

# International Hands-on Training on **Genome Editing Technologies**



Photo: L. Vidyasagar, ICRISAT

14-25 October 2019  
ICRISAT, Patancheru, Hyderabad, India



RESEARCH PROGRAM ON  
Grain Legumes and  
Dryland Cereals

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## **Foreword**

In recent years, biotechnological approaches have been the central to scientific research in agriculture. Significant progress has been made in terms of advances both in conventional and modern technologies. The global human population is projected to exceed more than 9 billion by 2050 requiring crops with higher quality yields with multiple beneficial traits. In this context, new breeding technologies like genome editing offers the potential to transform crop and livestock breeding programs at an astonishingly rapid rate without any adverse impact on the native phenotypes. Plants developed through genome editing can be directly released for cultivation with less regulatory efforts, as these are not classified as genetically modified organisms (GMO) in most of the countries. Hence, genome editing technologies have revolutionized the process of making DNA-level changes and the implications of this technology reach far beyond standard molecular biology applications. This introductory 10 – day comprehensive training program is ideal for researchers who are looking for a balanced theoretical vs hands-on introduction to gene editing.

The program is structured as a combination of lectures, discussions with hands-on laboratory instructions and technology demonstrations for helping the participants stay ahead imparting both theory and practical aspects of CRISPR based genome-editing technologies. The course will walk you through a basic gene editing workflows, from design and cloning of target specific guide RNAs (gRNAs), delivery of gRNAs in plant cells, detection through to analysis of gene editing efficiencies. This is a great opportunity to interact and benefit from the expertise of our scientists and collaborators across the globe and private sectors, as they help you design your first genome editing experiments. It is envisaged that at the end of the workshop, participants will be able to design their own experimental workflows.

We anticipate that workshops of this kind would help in knowledge dissemination and stimulating researchers for precise genome editing of priority traits in crop plants globally for the benefit of mankind.

**Pooja Bhatnagar-Mathur**

## **Experiments schedule**

### **Monday (14 October 2019)**

- Bioinformatics tools for guide RNA designing, off-target prediction

### **Tuesday (15 October 2019)**

- Strategy, cloning of the guideRNA and Cas9 into the plant transformation vector

### **Wednesday (16 October 2019)**

- Genetic transformation

### **Thursday (17 October 2019)**

- Confirmation of the recombinant clones by colony PCR
- Genetic transformation
- Bacterial transformation for the Gateway LR ligated reactions

### **Friday (18 October 2019)**

- Plasmid DNA isolation for the colony PCR positive colonies
- *Agrobacterium* transformation for the confirmed recombinant plasmids by electroporation
- Genetic transformation

### **Saturday leisure trip (19 October 2019)**

### **Sunday Holiday (20 October 2019)**

### **Monday (21 October 2019)**

- Demo Genetic transformation

### **Tuesday (22 October 2019)**

- Visit to the Central and Cellular Molecular Biology (CCMB) and National Institute of Animal Biotechnology (NIAB) for gene editing facilities

### **Wednesday (23 October 2019)**

- Functional characterization of the gene edited plants
- Genomic DNA isolation for the putative gene edited plants

**Thursday (24 October 2019)**

- Surveyor assay/ Nanopore sequencing for identifying the Indels in gene edited plants
- Library preparation
- Loading and sequencing

**Friday (25 October 2019)**

- Surveyor assay/Nano pore sequencing data analysis
- Brief presentations - participants

## List of speakers

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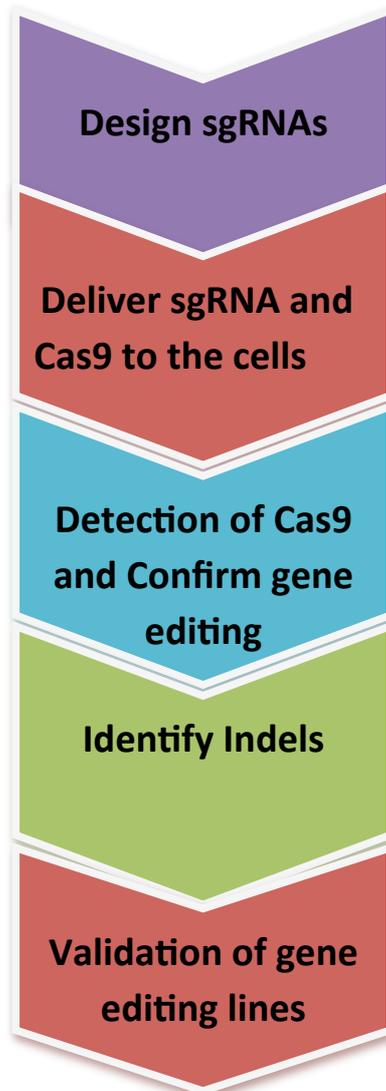
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## **Theme of the course**

The CRISPR-Cas9 system is being widely used for genome engineering in many different biological applications. CRISPR-mediated genome editing techniques greatly facilitate targeted genome modifications, such as deletions or insertions, in complex organisms including plants. It was originally adapted from the bacterial Type II CRISPR system and utilizes a Cas9 endonuclease guided by RNA to introduce site-specific double-strand breaks (DSBs) at specific sites in the targeted genome. The induced double-strand breaks are repaired either through non-homologous end joining (NHEJ) or homologous recombination (HR), resulting in specific and targeted mutations. In recent years, the CRISPR-Cas9 system has been established as the editing technique of choice due to ease of use, cost effectiveness, and the high-efficacy of desired targeted edits. In this training course, we demonstrate a complete workflow from design and synthesis of target specific single guide RNAs (sgRNAs), delivery of sgRNAs into plant cells and analysis of gene editing efficiencies (Fig. 1).



**Fig 1.** Overall workflow for CRISPR-mediated genome editing

## **Bioinformatics tools for guide RNA designing**

CRISPR/Cas9 system has been successfully used to generate precise mutations in multiple crops including sorghum, cotton, rice, wheat and potato. Most mutations generated by the CRISPR/Cas9 system are either insertions or deletions usually located close to the DSB (double strand break) site that occur 3 bp upstream of the proto-spacer adjacent motif (PAM) (Fig. 2). Target design is a crucial step to avoid off-target cleavage in the genome. As a result, access to genome databases is very important for genome editing using CRISPR/Cas9 technology. However, not all plant species have complete or fully accessible genomic databases. CRISPR/Cas9 may also be used for functional analysis of genes in plants where full genome is not sequenced yet. Nevertheless, access to full genome sequence may facilitate the design of reliable targets for fine-tuning mutations.

### ***sgRNA designing***

CRISPR/Cas9 gene targeting requires a custom single guide RNA (sgRNA) specific to the DNA target and a tracrRNA sequence which binds to a recombinant form of Cas9 protein that has DNA endonuclease activity. The resulting complex will cause target-specific double-stranded DNA cleavage. The cleavage site will be repaired by the nonhomologous end joining DNA repair pathway, an error-prone process that may result in insertions/deletions (INDELS) that may disrupt gene function.

### **Parameters to consider:**

1. Unique to whole genome (100% identity at nucleotide level).
2. The length of the sgRNA target site, excluding the PAM sequence, can be from 15 to 23 bases.
3. Identify a PAM sequence (5'-NGG-3') in the 3' end of the DNA sequence you would like to target.

*There are several online tools listed in Table 1 that detect PAM sequences and list possible crRNA sequences within a specific DNA region. These algorithms also predict off-target effects elsewhere in the genome, allowing to choose the most specific crRNA for your research application.*

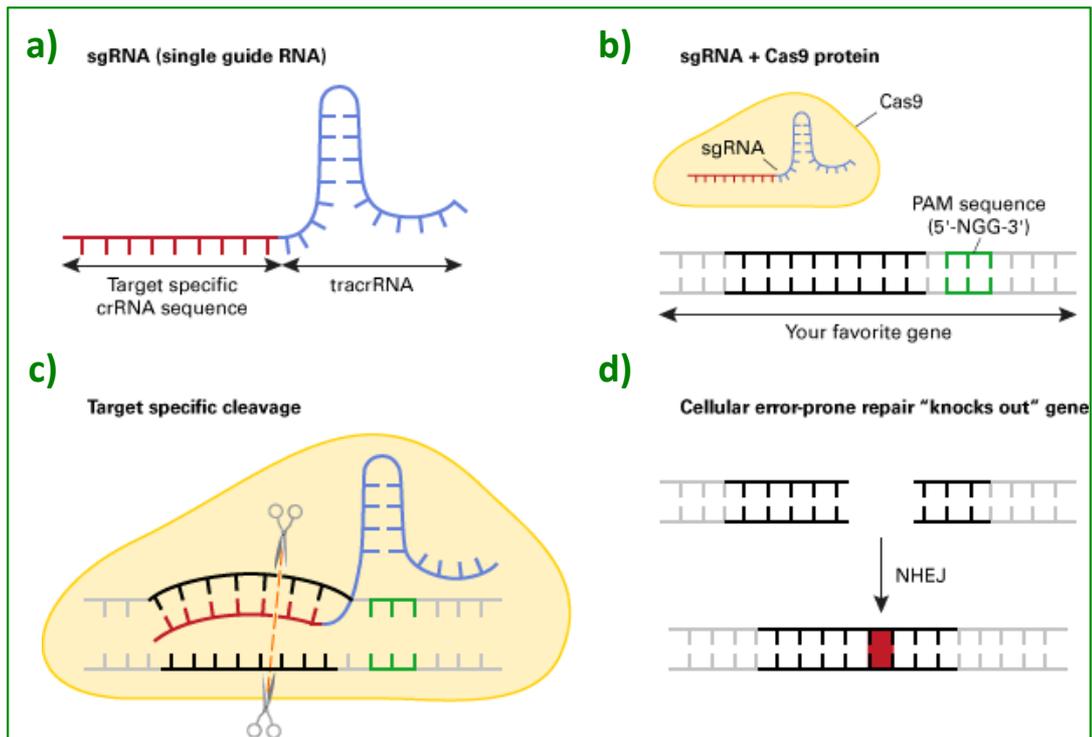
Name	Url	Output
CHOPCHOP	<a href="https://chopchop.cbu.uib.no">https://chopchop.cbu.uib.no</a>	Candidate guide sequences and off-target loci
CasFinder	<a href="https://omictools.com/casfinder-tool">https://omictools.com/casfinder-tool</a>	Candidate guide sequences and off-target loci
Cas-OFFinder	<a href="http://www.rgenome.net/cas-offinder/">http://www.rgenome.net/cas-offinder/</a>	Off-target loci for guide sequences
flyCRISPR	<a href="https://flycrispr.org/">https://flycrispr.org/</a>	Candidate guide sequences and off-target loci
E-CRISP	<a href="http://www.e-crisp.org/E-CRISP/">http://www.e-crisp.org/E-CRISP/</a>	Candidate guide sequences and off-target loci
Guide RNA Sequence Design Platform	<a href="https://www.hindawi.com/journals/bmri/2013/270805/">https://www.hindawi.com/journals/bmri/2013/270805/</a>	Candidate guide sequences and off-target loci
CasOT	<a href="https://omictools.com/casot-tool">https://omictools.com/casot-tool</a>	Off-target loci and additional guide sequences
CRISPR-ERA	<a href="http://crispr-era.stanford.edu/">http://crispr-era.stanford.edu/</a>	Candidate guide sequences and distances to TSS
Benchling	<a href="https://www.benchling.com/crispr/">https://www.benchling.com/crispr/</a>	Candidate guide sequences and off-target loci

**Table 1.** List of the Web-based sgRNA design tools

4. The 20 nt upstream of the PAM sequence will be target sequence (crRNA) and Cas9 nuclease will cleave approximately 3 bases upstream of the PAM.
5. Target sequence GC content should be between 45-65%.
6. The PAM sequence itself is absolutely required for cleavage, but it is NOT part of the sgRNA sequence and therefore should not be included in the sgRNA.

### **Tips**

- The target sequence can be on either of the DNA strands.
- Should not match 100% with any region other than target around “PAM + PAM proximal 7-10 nucleotides”
- RNA Pol III promoter - U3 (for guide starting with “A”) and U6 (for guide starting with “G”)
- Dual guides shall be used in FG (forward guide) & RG (reverse guide) combination to delete internal fragment
- Genomic BLAST can be used for finding the off-targets in addition to the online tools.
- When designing the target sequence, introns and the region near the 3' end of the coding sequence should be avoided.



**Fig 2. The principle of CRISPR/Cas9-mediated gene disruption.** A single guide RNA (sgRNA), consisting of a crRNA and a tracrRNA sequence that interacts with the Cas9 protein (a), binds to a recombinant form of Cas9 protein that has DNA endonuclease activity (b). The resulting complex will cause target-specific double-stranded DNA cleavage (c). The cleavage site will be repaired by the nonhomologous end joining DNA repair pathway, an error-prone process that may result in insertions/deletions (INDELs) that may disrupt gene function (d). (Courtesy: Takara)

The sequence of the 20 nt target site selected as sgRNA may not be unique across the entire genome of the organism. A given sgRNA targeting sequence will have additional sites throughout the genome where partial homology exists due to which that target sequence will direct the Cas9 nuclease toward that sites resulting in double strand break (DSB) in the DNA. To minimize off-target effects, the presence of such target sequences should be monitored in whole genome and selection of those target sequences should be avoided. Off-target effects could be minimized by using web-based sgRNA design tools and doing BLAST search of whole genome.

#### Off-target effect can cause

- Detrimental phenotypes
- Undetected accumulations

- Adverse effects in future generations/passages
- Excessive DNA breaks
- Genome rearrangements
- Cell cycle arrest
- Apoptosis

### **Off-target Analysis**

The Cas-OFFinder was used to identify the potential off-target sites. Several parameters from higher stringency to lower stringency were employed according to (Xin et al., 2017), as shown below:

- Mismatch=1; DNA Bulge Size=0; RNA Bulge Size=0
- Mismatch=2; DNA Bulge Size=0; RNA Bulge Size=0
- Mismatch=3; DNA Bulge Size=0; RNA Bulge Size=0
- Mismatch=0; DNA Bulge Size=1; RNA Bulge Size=1
- Mismatch=1; DNA Bulge Size=1; RNA Bulge Size=1
- Mismatch=2; DNA Bulge Size=1; RNA Bulge Size=1
- Mismatch=3; DNA Bulge Size=1; RNA Bulge Size=1
- Mismatch=0; DNA Bulge Size=2; RNA Bulge Size=2
- Mismatch=1; DNA Bulge Size=2; RNA Bulge Size=2

### **Tips**

- Once the 20 nt target sequence has been identified, off targets should be identified using either NCBI Blast search (<http://blast.ncbi.nlm.nih.gov>) or any web based tools such as Cas-OFFinder (<http://www.rgenome.net/cas-offinder/>) or CRISPR-P (<http://cbi.hzau.edu.cn/crispr/>).
- Max. total mismatches: can be set to 0-5. Note: more than four mismatches in total prevent DSB induction.
- Mismatches of the one to five base pairs at the 5' end of sgRNAs are better tolerated than those at the 3' end.
- Single and double mismatches are tolerated to various degrees depending on their position along the guide RNA-DNA interface.

- Mismatches at a distance to the PAM (non-seed), will still allow the introduction of a DSB, while mismatches close to the PAM region (seed region) will abolish the introduction of a DSB.
- Max. core mismatches: can be set to 0-2. Note: in the core, more than 2 mismatches abolishes DSB introduction at the potential off-target position.

# Cloning of the single guide RNA and Cas9 into the plant transformation vector

## Chemicals and materials

- Donor Vector1 (U6Pro-sgRNA cassette)
- Donor Vector2 (35s-Cas9-PolyA)
- Destination Vector (Expression vector)
- In-Fusion Cloning kit (Takara)
- LR Clonase™ II Plus Enzyme Mix (Invitrogen)
- Proteinase K solution 2 µg/µl (Invitrogen)
- SOC Medium
- Mach1™ T1R chemically competent cells
- Ampicillin (10 mg/ml)
- Kanamycin Sulfate (10 mg/ml)
- PCR components

## Step 1: Cloning of the sgRNA (20bp) into Donor vector I

### *PCR amplification for the Fragment 1 and 2*

- **Fragment 1** PCR amplified using forward primer from SbU6 promoter F (BstBI/AfeI sites) and sgRNA reverse primer including last 20 bp of SbU6 and 20 bases of gRNA (target).
- **Fragment 2** PCR amplified using forward primer including 20 bases of gRNA and first 20 bp of gRNA scaffold, reverse primer from donor vector at SbfI/ HpaI site.

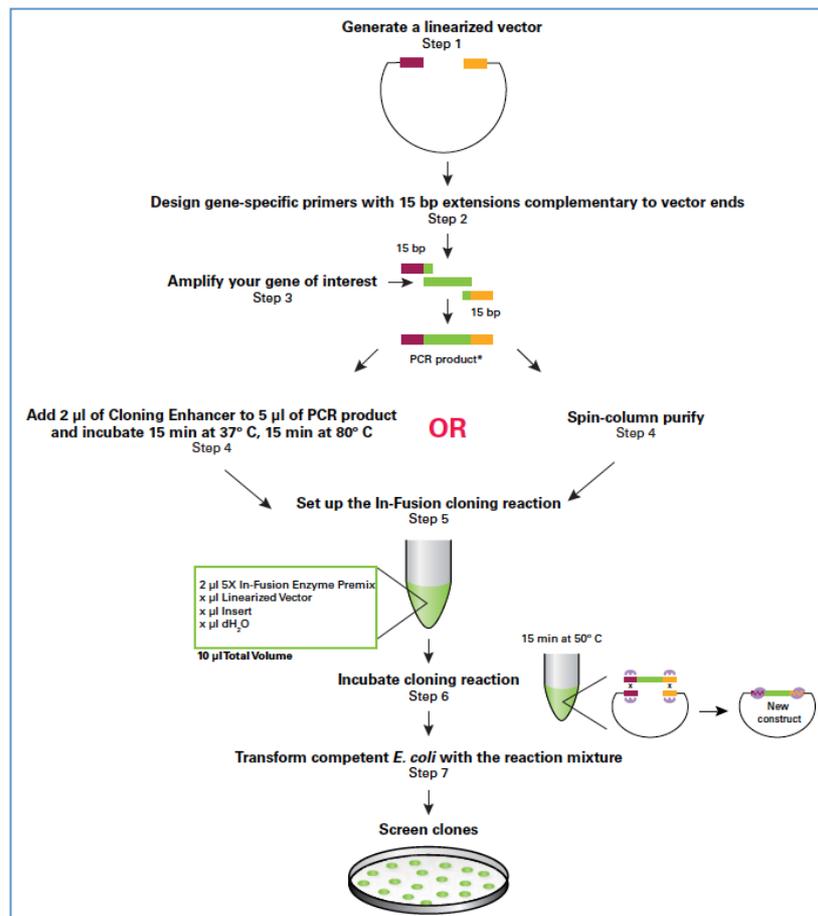
### *Overlap extension fusion PCR*

- Gel purified PCR fragments 1 and 2 mixed in equal molar concentrations and will be used as template for overlap extension fusion PCR using forward primer from SbU6 promoter (at BstBI/ AfeI site) and reverse primer from donor vector at SbfI/HpaI site.

## Step 2: Infusion cloning of complete fragment into donor vector 1

Typical single guide RNA (sgRNA) cloning into Cas9 vectors entails a lengthy process that requires multiple cloning steps such as incorporation of new restriction sites, restriction

digests, and ligations. The traditional approach is confounded by low cloning efficiency, resulting in additional time-consuming screens to identify a colony containing your sgRNA of interest. These bottlenecks also render it difficult to move sgRNAs from one vector to another and make it nearly impossible to multiplex several sgRNAs in one plasmid. Hence, infusion cloning is used for fast, directional cloning of one or more fragments of DNA into any vector, which fuses DNA fragments (e.g., PCR-generated inserts and linearized vectors) efficiently and precisely by recognizing 15-bp overlaps at their ends. These 15-bp overlaps can be engineered by designing primers for amplification of the desired sequences (Fig. 3).

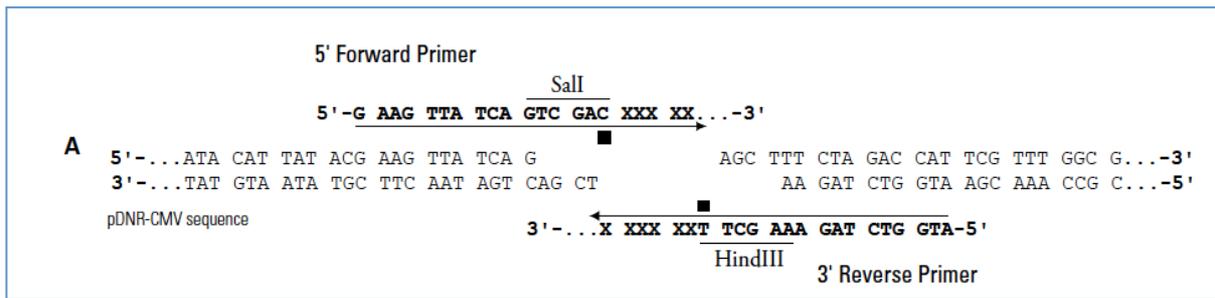


Courtesy: Takara

**Fig 3. Steps involved in In-Fusion cloning.**

### ***Primer designing***

Primer design and quality are critical for the success of the In-Fusion reaction. In-Fusion can join two or more fragments, e.g. vector and insert (or multiple fragments), as long as they share 15 bases of homology at each end. Therefore, In-Fusion PCR primers must be designed in such a way that they generate PCR products containing ends that are homologous to those of the vector (or each other) (Fig. 4).



**Fig 4.** Designing the primers for In-Fusion cloning. The above figure shows examples of primers designed with recognition sites for restriction enzymes that generate: 5' overhangs.

### ***Preparation of a linearized vector by restriction digestion***

Donor vector 1 will be linearized with restriction enzymes BstBI and SbfI, and the linear vector is purified. Fused purified PCR amplicon from step2 is then cloned into linearized donor vector using infusion cloning.

### ***Infusion reaction setup***

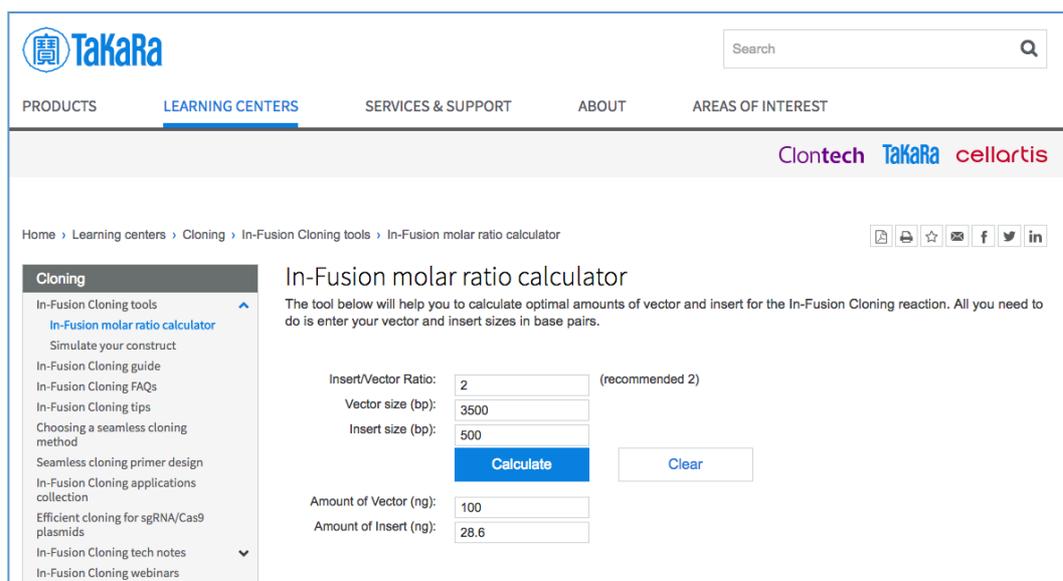
When performing a cloning reaction with more than two fragments (including the linearized vector), the molar ratio of each of the multiple inserts should be 2:1 with regards to the linearized vector, i.e., two moles of each insert for each mole of linearized vector (Fig. 5) (<https://www.takarabio.com/learning-centers/cloning/in-fusion-cloning-tools/in-fusion-molar-ratio-calculator>).

### **Components**

### **Volume**

5X In-Fusion HD Enzyme Premix	2 µl
Linearized vector (100 ng of 3.5kb linearized donor vector)	X µl*
Purified PCR fragment (28.6ng of 500bp insert)	X µl*
dH2O (as needed)	X µl
Total volume	10 µl

Adjust the total reaction volume to 10 µl using deionized H<sub>2</sub>O and mix the reaction.



**Fig 5.** Tool used for calculation of the insert and vector molar ratio for infusion cloning.

Incubate the reaction for 15 min at 50 °C, then place on ice, followed by bacterial transformation and recombinant selection by colony PCR.

### **Preparation of binary vector using gateway cloning strategy**

gRNA cassette (Donor vector 1), Cas9 (Donor vector 2) along with the destination vector (Binary) is used in LR recombination reaction.

**Gateway cloning** Gateway cloning Technology is a universal cloning method that takes advantage of the site-specific recombination properties of bacteriophage lambda to provide a rapid and highly efficient way to move your gene of interest into multiple vector systems.

### **Gateway reaction set up**

Add the following components to a 1.5 ml micro centrifuge tube at room temperature and mix:

<b><u>Components</u></b>	<b><u>Volume</u></b>
LR Clonase II Enzyme Premix	2 µl
Donor Vector1Guide (10 f.mol)	X µl*
Donor Vector2 cas9 (10 f.mol)	X µl*
Destination Vector (20 f.mol)	X µl
1x TE (as needed)	X µl
Total volume	10 µl

Incubate the reaction at 25°C for 16h/overnight.

After that add 1  $\mu$ l proteinase K enzyme to the reaction mix and incubate at 37°C for 15 min followed by bacterial transformation.

### ***Bacterial transformation***

- 1 Take the competent *E. coli* cells (supplied with the kit) from -80°C and thaw slowly on ice.
- 2 Add the ligation/cloning mix (1-3  $\mu$ l out of 6  $\mu$ l) to the competent *E. coli* cells and mix by tapping the tube gently and incubate on ice for 30 min.
- 3 Heat-shock the cells for 60 sec at 42°C.
- 4 After heat-shock immediately keep the *E. coli* cells on ice for 5 min.
- 5 Add 250  $\mu$ l/800  $\mu$ l of room temperature SOC/LB medium and keep the tube at 37 °C for 60 min on a rotatory shaker at 200 rpm.
- 6 Plate (spread) at least two volumes (i.e. 50 and 100  $\mu$ l) of the transformed *E. coli*. cells on pre-warmed LB agar plate containing antibiotic (kanamycin 50  $\mu$ g/ml) selection.
- 7 Incubate the plates overnight at 37 °C.
- 8 Check for colonies and perform colony PCR.

### ***Colony PCR***

- 1 Select colonies to pick. Pick colony with a sterile pipette tip. Dab the pipette tip into a properly labelled master plate (containing the appropriate selection) then place the pipette tip in a correspondingly labelled tube containing 50  $\mu$ l of sterile water. You may pick as many as you wish, typically 8-16 colonies from each transformation plate. The master plate may contain colonies from many transformation plates. Incubate master plate at 37°C. Rinse the tip in the H<sub>2</sub>O in the tube by pipetting up and down a few times.
- 2 Keep the labelled tubes that contain the bacterial suspension in boiling water bath for 3 min or heat @ 95°C for 10 min. Allow to cool to room temperature.
- 3 Prepare 50  $\mu$ l of PCR reaction mixture containing 1X PCR buffer, 200  $\mu$ M dNTPs, 2.5 U of Taq DNA polymerase, (enzyme is added just before use) and 150 ng each of the required primers.
- 4 Add 5  $\mu$ l of the cell suspension to the reaction mixture and start the PCR.
- 5 Keep the PCR conditions as 94°C, 1 min; 55°C, 1 min; and 72°C, 1 min for 30 cycles.
- 6 When finished, run 10  $\mu$ l of reaction mixture on a 0.8% agarose gel.

### ***Agrobacterium* transformation**

The isolated expression vector plasmid from *E.coli* is then transferred to *Agrobacterium* strain to make it ready for *Agro-transformation*.

#### **Chemicals/materials**

- Expression cassette (Binary vector)
- Spectinomycin
- PCR components
- PCR machine

#### **Electro-transformation of *Agrobacterium tumefaciens***

- Transformation was initiated by adding 10 -100ng of plasmid DNA to the frozen *Agrobacterium*.
- The suspension was carefully mixed with a pipette tip and transferred to a pre-chilled 2 mm cuvette. Electroporation was carried out at 2.5 KV, 25  $\mu$ F capacitance and 201  $\Omega$  resistance for nearly 5 milli seconds.
- For all the successive electroporations, a pulse time of 5.25 milli sec was recorded. Immediately after electroporation, 1 ml of fresh LB medium was added and the bacterial suspension was incubated at 28 °C with shaking for 2 h.
- After 2 h of incubation, 100  $\mu$ l of the transformed cells were then plated on LB semi-solid plates with kanamycin for selection of transformed clones, and the plates were incubated at 28 °C for 48 h. Single colonies were picked up from the plate and incubated for plasmid mini preparation.

#### ***Agrobacterium* culture preparation for the transformation**

- Inoculate *Agrobacterium* strain C58 harbouring expression construct grown overnight at 28°C in 30 mL of LB medium with antibiotics rifampicin, and kanamycin at concentrations of 50  $\mu$ g/mL.
- Prepare the secondary culture by adding the overnight culture to 300 mL of fresh medium with the same antibiotics and grown to the stationary phase (OD600 ~2.0).
- Harvest the cells by centrifuging at 5500g for 20 min and resuspend the pellet in infiltration medium (0.5 $\times$  MS, 5% sucrose, 0.05% Silwett L-77) to obtain the desired density (OD600 of 0.8 or >2.0).

- Infect plants by floral dip/submersing inflorescences in the bacterial suspension (Clough and Bent, 1998) or performing directly on inflorescence by adding drop-by-drop agro culture using micropipette.

## **Genetic transformation into Chickpea/ Tobacco/ Arabidopsis**

Irrespective of the plant part used as an explant, the plant material should be surface sterilized to remove the surface borne micro-organisms. The explants are cleaned with a liquid detergent in running water and surface sterilized with disinfectant solutions like sodium hypochlorite 0.5 to 5%, calcium hypochlorite 9 to 10%, hydrogen peroxide 10 to 12 %, bromine water 2%, mercuric chloride 0.1 to 1%, benzalkonium chloride 0.01 to 0.1%, ethanol 75 to 95%. Each plant species has specific sterilization procedure for explant preparation.

### **Chickpea (*Sharma et al., 2016*)**

#### *Seed Sterilization*

1. Healthy and mature seeds of chickpea were surface sterilized using 70% (v/v) ethanol for 1 min followed by 0.1% mercuric chloride for 10 mins.
2. The seeds were then washed with distilled water twice and then soaked overnight in sterile water.

#### *Explant preparation*

3. The seeds were then decoated and placed on a germination media containing MS, BAP and kinetin. The plates were incubated at  $26 \pm 1^\circ\text{C}$  under continuous light conditions (60 PE/m<sup>2</sup>/s light intensity provided by cool white fluorescent lamps) for 5 to 7 days until the axillary buds are prominent.
4. The axillary buds are removed carefully from the base followed by two cuts made through the axillary meristem to remove the root and shoot tips up to the epicotyl and hypocotyl regions.
5. These axillary meristem explants are then incubated on a plate containing MS, BAP and kinetin for another 7 days under similar conditions as earlier.
6. The emerging shoot buds are carefully removed from the emerging shoot buds from the enlarged base of the axillary bud by scraping with a sterile scalpel to obtain explant for transformation.

### ***Agrobacterium* culture preparation and infection**

7. A single colony of *Agrobacterium tumefaciens* strain harbouring the gene of interest is grown in 25 ml of YEB liquid medium containing desired antibiotics and allowed to grow overnight at 28°C in an incubator-shaker.
8. The culture is allowed to grow until an OD. of 0.6-1 and centrifuged at 6000g for 5 min to obtain pellet.
9. The pellet is then resuspended in 25 ml half strength MS medium. This step is repeated once.
10. The culture is then poured on a sterile plate for infecting the prepared explants.
11. The explants are dipped in the agrobacterium culture for 1-2 s and placed on MS media ensuring that the base of the cotyledon is embedded in the medium.
12. The explants are co-cultivated with the bacteria for 48h, at  $26 \pm 1^\circ\text{C}$  under continuous cool white light provided by fluorescent lamps (60 PE/m<sup>2</sup>/s).

## **Tobacco (*Horsch et al., 1989*)**

### ***Explant preparation***

- The leaves of Tobacco are surface-sterilized by sequential treatments by wiping them with 70% ethanol and then with sterile tissue paper.
- Transfer to 15% clorax solution for 10 min. These are then washed in a series of sterile water for three times.
- Sterilize with Bivastin for 30s and wash with distilled water and then, sterilize the leaves with 0.1% mercuric chloride for 2-5 min.
- Rinse the leaves two to three times with sterile distilled water. Then, leaf discs are cut with a sterile leaf disc borer.

### ***Co-cultivation and selection of transformed tobacco leaf explants***

- The tobacco leaf discs are infected by dipping for one minute in the *Agrobacterium* culture prepared as described above.
- The explants are placed on MS<sub>4</sub> media.
- The explants are plated as 10 leaf discs per petri plate and sealed with parafilm.
- After 2 days of co-cultivation, the explants are transferred into MS<sub>4</sub> containing 250mg/l cefotaxime and 20-50mg/l Hygromycin.
- After induction of 2-3weeks, the explants are transferred to the selection media of same composition but supplemented with 30 and 50 mg/l Hygromycin to facilitate the growth of only transformed plants.

### ***Shoot elongation, rooting hardening and acclimatization of the explants***

- Once the shoot initiation was observed, the leaf explants forming shoots were placed on plane MS medium and 250mg/l cefotaxime. The explants with differentiated and proliferated multiple shoots were transferred to MS medium.
- The shoots over 3 cm in length were transferred to root induction medium (RIM) consisting of MS basal medium.
- The *in vitro* regenerated plantlets with well-developed roots were transferred to green house for acclimatization.

### ***Arabidopsis thaliana* (Clough and Bent 1998)**

- Sow the seeds of *Arabidopsis* in 4-cm pots containing sand/soil/Farmyard manure (3:2:1) mixture and incubate in the culture room at 22°C temperature for germination.
- Plants at the four-leaf stage transfer to the glasshouse and irrigate every 2 days until inflorescence appeared.
- Plants with inflorescences of about 5 cm transform with suspension cultures of *A. tumefaciens* harbouring the binary plasmids by dipping inflorescence for 5 mins or plants were inoculated by direct drop-by-drop inoculation to every flower by using a micropipette.
- Cover the plants with plastic bags and incubate in dark for 10–24 h. Inoculation with the *Agrobacterium* was repeated twice at 3-day intervals and the seeds were collected when all the siliques dried.
- Collect seeds from plant once siliques get dried.

### ***Selection***

- Incubate *Arabidopsis* seeds in *Arabidopsis* sterilization buffer for 5 min and immediately wash the seeds for 4 to 5 times with sterile water.
- Place the 300-400 seeds in petri dish containing selection medium with kanamycin (50 µg/mL).
- Incubate the plates at 28°C with a photoperiod of 16h light and 8h dark for approximately 10 days, until plants reached the 4-leaf stage, to ensure kanamycin resistance.
- The plants that are bleached out are considered negative while healthy plants are transferred to glasshouse.

## **Functional characterization of gene edited plants**

To confirm the genome editing at molecular level and to identify and characterize the mutations, we need to do polymerase chain reaction (PCR) using primers designed for flanking sgRNA to identify the predicted Cas/sgRNA- DNA cleavages followed by the analysis of the PCR products by T7E1 and surveyor assays. To further confirm the indels (insertions or deletions), PCR products can be cloned and sequenced with Sanger sequencing/nanopore sequencing.

### **Genomic DNA isolation**

Obtaining high quality intact DNA is the first and the critical step in performing PCR. Many procedures are currently available for the isolation of total DNA from prokaryotes and eukaryotes. Different plant species have varying levels of polysaccharides, polyphenols, and other secondary metabolites which affect the quality of the extracted DNA. To isolate high quality total DNA from small amounts of starting material, the DNeasy® Plant Mini Kit protocol can be used for different plant species. The procedure is simple and rapid (<30 min). The DNeasy membrane from QIAGEN combines the binding properties of a silica-gel based membrane with micro spin technology. DNA is adsorbed to the DNeasy membrane in the presence of high salt concentrations of chaotropic salt, which removes water from hydrated molecules in solution. In DNeasy extraction procedure, buffer conditions are designed to allow adsorption of DNA specific to the silica-gel membrane and offer an optimal removal of carbohydrates, polyphenols and other plant metabolites.

### **Chemicals/materials**

- Plant DNeasy mini kit
- Sterile 1.5 ml and 2 ml eppendorf tubes
- Sterile mortars and pestles
- Liquid nitrogen
- Absolute ethanol (molecular biology grade)

### **Notes**

- Perform all centrifugation steps at room temperature (15–25°C)
- If necessary, redissolve any precipitates in Buffer AP1 and Buffer AW1 concentrates
- Add ethanol to Buffer AW1 and Buffer AW2 concentrates
- Preheat a water bath or heating block to 65°C

## Procedure

1. Disrupt samples ( $\leq 100$  mg fresh tissue or  $\leq 20$  mg lyophilized tissue) using the clean, sterile mortar and pestle or micro pestle.
2. Add 400  $\mu$ l Buffer AP1 and 4  $\mu$ l RNase A (100mg/mL). Vortex and incubate for 10 min at 65°C. Invert the tube 2-3 times during incubation.  
*Note: Do not mix Buffer AP1 and RNase A before use.*
3. Add 130  $\mu$ l Buffer P3. Mix and incubate for 5 min on ice.
4. Centrifuge the lysate for 5 min at 20,000 x g (14,000 rpm). (Recommended)
5. Pipet the lysate into a QIAshredder spin column placed in a 2 ml collection tube. Centrifuge for 2 min at 20,000 x g.
6. Transfer the flow-through into a new tube without disturbing the pellet if present. Add 1.5 volumes of Buffer AW1, and mix by pipetting.
7. Transfer 650  $\mu$ l of the mixture into a DNeasy Mini spin column placed in a 2 ml collection tube. Centrifuge for 1 min at  $\geq 6000$  x g ( $\geq 8000$  rpm). Discard the flow through. Repeat this step with the remaining sample.
8. Place the spin column into a new 2 ml collection tube. Add 500  $\mu$ l Buffer AW2, and centrifuge for 1 min at  $\geq 6000$  x g. Discard the flow-through.
9. Add another 500  $\mu$ l Buffer AW2. Centrifuge for 2 min at 20,000 x g. Note: Remove the spin column from the collection tube carefully so that the column does not come into contact with the flow-through.
10. Transfer the spin column to a new 1.5 ml or 2 ml microcentrifuge tube.
11. Add 50  $\mu$ l Buffer AE for elution. Incubate for 5 min at room temperature (15–25°C). Centrifuge for 1 min at  $\geq 6000$  x g.
12. Repeat step 11.

## **Polymerase Chain Reaction (PCR)**

The PCR is a technique for the *in vitro* amplification of specific DNA sequences by the simultaneous primer extension of complementary strands of DNA. This involves repeated cycles of heat denaturation of the DNA, annealing of primers to the complementary sequences and extension of the annealed primers with thermostable *Taq* DNA polymerase in the presence of four deoxyribonucleotides (dNTPs). Since the extension products are complementary to and capable of binding primers, subsequent cycles of amplification double the amount of target DNA synthesized in the previous cycle. The result is exponential accumulation of the specific target DNA. In gene editing, PCR is used for confirmation of the target region in the transformed plants.

### **Chemicals/materials**

- Thermal cycler
- Sterile 0.2 ml, 0.5 ml and 1.5 ml Eppendorf tubes
- Oligonucleotide primers (forward and reverse)
- Template DNA (genomic DNA)
- *Taq DNA* Polymerase
- dNTP mix
- Nuclease free water (Invitrogen)
- Micropipettes (10  $\mu$ l, 20  $\mu$ l, 100  $\mu$ l, 200  $\mu$ l and 1000  $\mu$ l single channel pipettes)
- Microfuge tubes or PCR strips

### **Precautions**

PCR is one of the most highly sensitive techniques. Care must be taken to avoid cross contamination and carryover of template, to prevent false amplifications. The following tips may help in getting good results with PCR.

- Autoclave all solutions used in PCR. This degrades any extraneous DNA/RNA and nucleases.
- Divide reagents into aliquots to minimize the number of repeated samplings necessary.
- Ensure that all the reaction components are added as per the required concentration.

- Wherever possible, prepare master reaction mixture by premixing all reagents except template. Distribute into individual reactions, then add the template directly into each tube.
- Always use a positive control (known positive) and a negative control (no 'template' control) to ensure the specificity of the PCR reaction.

### PCR Reaction Setup

1. Add the following in a sterile 0.2 ml tubes.  
(Composition given is for one reaction)

<u>Component</u>	<u>Volume</u>
Autoclave distilled water	upto 50 $\mu$ L
10x PCR buffer, -Mg <sup>++</sup>	5 $\mu$ L
50 mM MgCl <sub>2</sub>	1.5 $\mu$ L
10 mM dNTP	1 $\mu$ L
10 $\mu$ M Forward Primer	2.5 $\mu$ L
10 $\mu$ M Reverse Primer	2.5 $\mu$ L
Template DNA	varies
<i>Taq</i> DNA Polymerase (5 U/ $\mu$ L)	0.2 $\mu$ L

\*Use upto 2.5U for longer targets

2. Place the PCR tubes in the thermal cycler and use the following PCR programme for the amplification.

Step		Temperature (°C)	Time
Initial denaturation		94	3 min
25-35 PCR Cycles	Denature	94	1 min
	Annealing	~55 (depending on primer T <sub>m</sub> )	1 min
	Extension	72	~60 sec/kb
Final extension		72	10 min
Hold		4	forever

3. Analyze 10  $\mu$ L of PCR product by using agarose gel electrophoresis by loading controls together as described in the subsequent section.
4. Use PCR product immediately for down-stream applications such as for TA-cloning, Surveyor assay and nanopore sequencing.

## **Cloning of PCR amplified DNA fragments into TOPO-TA vector**

TOPO TA Cloning Kits (Invitrogen, Catalogue numbers K4575 and K4500) combine the TOPO and TA Cloning technologies to enable fast, efficient cloning of *Taq*-amplified PCR products. The DNA topoisomerase-I, functions both as a restriction enzyme and as a ligase. Its biological role is to cleave and re-join DNA strands to relieve the topological constraints of double helical DNA during replication. *Vaccinia* virus has a site specific topoisomerase I that recognizes the pentameric sequence 5'-(C/T)CCTT-3' and cleaves between the C/T and covalently bond to the phosphate group of the 3' thymidine. TOPO vectors are provided linearized with topoisomerase I covalently bound to each 3' phosphate. *Taq* DNA polymerase has a terminal transferase activity that adds a single 3'-A overhang to each end of the PCR product. TOPO TA Cloning vectors contain 3'-T overhangs with activated topoisomerase-I that enable the direct ligation to 3'-A overhang of the PCR product within 5 min at room temperature. The TOPO-TA cloning system also utilizes a unique Zero Background technology by using the lethal *ccdB* (control of cell death) gene for positive selection of only recombinant clones. The *ccdB* protein poisons bacterial DNA gyrase, causing degradation of the host chromosome and results in cell death. When an insert is ligated into the vector, the *ccdB* gene is disrupted, enabling only recombinant colonies to grow. By eliminating high vector background yields are nearly 100% recombinants.

Once you have PCR amplified the desired DNA product, you are ready to clone into TOPO TA Cloning vector.

### Setting Up the TOPO Cloning Reaction:

<b><u>Component</u></b>	<b><u>Volume</u></b>
PCR amplified DNA fragment	0.5 to 4.0 $\mu$ l
Salt Solution (supplied with the kit)	1.0 $\mu$ l
Sterile water	up to 5 $\mu$ l
TOPO vector	1.0 $\mu$ l
Total	6.0 $\mu$ l

Mix gently and incubate for 5-15 min at room temperature and then place the tube on ice and proceed for transformation and screening of recombinant clones by colony PCR. The recombinant plasmids will be used for sequencing.

## Surveyor Assay

T7 surveyor assay should be used when no appropriate restriction enzyme site exists.

### Chemicals and materials

- Genomic DNA of test samples and wild type (WT) plants
- PCR machine and PCR kit
- Surveyor Nuclease kit (IDT)
- Agarose gel electrophoresis

### Step 1: Amplification reactions

This step is critical to the success of the surveyor nuclease digestion.

1. PCR was performed using Phusion® High-Fidelity DNA Polymerase (NEB) with isolated genomic DNA of T<sub>0</sub> transformed and wild type plants to check the expected product size using specific primers. For every PCR that was performed for transformed plant, a WTcontrol PCR should be performed alongside under the similar conditions.
2. After the completion of the PCR, 3.5 µL from each reaction was loaded on agarose gel to confirm the success of PCR reaction.
3. PCR yield should be sufficiently high (>25 ng/µL) with low background and free of primer dimer artifacts.

Set up the PCR reaction in 0.2 ml tube by adding the following components in the order listed (keep on ice.). One tube will be used for test sample DNA and the other for reference DNA:

<u>Reagents</u>	<u>Transformed</u>	<u>WT</u>
Polymerase buffer (10X)	5.0 ml	5.0 ml
dNTPs (10 mM)	8.0 ml	8.0 ml
Template DNA (100-300ng)	1.0 ml	1.0 ml
Forward primer (150 ng/ml)	1.0 ml	1.0 ml
Reverse primer (150 ng/ul)	1.0 ml	1.0 ml
H <sub>2</sub> O	33.5 ml	33.5 ml
Taq polymerase (5 U/ml)	0.5 ml	0.5 ml
Total	50 ml	50 ml

Carry out PCR using the following conditions:

94 °C	55 °C	72 °C
1 min	1 min	2 min

### **Heteroduplex formation**

The products of the PCR reaction must be denatured and annealed in order to allow formation of heteroduplex between sample and reference PCR products. It is important to include sample alone and/or reference DNA alone in separate tubes to serve as negative controls.

Heteroduplex formation using a thermal cycler:

- Mix equal amounts of test sample and reference PCR products in a 0.2 mL tube. The concentration of test sample DNA and WT DNA should be in the range 25–80 ng/μL (ideally 50 ng/μL). About 200–400 ng of hybridized DNA is recommended for treatment with Surveyor Nuclease S, so that each tube contains  $\geq 200$  ng total DNA.
- Place WT DNA alone in a separate 0.2 mL tube.
- For efficient annealing, the final volume should be at least 10 μL.

- Heterogeneous test sample DNA that does not require the addition of wildtype, or reference DNA should still undergo the following hybridization procedure to ensure the formation of cleavable mismatches.
- Place the tube in a thermal cycler and run the following program:

<b>Temperature</b>	<b>Time/ Ramp</b>
95°C	10 min
95°C to 85°C	(-2.0°C/sec)
85°C	1 min
85°C to 75°C	(-0.3°C/sec)
75°C	1 min
75°C to 65°C	(-0.3°C/sec)
65°C	1 min
65°C to 55°C	(-0.3°C/sec)
55°C	1 min
55°C to 45°C	(-0.3°C/sec)
45°C	1 min
45°C to 35°C	(-0.3°C/sec)
35°C	1 min
35°C to 25°C	(-0.3°C/sec)
25°C	1 min
4°C	Hold ∞

The product is now ready to be treated with Surveyor Nuclease for heteroduplex analysis. Continue with step 3.

### **Step 3: Heteroduplex digestion**

In this step, the heteroduplex test sample DNA from step 2 is cleaved by the Surveyor Nuclease, along with necessary reference DNA and controls. Optimized Mg<sup>++</sup> concentration is essential to Surveyor Nuclease function.

### Reaction setup:

Digest the hetero/homoduplex DNA experimental samples and any reference DNA in separate tubes.

1. The volumes of 0.15 M MgCl<sub>2</sub> solution required should be calculated based on the volume of the PCR product.
2. For each digestion, add the following components in a nuclease free 0.2 mL tube (keep on ice) in the order shown below:

<u>Component</u>	<u>Amount</u>
Hybridized DNA*	200–400 ng
0.15 M MgCl <sub>2</sub> Solution†	1/10th volume
Surveyor Enhancer S	1 μL
Surveyor Nuclease S	1 μL
* DNA volume should be	8–40 μL

† This additional magnesium is required even if PCR buffer also contains some magnesium

3. Mix by vortexing gently, by agitation or by aspiration/expulsion in a pipette tip using a micropipette.
4. Incubate at 42°C for 45-60 min. Increasing reaction time beyond 60 min may increase DNA degradation due to exonuclease activity of the Surveyor enzyme
5. Add 1/10th volume of Stop Solution and mix. Store the digestion products at –20°C if not analyzed immediately.
6. Proceed with fragment analysis by analyzing on an agarose or polyacrylamide gel or store at -20°C until ready.

### Indel identification

The T<sub>0</sub> plants that shows positive results in the Surveyor Assay are further confirmed by Sanger sequencing/Nanopore sequencing using the PCR product. The successive progenies are also analysed to study the segregation in order to identify homozygous mutation at target locus. Each PCR reaction had a volume of 40 μL and was performed using Q5® High-Fidelity DNA Polymerase (NEB). PCR products were purified from agarose gel (QIAquick Gel Extraction Kit, QIAGEN) and were sequenced directly by Sanger sequencing or nanopore sequencing.

## Nanopore sequencing

Nanopore sequencing is a third generation approach used in the sequencing of polynucleotides in the form of DNA or RNA and biopolymers. Using nanopore sequencing, a single molecule of DNA or RNA can be sequenced without the need for PCR amplification or chemical labelling of the sample. At least one of these aforementioned steps is necessary in the procedure of any previously developed sequencing approach. Nanopore sequencing has the potential to offer relatively low-cost genotyping, high mobility for testing, and rapid processing of samples with the ability to display results in real-time. The applications of nanopore sequencing including rapid identification of viral pathogens, monitoring ebola, environmental monitoring, food safety monitoring, human genome sequencing, plant genome sequencing, monitoring of antibiotic resistance, haplotyping and other applications.

### Chemicals/Materials

- DNA samples (PCR products)
- Nano-pore sequencing kit including Bar coding kit
- PCR Machine
- Magnetic stand
- AMPure XP beads
- Qubit High sensitivity DNA estimation kit

### Library preparation

#### *Sample DNA preparation*

#### *End-prep*

Perform end-repair/dA-tailing of fragmented DNA as follows:

1 µg	End-repaired DNA
7 µl	Ultra II End-prep reaction buffer
3 µl	Ultra II End-prep enzyme mix

Make up to 60 µl Nuclease-free water

- Mix gently by flicking the tube, and spin down. Transfer the sample to a 0.2 ml PCR tube, and incubate for 5 minutes at 20°C and 5 minutes at 65°C using the thermal cycler, hold at 4°C.

- Transfer the sample to a 1.5 ml DNA LoBind Eppendorf tube. Add 60  $\mu$ l of resuspended AMPure XP beads to the end-prep reaction and mix by pipetting. Incubate on a rotator mixer for 5 minutes at room temperature.
- Spin down the sample and keep the tube on the magnet, and pipette off the supernatant.
- Keep on magnet, wash beads with 200  $\mu$ l of freshly prepared 70% ethanol without disturbing the pellet for 30s to 1min. Remove the 70% ethanol using a pipette and discard. (Repeat the step).
- Spin down and place the tube back on the magnet. Pipette off any residual 70% ethanol. Briefly allow to dry.
- Remove the tube from the magnetic rack and resuspend pellet in 24  $\mu$ l Nuclease-free water. Incubate for 5 min at RT.
- Pellet the beads on a magnet until the elute is clear and colourless. Remove and retain 24  $\mu$ l of elute into a clean 1.5 ml Eppendorf DNA LoBind tube.
- Quantify 1  $\mu$ l of end-prepped DNA using a Qubit fluorometer - recovery aim > 700 ng. Take forward approximately 700 ng of end-prepped DNA in 30  $\mu$ l into adapter ligation.

### **Ligation of barcode adapter**

Add the reagents in the order given below, mixing by flicking the tube between each sequential addition:

23 $\mu$ l	End prep DNA
2.5 $\mu$ l	Native Barcode Adapter
25.5 $\mu$ l	Blunt/TA Ligase Master Mix

Mix gently by flicking the tube, and spin down. Incubate the reaction for 10 min at RT.

- Add 51  $\mu$ l of resuspended AMPure XP beads to the reaction and mix by pipetting. Incubate on a rotator mixer for 5 minutes at RT.
- Spin down the sample and keep the tube on the magnet, and pipette off the supernatant.
- Wash beads twice with 200  $\mu$ l of freshly prepared 70% ethanol. Pipette off any residual 70% ethanol. Briefly allow to dry.
- Remove the tube from the magnetic rack and resuspend pellet in 25  $\mu$ l Nuclease-free water. Incubate for 10 min at RT.
- Pellet the beads on a magnet, Remove and retain the eluate which contains the DNA library in a clean 1.5 ml Eppendorf DNA LoBind tube. Dispose of the pelleted beads

- Quantify 1  $\mu\text{l}$  of end-prepped DNA using a Qubit fluorometer. Dilute the library to a concentration of 10 ng/ $\mu\text{l}$  with Nuclease-free water or 10 mM Tris-HCl pH 8.5.
- Pool all the barcoded samples in equimolar ratio to a single microcentrifuge tube.

### **Sequencing adapter ligation**

To the pooled barcoded samples (equal to 50  $\mu\text{l}$ ) add 20  $\mu\text{l}$  of sequencing adapter (Bam1D) and 70  $\mu\text{l}$  Blunt/TA ligation master mix

- Mix gently by flicking the tube, and spin down. Incubate the reaction for 10 minutes at RT.

### **AMPure XP bead binding**

- Add 56  $\mu\text{l}$  (0.6 v of reaction mix) of resuspended AMPure XP beads to the adapter ligation reaction from the previous step and mix by pipetting. Incubate on a rotator mixer for 5 min at RT. Place on magnetic rack, allow beads to pellet and pipette off supernatant.
- Add 250  $\mu\text{l}$  of LFB Buffer (Long fragment buffer)/SFB (Short fragment buffer) to the beads. Close the tube lid, and resuspend the beads by flicking the tube. Return the tube to the magnetic rack, allow beads to pellet and pipette off the supernatant. (Repeat the step).
- Remove the tube from the magnetic rack and resuspend pellet in 14  $\mu\text{l}$  elution buffer. Incubate for 10 min at RT.
- Pellet the beads on a magnet, Remove and retain the eluate which contains the DNA library in a clean 1.5 ml Eppendorf DNA LoBind tube, Dispose of the pelleted beads.
- The prepared library is used for loading into the flow cell. Store the library on ice until ready to load.

### **Flow cell priming**

- After opening the priming port check for a small bubble under the cover. Draw back a small volume to remove the air bubble.
- Set a P1000 pipette to 200 $\mu\text{l}$
- Insert the tip in to priming port
- Turn the wheel until the dial shows 220-230 $\mu\text{l}$  or until you can see a small volume of buffer at entering the pipette tip.

### **Flow cell priming mix**

From the mix (30µl of FLT and 1.3 ml of FLB) add 800µl this flow cell priming mix to the flow cell through priming port by drop by drop.

Set up the library for loading as follows

Library	12 µl
Sequencing buffer (SQB)	37.5 µl
Library loading beads (LB)	25.5 µl

Total volume 75 µl.

Load 75µl of library on spot on port drop by drop. Close the port and go for run.

### **Sequencing data analysis**

MinION device control, data acquisition and real-time base calling are carried out by the MinKNOW software. There are three options for how to carry out sequencing on the MinION:

1. Data acquisition and basecalling in real-time using MinKNOW on a computer/laptop
2. Data acquisition and basecalling in real-time using the MinIT device
3. Data acquisition using MinKNOW on a computer/laptop and basecalling at a later time using Albacore.

#### **1) Basecalling of raw data to fastq / fasta file formate using Albacore:**

**MinKNOW produces two types of .fast5 files**





you can optionally save the index with option -d and replace the reference sequence file with the index file on the minimap2 command line:

Step 1	Alignment – Map to Reference
Tool	Minimap2
Input	.fastq files, reference genome
Output	aln.sam Intermediary file, removed from final output
Notes	Alternate alignment tools: BWA-MEM, NGMLR
Command	./minimap2 -ax map-ont ref.fa ont.fq.gz > aln.sam

Step 2	Sort SAM file by coordinate, convert to BAM
Tool	Picard Tools
Input	aligned_reads.sam
Output	sorted_reads.bam Intermediary file, removed from final output
Command	java -jar picard.jar SortSam INPUT=aligned_reads.sam OUTPUT=sorted_reads.bam SORT_ORDER=coordinate

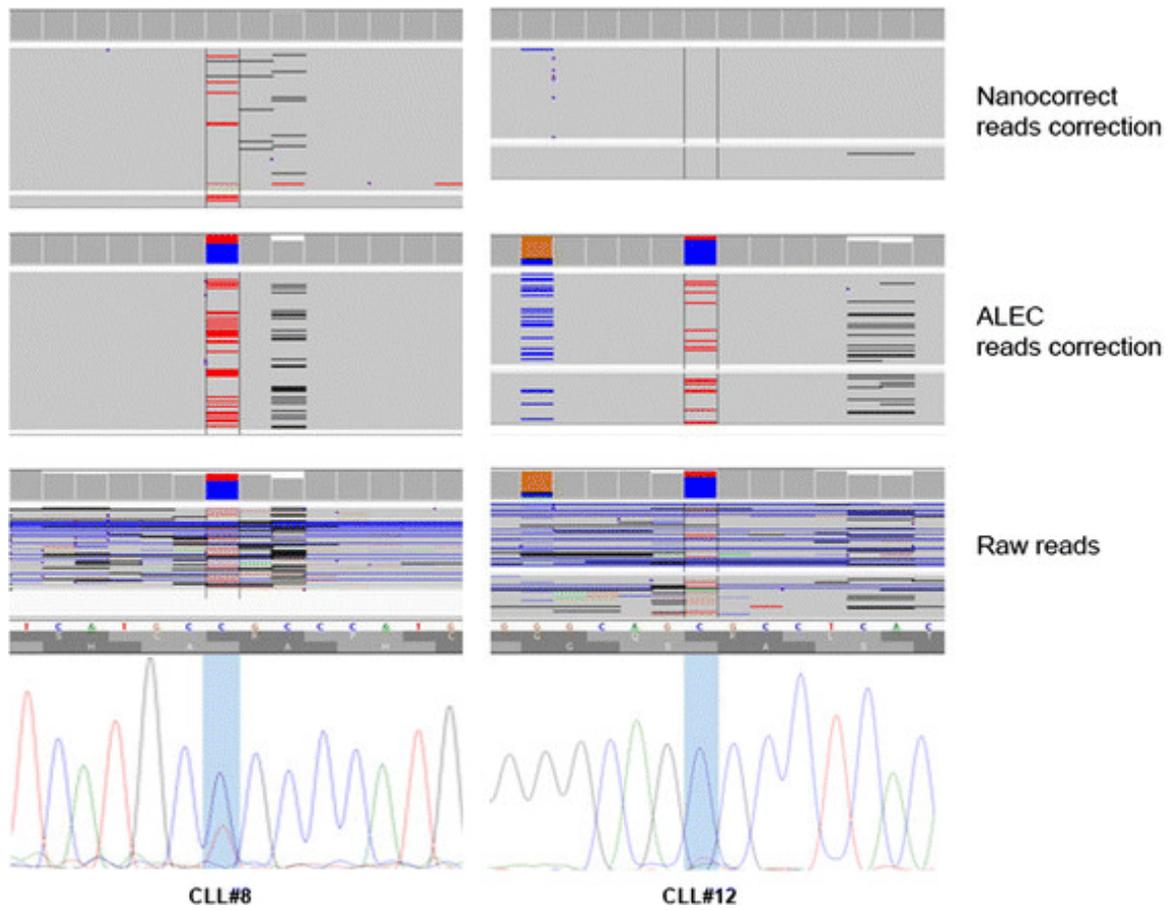
Step 3	Collect Alignment & Insert Size Metrics
Tool	Picard Tools, R, Samtools
Input	sorted_reads.bam, reference genome
Output	alignment_metrics.txt, insert_metrics.txt, insert_size_histogram.pdf, depth_out.txt

Command	java -jar picard.jar CollectAlignmentSummaryMetrics R=ref I=sorted_reads.bam O=alignment_metrics.txt
	java -jar picard.jar CollectInsertSizeMetrics INPUT=sorted_reads.bam OUTPUT=insert_metrics.txt HISTOGRAM_FILE=insert_size_histogram.pdf
	samtools depth -a sorted_reads.bam > depth_out.txt

Step 4	Mark Duplicates
Tool	Picard Tools
Input	sorted_reads.bam
Output	dedup_reads.bam metrics.txt
Command	java -jar picard.jar MarkDuplicates INPUT=sorted_reads.bam OUTPUT=dedup_reads.bam METRICS_FILE=metrics.txt

Step 5	Call Variants
Tool	Nanopolish / Varscan
Input	Reads.sorted.bam, reference genome
Output	polished.vcf
Notes	Then we run the consensus algorithm. For larger datasets we use nanopolish_makerange.py to split the draft genome assembly into 50kb segments, so that we can run the consensus algorithm on each segment in parallel. The output would be the polished segments in fasta format.
Command	nanopolish variants --consensus -o polished.vcf \ -w "tig00000001:200000-202000" \ -r reads.fasta \ 





**Figure 9.** Example of mutations detected in two samples (CLL8 and CLL12). The mutation detected in CLL8 was not found from nanocorrect corrected reads. CLL12 mutation was detected only from the analysis with raw and ALEC corrected reads. Aligned reads are visualized by IGV

## Supplementary information

### ***E. coli* competent cell preparation and transformation**

#### ***Competent cell preparation***

- *E. coli* DH5 $\alpha$  cells were streaked on a LB agar plate and a single colony was grown in Luria-Bertani (LB) medium (100 ml) at 37 °C @ 250 rpm till the OD<sub>600</sub> reaches to 0.4-0.5.
- Cells were harvested by centrifugation at 4000 rpm for 10 min at 4 °C.
- Pellet was suspended in 20 ml of 100 mM CaCl<sub>2</sub> and incubated on ice for 30 min.
- Cells were collected by centrifugation at 3000 rpm for 10 min at 4 °C, re-suspended in 2 ml of CaCl<sub>2</sub> and incubated at 4 °C for overnight.
- Sterile glycerol (1 ml) was added and 0.1 ml of cell suspension was aliquoted into eppendorf tubes and stored at -80 °C.

#### **Preparation of *Agrobacterium* electro-competent cells**

- A starter culture of *Agrobacterium tumefaciens* (C58) strain was raised in 5 ml liquid Yeast Extract Peptone (YEP) medium containing rifampicin (50 mg/L).
- *Agrobacterium* was cultured for 24 h at 28 °C in an orbital shaker maintained at 220 rpm.
- 1 ml of inoculum was added to 100 ml of fresh YEP medium containing rifampicin (50 mg/L) and the culture was allowed to grow till log phase (O.D<sub>600</sub> 0.4 - 0.6).
- The log phase culture was chilled on ice for 30 min and centrifuged at 5000 rpm for 15 min at 4 °C.
- The pellet was suspended in 20 ml of ice-cold sterile double distilled water and centrifuged at 5000 rpm for 5 min at 4 °C. Repeat this step again.
- The obtained bacterial pellet was suspended in 20 ml of 10% ice-cold glycerol and centrifuged at 4000 rpm for 10 min at 4 °C.
- Finally the pellet was suspended in 2ml of 10% glycerol and aliquots of 50  $\mu$ l were made and stored at -70 °C till further use.

### **Quantification**

The Invitrogen Qubit 3 Fluorometer is used to quickly and specifically quantitate DNA or RNA.

- Qubit assay dyes bind selectively to DNA, RNA, or protein, making it more sensitive than UV absorbance
- Uses as little as 1  $\mu$ L of sample, even with very dilute samples

### **Procedure**

1. Prepare one 0.5 ml thin-walled PCR tube for each sample you want to quantify, plus those needed for the standards (2 for a nucleic acid assay\* technical replicates).
2. Make working solution by combining (1 x n)  $\mu$ l Invitrogen™ Qubit™ BR Reagent with (199 x n)  $\mu$ l Invitrogen™ Qubit™ BR buffer where \*n is equal to (number of samples + 2) for nucleic acid assay. This must be done in a clean plastic tube. Vortex for 2-3 seconds being careful not to create bubbles.
3. Make standard solutions 1 and 2 for a nucleic acid assay by combining 190  $\mu$ l working solution with 10  $\mu$ l of the appropriate stock solution from kit.

*For example, if you are using the dsDNA BR assay kit to measure the concentration of DNA, you will prepare 2 standard solutions. The first will contain 190 µl of working solution and 10 µl of standard #1 (0 ng/µl) and the second will contain 190 µl of working solution + 10 µl of standard #2 (10 ng/µl).*

4. Combine 1-20 µl of sample with 199-180 µl of the working solution to obtain a final volume of 200 µl.

*The Qubit can only quantify from 0.5 ng/ml to 600 ng/ml; keep this in mind as you are diluting your samples. Use more of your sample when it is a low concentration and less of your sample when it is a high concentration*

5. Vortex all tubes for 2-3 seconds being careful not to create bubbles.
6. Incubate the tubes for 2 min at room temperature.
7. Turn on Qubit by pressing any of the four buttons present on the instrument.
8. Use the arrows to select the appropriate assay and press "GO."
9. On the calibration screen, highlight either "Run new calibration" or "Use last calibration" and press "GO". If you are not re-calibrating, skip to step 10.
10. Calibration
  - Insert the tube containing Standard #1 in the Qubit fluorometer, close the lid, and press "GO."
  - Remove Standard #1.
  - Repeat for remaining standard solution(s).

11. Insert a sample tube into the Qubit fluorometer, close the lid, and press "GO."

12. Record the reading given by the Qubit fluorometer.

*This value is the concentration of the nucleic acid after it has been diluted with the working sample in the sample tube. To find the concentration of the original sample, choose "Calculate sample concentration" and select the number of microliters of sample added to the assay tube.*

13. Continue reading samples and recording values until all samples are read.

*If you did not choose "Calculate sample concentration" in step 11, you must calculate the concentration of your original sample by using the equation below.*

$$\text{Concentration of sample} = (\text{QF Value}) \times 200 / x$$

QF value = the value given by the Qubit fluorometer

x = the number of microliters of sample added to the assay tube

**\*NOTE\*** *Do not leave samples in the Qubit fluorometer after readings have been made or hold it in your hand before the reading. The sample will heat up and the higher temperature will influence the accuracy of the array.*

**\*NOTE\*** *The fluorescence signal for your samples will be stable for 3 hours after incubation.*

### **Purification of DNA Fragments from Agarose Gel using QIAquick Gel Extraction Kit**

1. Excise the DNA fragment from the agarose gel with a clean, sharp scalpel. Minimize the size of the gel slice by removing extra agarose.
2. Weigh the gel slice in a colorless tube. Add 3 volumes of Buffer QG to 1 volume of gel (100 mg ~ 100 µl).

For example, add 300 µl of Buffer QG to each 100 mg of gel. For >2% agarose gels, add 6 volumes of Buffer QG. The maximum amount of gel slice per QIAquick column is 400 mg; for gel slices >400 mg use more than one QIAquick column.

3. Incubate at 50°C for 10 min (or until the gel slice has completely dissolved). To help dissolve gel, mix by vortexing the tube every 2–3 min during the incubation.

IMPORTANT: Solubilize agarose completely. For >2% gels, increase incubation time.

4. After the gel slice has dissolved completely, check that the color of the mixture is yellow (similar to Buffer QG without dissolved agarose). If the color of the mixture is orange or violet, add 10 µl of 3 M sodium acetate, pH 5.0, and mix. The color of the mixture will turn to yellow.

The adsorption of DNA to the QIAquick membrane is efficient only at pH ≤7.5.

Buffer QG contains a pH indicator which is yellow at pH ≤7.5 and orange or violet at higher pH, allowing easy determination of the optimal pH for DNA binding.

5. Add 1 gel volume of isopropanol to the sample and mix.

For example, if the agarose gel slice is 100 mg, add 100 µl isopropanol. This step increases the yield of DNA fragments <500 bp and >4 kb. For DNA fragments between 500 bp and 4 kb, addition of isopropanol has no effect on yield.

Do not centrifuge the sample at this stage.

6. Place a QIAquick spin column in a provided 2 ml collection tube.

7. To bind DNA, apply the sample to the QIAquick column, and centrifuge for 1 min.

The maximum volume of the column reservoir is 800 µl. For sample volumes of more than 800 µl, simply load and spin again.

8. Discard flow-through and place QIAquick column back in the same collection tube.

9. Recommended: Add 0.5 ml of Buffer QG to QIAquick column and centrifuge for 1 min.

This step will remove all traces of agarose. It is only required when the DNA will subsequently be used for direct sequencing or cloning.

10. To wash, add 0.75 ml of Buffer PE to QIAquick column and centrifuge for 1 min.

Note: If the DNA will be used for salt-sensitive applications, such as blunt-end ligation and direct sequencing, let the column stand 2–5 min after addition of Buffer PE, before centrifuging.

11. Discard the flow-through and centrifuge the QIAquick column for an additional 1 min at 17,900 x g (13,000 rpm).

IMPORTANT: Residual ethanol from Buffer PE will not be completely removed unless the flow-through is discarded before this additional centrifugation.

12. Place QIAquick column into a clean 1.5 ml microcentrifuge tube.

13. To elute DNA, add 50 µl of Buffer EB (10 mM Tris·Cl, pH 8.5) or water (pH 7.0–8.5) to the center of the QIAquick membrane and centrifuge the column for 1 min.

Alternatively, for increased DNA concentration, add 30 µl elution buffer to the center of the QIAquick membrane, let the column stand for 1 min, and then centrifuge for 1 min.

IMPORTANT: Ensure that the elution buffer is dispensed directly onto the QIAquick membrane for complete elution of bound DNA. The average eluate volume is 48  $\mu$ l from 50  $\mu$ l elution buffer volume, and 28  $\mu$ l from 30  $\mu$ l.

14. If the purified DNA is to be analyzed on a gel, add 1  $\mu$ l of purified DNA with running dye and water mix and load on gel.

## References

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## Notes











