsetting, the consent document explicitly details the autopsy procedure (**Supplementary Fig. 1**) in accordance with guidelines from the Consensus Panel on Research with the Recently Dead (CPRRD)[14] and specifically seeks advanced consent for the tissue donation. However, the study also states that CASCADE participation will be terminated if the family does not wish to proceed with tissue donation at the time of death. Though somewhat controversial, honoring family members' wishes at this time, even if they conflict with those expressed by the participant, derives from our wish to avoid causing undue distress to grieving survivors. An additional consideration to rapid autopsy for research is local laws and guidelines governing consent for post-mortem procedures, including whether final consent of family members is required. CASCADE has not been publicized, and none of the patients we approached were aware of the program before the discussion. Notably, many participants initiated a discussion by independently expressing a wish to donate their body for research, suggesting a willingness among late-stage cancer patients to participate in this type of research. Previous studies have found that the opportunity to participate in a donation program, when properly instituted, can help to provide meaning at the end of life for patients and their families[15,16], and this is consistent with our experience. We ensure that participants and their family are aware that CASCADE involves tissue rather body donation, and the deceased will be returned to the family for burial or cremation.

Our model ensures that autopsies are performed to a high professional standard as soon as is practically possible, without the overhead costs of an extensive study-specific infrastructure. Tobin Brothers provides its services pro bono as a community contribution, and VIFM performs autopsies at cost. The staff of Tobin Brothers and VIFM are highly engaged and understand that the promptness of starting an autopsy can affect the quality of the tissue obtained. Investigators share the cost of the program coordinator among the various disease streams. In 2014 the cost of the study was ~AU$5,400 (US$4,122) per participant, including the direct cost of the autopsy and amortized cost of the coordinator. We note that there is substantial in-kind support of the work, including biobanking costs. To our knowledge, the costs of running other rapid cancer-autopsy studies have not previously been published, although the Australian brain biobanking experience estimates costs of AU$15,000 per successful donation[17].

Close liaisons with community and hospice palliative care staff are essential to track participants without intruding on the family. Advance warning of the imminent death of a participant is particularly useful in establishing the processes for the timely procurement of a death certificate and awareness of the most current wishes of the family. A program coordinator is essential, as is active involvement and 'load sharing' of research investigators. The confronting nature of the autopsy procedure and the potential for fatigue owing to the constant nature of the program represent substantial challenges for researchers and the program coordinator, respectively. An additional program coordinator would be desirable to share the load throughout the year.

It is appropriate to reflect on the place of autopsy versus biopsy in the analysis of

recurrent disease and acquired resistance. Our view is that both approaches have strengths and weaknesses and should be regarded as complementary. Patient discomfort, the risk of complications (such as hemorrhage), the small amounts of material acquired and the difficulty of biopsying sites such as the brain or thoracic space are limitations to research-driven biopsy. In contrast, autopsies allow the collection of large samples of tumor tissue from multiple metastatic sites, including those that are typically inaccessible during life. Autopsy allows comprehensive mapping of the diversity of disease in individual patients. A small number of patients have provided unparalleled insight into the timing and pattern of disease progression, metastatic spread and polyclonal seeding[7,18,19]. For example, autopsy-enabled genomic studies have shown how minor clones found in primary tumors can dominate disease progression and ultimately lead to the death of the patient[20,21].

We performed 30 autopsies in our initial 3-year period, and it is possible to expand collection further and create a larger repository of accessible metastatic tumor tissue. However, autopsies lack the throughput of biopsy collection or the ability to collect closely timed samples. Because few patients are sampled at autopsy, the power to distinguish driver from passenger events based on statistical analyses is typically low; therefore, the development of PDXs to functionally evaluate findings is important[22,23].

CASCADE was established to address the need for extensive sampling of metastatic disease for research purposes. Routine autopsy of cancer patients has virtually disappeared from clinical practice in hospital systems. Neurological researchers have established post-mortem brain banks, necessitated by the complexities of obtaining tissue samples for research purposes while patients are alive[17,24,25]. In contrast, few cancer-autopsy studies have been described in detail[26,27]. Recently, a large-scale brain- and body-donation program was described[28] for a range of diseases, including cancer, and there has also been a focus on the collection of normal tissues, including through rapid autopsy, for research purposes[29]. Models of research-driven autopsy often rely on an extensive, dedicated infrastructure that is costly and time-consuming to maintain. We believe our model is transferable to other research environments seeking to study end-stage cancer, efficiently providing an enabling platform for several disease streams simultaneously.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper (doi:10.1038/nbt.3674).

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COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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1. Collins, F.S. & Barker, A.D. *Sci. Am.* **296**, 50–57 (2007).
2. Hudson, T.J. *et al. Nature* **464**, 993–998 (2010).
3. Gerlinger, M. *et al. N. Engl. J. Med.* **366**, 883–892 (2012).
4. Andersson, A.K. *et al. Nat. Genet.* **47**, 330–337 (2015).
5. Keats, J.J. *et al. Blood* **120**, 1067–1076 (2012).
6. van Thuijl, H.F. *et al. Acta Neuropathol.* **129**, 597–607 (2015).
7. Patch, A.M. *et al. Nature* **521**, 489–494 (2015).
8. Topp, M.D. *et al. Mol. Oncol.* **8**, 656–668 (2014).
9. Quintana, E. *et al. Cancer Cell* **18**, 510–523 (2010).
10. Read, M. *et al. Ann. Surg. Oncol.* **23**, 305–311 (2016).
11. Lawrence, M.G. *et al. Nat. Protoc.* **8**, 836–848 (2013).
12. Laidlaw, I.J. *et al. Endocrinology* **136**, 164–171 (1995).
13. van der Linden, A. *et al. PLoS One* **9**, e115675 (2014).
14. Pentz, R.D. *et al. Nat. Med.* **11**, 1145–1149 (2005).
15. Lindell, K.O., Erlen, J.A. & Kaminski, N. *PLoS Med.* **3**, e234 (2006).
16. Steinhauser, K.E. *et al. Ann. Intern. Med.* **132**, 825–832 (2000).
17. Dedova, I. *et al. Int. J. Mol. Sci.* **10**, 366–384 (2009).
18. Gundem, G. *et al. Nature* **520**, 353–357 (2015).
19. Campbell, P.J. *et al. Nature* **467**, 1109–1113 (2010).
20. Shah, S.P. *et al. Nature* **461**, 809–813 (2009).
21. Krøigård, A.B. *et al. Oncotarget* **6**, 5634–5649 (2015).
22. Cassidy, J.W., Caldas, C. & Bruna, A. *Cancer Res.* **75**, 2963–2968 (2015).
23. Eirew, P. *et al. Nature* **518**, 422–426 (2015).
24. Hulette, C.M. *J. Neuropathol. Exp. Neurol.* **62**, 715–722 (2003).
25. Kretzschmar, H. *Nat. Rev. Neurosci.* **10**, 70–78 (2009).
26. Embuscado, E.E. *et al. Cancer Biol. Ther.* **4**, 548–554 (2005).
27. Rubin, M.A. *et al. Clin. Cancer Res.* **6**, 1038–1045 (2000).
28. Beach, T.G. *et al. Neuropathology* **35**, 354–389 (2015).
29. Carithers, L.J. *et al. Biopreserv. Biobank.* **13**, 311–319 (2015).

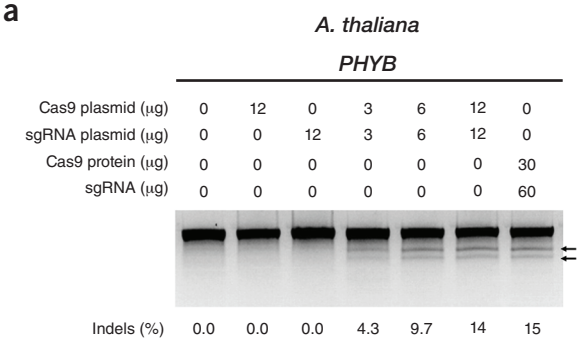
Bypassing GMO regulations with CRISPR gene editing

To the Editor:

The US Department of Agriculture (USDA) recently announced that regulation of a CRISPR-Cas9 gene-edited mushroom, in which a polyphenol oxidase (*PPO*) gene had been mutated to avoid (or delay) browning, fell outside of genetically modified organism (GMO) legislation[1]. However, we remain concerned that the approved mushroom may still contain tiny fragments of foreign DNA in its genome. If foreign DNA is present after CRISPR-Cas9 editing, regulatory approval would be required under current GMO legislation.

In a letter (available in ref. 1 and at https://www.aphis.usda.gov/aphis/home/) submitted to the USDA, Yinong Yang, a researcher at Pennsylvania State University, outlined how the gene-edited mushroom was produced. Mushroom protoplasts (fungal cells with no cell wall) were transfected with plasmids encoding Cas9 and a guide RNA (gRNA) specific for a *PPO* gene. Transfected protoplasts were regenerated to produce mushrooms that contained small deletions (1–14 base pairs (bp)) in the *PPO* gene. The researchers analyzed the genome of edited fungi using PCR and Southern blot analysis and found no evidence of foreign DNA in the genome. In response, the USDA decided that the gene-edited mushroom fell outside of GMO regulations and that the US government had no authority to regulate this product.

a



b

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CACTAGGAGCAACACCC----------AACGGG    WT
CACTAGGAGCAACACCgTAGTTATCTGAACGGG   +10 (AmpR)

CACTAGGAGCAACACCC-----------AACGGG    WT
CACTAGGAGCAACACCCGGAGGACCGAAAACGGG   +11 (AmpR)

CACTAGGAGCAACACCC------------AACGGG    WT
CACTAGGAGCAACACCCTAGGAGCAACACCcaacG   +12 (gRNA guide sequence)

CACTAGGAGCAACACCC---------------AACGGG    WT
CACTAGGAGCAACACCCCGCTCGCCGCAGCCGAACGaG   +15 (Cas9 plasmid backbone)

CACTAGGAGCAACACCC--------------------------------AACGGG    WT
CACTAGGAGCAACACCCAAGGCCTTTTGCTGGCCTTTTGCTCACATGTTCAACGGG   +33 (pUC origin)

CACTAGGAGCAACACCC-----------------------------------AACGGG    WT
CACTAGGAGCAACACCtGATGATCAGGTCCTTCTTCACCTCCTTGTAGCCCTAACGGG   +35 (Cas9 sequence)

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CACTAGGAGCAACACCCGGAGGACCGAAGGAGCTAACCGCTTTTTTGCACAACAACGGG   +36 (AmpR)

CACTAGGAGCAACACCC-------------------------------------------AACGGG    WT
CACTAGGAGCAACACCCGCCCTGATCAAGAAGTACCCCAAGCTGGAGAGCGAGTTCGTGTAACGGG   +43 (Cas9 sequence)

CACTAGGAGCAACACCC---------------------------------------------AACGGG    WT
CACTAGGAGCAACACCaGAGGTGGCGAAACCCGACAGGACTATAAAGATACCAGGCGTTTCCAACGGG   +45 (pUC origin)

CACTAGGAGCAACACCC-----------------------------------------------AACGGG    WT
CACTAGGAGCAACAACgATCGGAGGACCGAAGGAGCTAACCGCTTTTTTGCACAACATGGGGCAACGGG   +46 (AmpR)
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Figure 1 Insertions of Cas9 and gRNA plasmid-derived DNA sequences at the genomic target site in transiently transfected *Arabidopsis* protoplasts. (**a**) T7 endonuclease I (T7EI) assay showing targeted mutagenesis in *PHYB* in protoplasts. *Arabidopsis* protoplasts were transfected by variable amounts of Cas9 and gRNA plasmids. Arrows indicate the expected positions of DNA bands cleaved by T7EI. Mutation frequencies (indels (%)) were measured by targeted amplicon sequencing. (**b**) Mutant DNA sequences containing plasmid-derived DNA sequences. The numbers of inserted nucleotides are indicated. Inserted sequences are underlined. Origins of inserted sequences are indicated in parentheses. The protospacer-adjacent motif is shown in bold. WT, wild type.