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REVIEW ARTICLE

CRISP Points on Establishing *CRISPR-Cas9* In Vitro Culture Experiments in a Resource Constraint Haematology Oncology Research Lab

Jhumki Das[1] · Prateek Bhatia[2] · Aditya Singh[3]

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Abstract Gene editing research has seen rapid growth over the past decade or so, however with the discovery of *CRISPR-Cas9* gene editing tool in recent years, the same has witnessed a global interest with many scientists and research groups worldwide carrying out cutting edge experiments to target various diseases and cancers and develop a cure. This has been made possible partially due to the ease of use and flexibility of the *CRISPR-Cas9* system as compared to other conventional gene editing tools. Hence, *CRISPR-Cas9* has found its way into most basic molecular laboratories and within reach of most low-middle income research groups. Despite these favourable advantages, there exists a cost barrier and lack of proper knowledge and awareness on the correct work flow desired, especially in molecular laboratories looking forward to develop and experiment with high end research. This mini review attempts to iron out these factors and project an algorithmic approach to tide over and establish a workable in vitro gene editing experiment in a resource constraint haematology oncology laboratory setting. However, the basic principle and steps outlined in this review can also be translated for research in any other medical specialty laboratory setting.

✉ Prateek Bhatia
prateekbhatia@rediffmail.com

[1] Pediatric Allergy Immunology Unit, Post Graduate Institute of Medical Education and Research, Chandigarh, India

[2] Pediatric Hematology-Oncology Unit, Advanced Paediatric Centre, Post Graduate Institute of Medical Education and Research, Chandigarh 160012, India

[3] Pediatric Hematology-Oncology Unit, Department of Pediatrics, Post Graduate Institute of Medical Education and Research, Chandigarh, India

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Introduction

Genome editing is an important technique to study gene function (by observing the phenotypic outcome of DNA modification). Moreover, it can also be used as an effective therapeutic tool because of its potential to introduce ameliorative changes that can repair a defective gene and correspondingly initiate the exit from a diseased state. Genome editing is the process of modifying the nucleotide sequence of the genome by creating a Double stranded break (DSB) in the DNA using various engineered proteins (nucleases) that cleave DNA in a site-specific manner, such proteins being termed as 'molecular scissors'. These DSBs are then repaired by cell's endogenous repair machinery using one of the two basic mechanisms: nonhomologous end joining (NHEJ) or homology directed repair (HDR) [1]. In NHEJ, the two ends of the DSBs are randomly ligated back together by the host DNA repair mechanism which often leads to an insertion/deletion (indels) at the site of the break. The size of such indels usually ranges between 1 and 15 bp, but can be much larger, which might result in disruption of gene function by shifting of the translational frame of the coding sequence. This property can be used to delete defective genomic regions for therapeutic purposes. On the other hand, HDR mediated repair is achieved by recombination of the target locus with exogenously supplied DNA (donor template) which has homology to the site of the DSB. This property can be used to induce specific point mutations or to insert desired sequences.

The conventional gene editing tools like Meganucleases, zinc finger nucleases (*ZFNs*) and transcription activator-like effector nucleases (*TALENs*) generated a targeted DSB

in the genome based on their customized DNA binding specificities. Meganucleases are the engineered version of naturally occurring restriction enzymes having extended DNA recognition sequence of 14–40 bp. The *ZFN* and *TALEN* enzymes are generated by the fusion of a DNA binding domain with a nonspecific DNA cleavage domain from *Fok1* restriction endonuclease [2]. Both *ZFN*s and *TALENs* have been used in diverse fields like study of novel genes, development of disease trials, development of personalized medicines and gene therapy trials for diseases [3]. However, the assembly of both *ZFNs* and *TALENs* is very laborious owing to their protein nature; moreover multiplex targeting of different loci at the same time is also very difficult. Owing to these difficulties the discovery of the clustered regularly interspaced short palindromic repeats—*CRISPR* associated (*CRISPR-Cas*) system and its subsequent adaptation as a gene manipulation tool has provided a promising alternative. The comparative features of the four gene editing tools used are highlighted in Table 1.

CRISPR-Cas9 is a RNA guided gene editing tool, which relies on a single sgRNA that matches the targeted DNA sequence and thus can be used for various experimental purposes. Moreover, the ability of sgRNA to be efficiently multiplexed such that different genomic regions can be edited simultaneously makes *CRISPR-Cas9* the most popular method. The *Cas9* is insensitive to methylation of DNA bases, thus increasing the ease of editing genomic regions which are not amenable to changes.

In this mini review we embark upon the journey to highlight the *CRISPR-Cas9* system components, their selection and designing tools and steps required to conduct a successful in vitro experiment in a research lab within boundaries of resource constraints. We are appreciative of the fact that in the current era of rapidly growing genomics and targeted therapeutics, working with genome editing possesses a challenging thought to many budding young researchers and scientists. Hence with this "crisp" review on *CRISPR-Cas9* we wish to bridge this gap and try to design the basic workflow as well as estimate the approximate cost required for initiating in vitro experiments using this tool. This basic knowledge can help young researchers to design experiments and work in their labs on different genetic conditions or cancer cell lines and help them to move forward from cell based to animal model studies by exploring potential of *CRISPR-Cas9* gene editing technology.

What is CRISPR-Cas9?

The Clustered Regularly Interspaced Short Palindromic repeats—CRISPR associated (*CRISPR-Cas*) gene editing platform has been derived from a bacterial innate immune system, which protects the bacteria from invasion against bacteriophages by eliciting a robust response upon reinfection [4]. The *CRISPR-Cas* system is highly diverse and is categorized into 3 main types and several subtypes based on the differences in the *CRISPR* repeat sequence, *cas* gene sequence and the architecture of the *cas* operons. A particular type of CRISPR consist of a signature 'Cas protein' viz.: the type I system contain the *Cas3* nuclease-helicase, type II system consists of the *Cas9* nuclease and the type III system have *Cas10* proteins [5]. The type I and type III *CRISPR-Cas* systems are found in various phylogenetically diverse bacteria and archaea whereas the type II system is restricted to bacteria. Amongst the three types, the type II *CRISPR-Cas9* system is profoundly used in various targeted genome engineering techniques.

Components of CRISPR-Cas9 Complex and Mechanism of Action

The type II *CRISPR-Cas* complex consisting of the *Cas9* protein and associated RNA components called the crRNA and tans-acting RNA (tcrRNA), which binds to the complementary sequence (20 nucleotides) in the target DNA [6, 7]. The *Cas9* protein is composed of two lobes: a nuclease lobe containing the HNH and RuvC-like domains that cleave the target and non-target site respectively; and a α-helical lobe containing the bridge-helix, consisting of arginine residues, which hold the two lobes together and make contact with the guide RNA. The *Cas9* protein binds to a proto-spacer adjacent motif (usually 5′ NGG), subsequently initiating unwinding of the target DNA adjacent to it and eventually leading to a double stranded break in the DNA by the action of the HNH and RuvC domains [8].

Table 1 Contrasting characteristics of the four gene editing tools

Nuclease	Target site length	Mechanism of recognition	Ease of design	Engineering components
Engineered meganucleases (MNs)	> 18 bp	Protein-DNA	Extremely difficult	Restricted
ZFNs	18–36 bp	Protein-DNA	Difficult	Available
TALENs	24–10 bp	Protein-DNA	Easy	Freely available
CRISPR-Cas9	19–22 bp	RNA–DNA	Simple	Freely available

Mismatches in the 2 bp region adjacent to the PAM sequence abolish the *Cas9* binding. The overview of the working mechanism of *CRISPR-Cas9* system is shown in Fig. 1.

Stepwise Crisp Approach to Conduct an In Vitro Experiment Using CRISPR-Cas9

The flowchart in Fig. 2 highlights the step wise approach to be followed while carrying out in vitro experiments using *CRISPR-Cas9* system.

"CRISP" Points to Keep in Mind on CRISPR-Cas9 Tool Use in Practical Experiments

(a) Reducing off-target genomic modifications for sgRNA

The precision of gene editing by *Cas9*-gRNA complex faces a major hindrance by the recognition and cleavage of non specific regions in the genome, called the 'off-target' activity. Therefore, it is necessary to reduce the off-target activities, which can be achieved by various approaches, few of which are: (1) one method is to design a modified gRNA by adding two guanine nucleotides at the 5′end of the sgRNA or by truncating the 3′ends of the tracrRNA derived sequence [9]; (2) the use of paired nickases instead of the *Cas9* nuclease, which generates adjacent off-set nicks at the target site, is also useful in reducing off target effects [10]; (3) using low levels of gRNA-*Cas9* complex also helps in reducing off target effects [11]; (4) the use of tru-gRNAs that are trucated at the 5′ (17 or 18 nucleotides in length) have increased sensitivity to single or double mismatches at the gRNA:DNA interface also reduce off target mutagenesis significantly [12]. Various web based tools are freely available for designing sgRNA with probable off target predictions (refer Table 2). Such softwares can be used to generate sgRNAs with high sensitivities and desired specificities.

(b) Robust CRISPR-Cas9 construct

Designing a robust *CRISPR-Cas9* construct is necessary to achieve efficient editing with less chances of failure. The

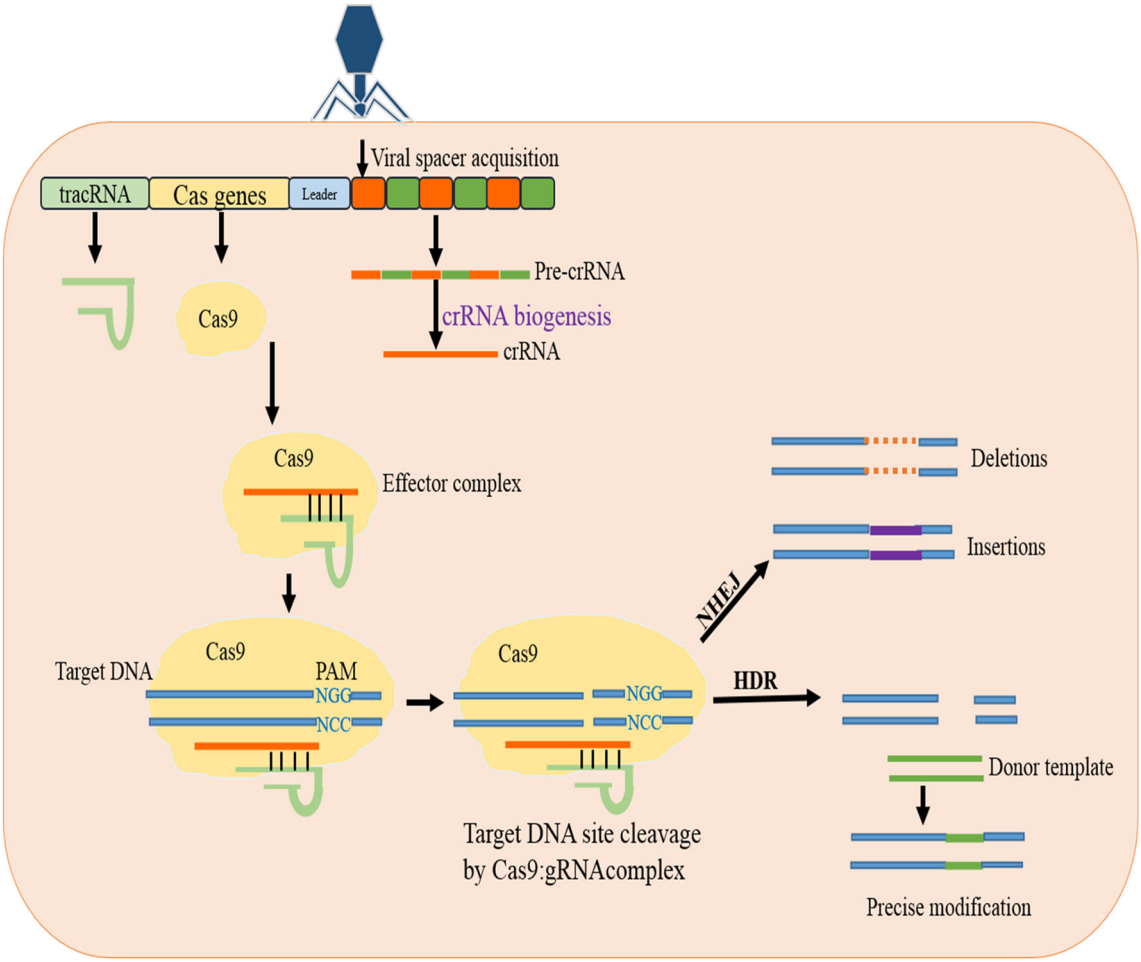

Fig. 1 Schematic overview of CRISPR-Cas9 mechanism

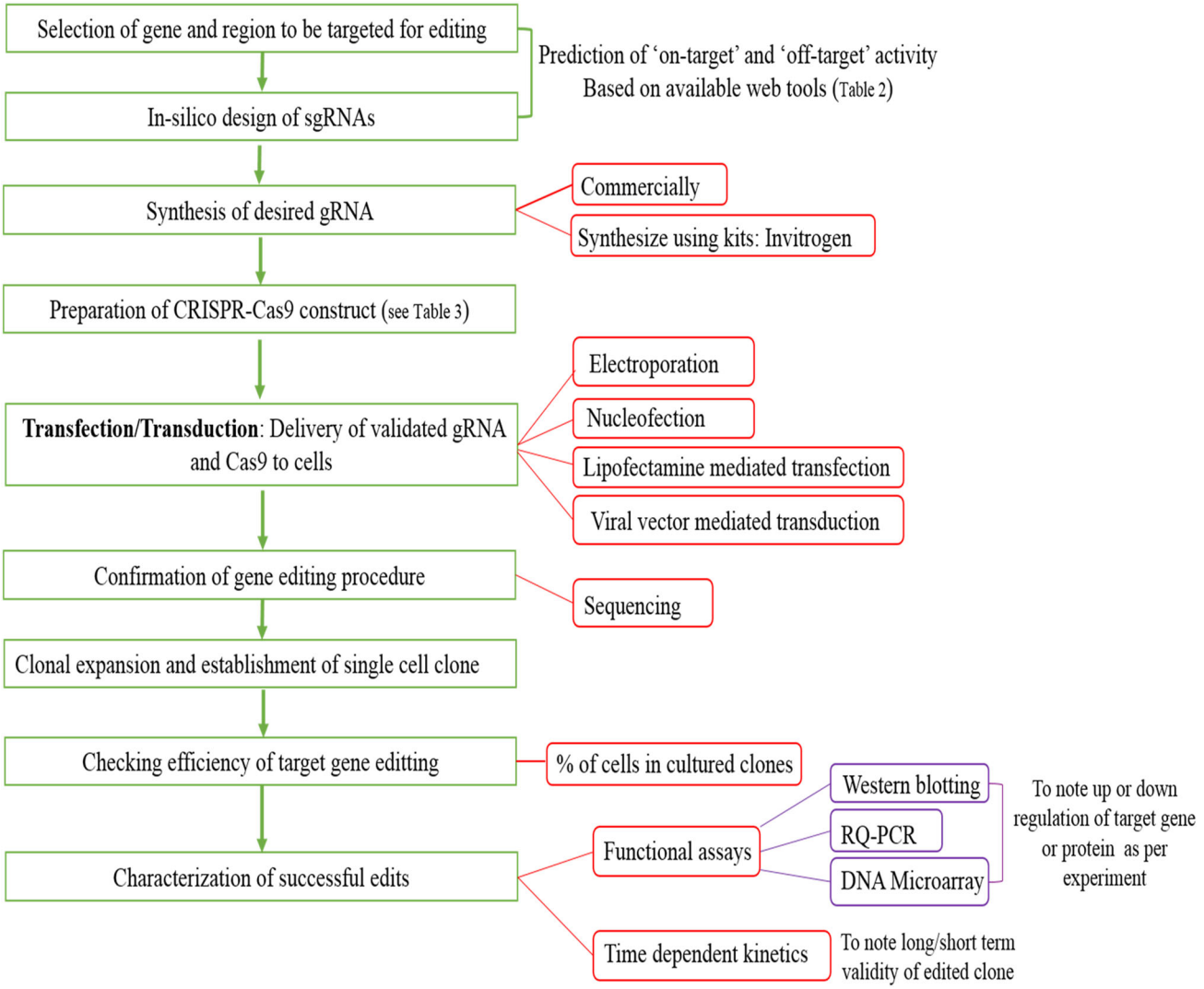

Fig. 2 Steps involved in the design of experiment involving CRISPR-Cas9

Table 2 Tools for design of guide RNA

Tool	Website	Input	Software
sgRNA designer	http://www.broadinstitute.org/rnai/public/analysistools/sgrna-design	Ensemble transcript IDs or nucleotide sequences	Web/local
CRISPR multitargeter	http://www.multicrispr.net/	Gene/transcript ID or sequence	Web
Cas9 design	http://cas9.cbi.pku.edu.cn	Input sequence or FASTA file	Web
SSFinder	https://code.google.com/p/ssfinder/	FASTA file	Python script
Cas OFFinder	http://www.rgenome.net/casoffinder/	FASTA file	Web/local

Table 3 Comparision of different Cas9 construct used in CRISPR experiment

Cas9 construct	Cleavage efficiency	Benefits
Cas9 plasmid	Low	Easy to procure or design and use in lab setting
Cas9 mRNA/gRNA	High	Lower off-target effects
Cas9 RNP	Very high	Faster targeted editing, lower off-target effects

various forms of *Cas9* construct are highlighted in Table 3 and we can note that amongst these the direct delivery of *Cas9* RNP construct consisting of the *Cas9*/gRNA complex into cells is best in terms of faster editing as no transcription or translation of gene is needed and it has minimal off-target effects [13]. However, constructing such a complex in lab with various reagents or consumables is technically challenging and if one intends to buy it from commercial sources it is costly. Alternative approach of CRISPR plasmid construct is the least costly, however, cleavage efficiencies are low ($\sim$ 30–40%) and need to be tuned and worked upon in lab with stringent control to achieve desirable results.

(c) Delivery of CRISPR-Cas components to cells

The generation of a germline mouse in which Cas9 expression is already established can be used a source of primary cell lines and animal model [14]. In hard to transfect cells such as primary cell lines, transduction of sgRNA and *Cas9* can be achieved by using viral vectors like Adeno associated virus (AAV) and Lentiviral vectors. For transfection in adherent cells lipofectamine mediated transfection is preffered whereas in case of transfection in suspension cells electroporation or nucleofection is beneficial.

(d) Validation of target genome editing

Introduction of *cas9* and gRNA in the cells results in a mixed population of cells of which some will have one allele edited, some having both alleles edited and some with no editing. Thus, it is necessary to asses whether significant number of cells are edited or not. This validation can be done by either sequencing (to detect off-target) or by performing cleavage detection assay using kits employing repoter dyes (refer Fig. 3).

(e) Functionality assay of nuclease cleavage

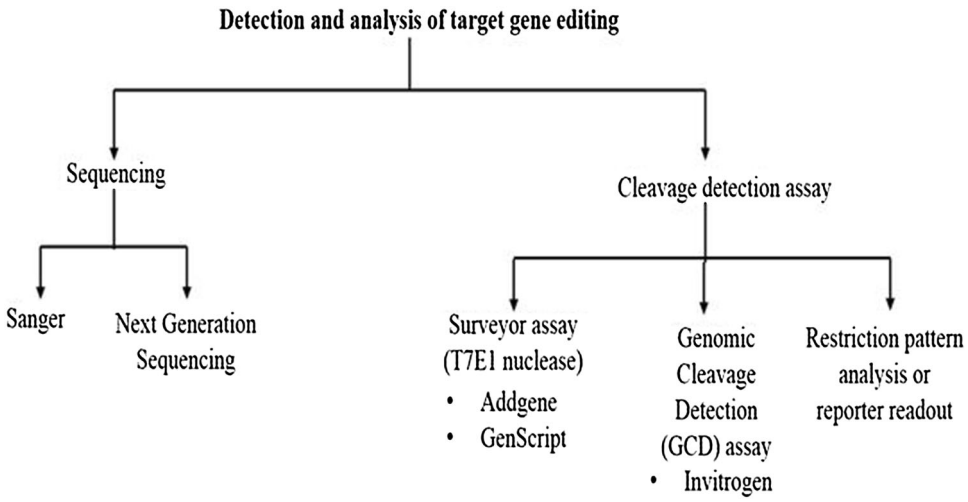

Fig. 3 Methods to asses and analyze target gene edit

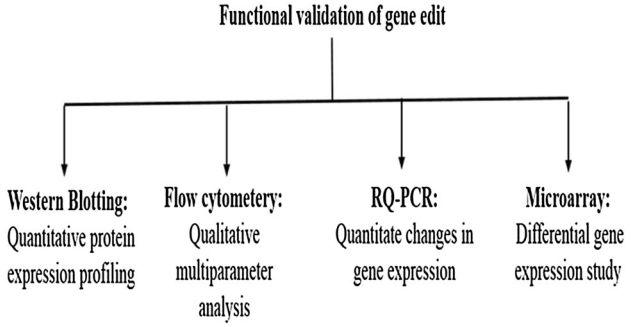

Fig. 4 Methods to asses functional validity of target gene edit

The effect of gene editing in transfected cells can be assayed by various functional screening methods using the cell lysates or by using fluroscent imaging in live or fixed cells (refer Fig. 4). These functional assays provide real time information on expression level of target gene that has been edited or its downsream surrogate genes, the expression of which is dependent upon the target gene.

Approximate Costing for Various Reagents/ Consumables Needed for Establishing CRISPR-*Cas9* In Vitro Experiments in a Lab

Varoius companies provide all-in-one tools for *CRISPR-Cas9* experiments, a list of these companies is given in Table 4. The detailed costs likely to be incured while doing a *CRISPR-Cas9* based in vitro experiment is highlighted in Table 5. This list though exhaustive covers nearly all costs related to consumables or reagents required, however it does not cover cost of cell lines needed per experiment and the costs for equipment required to have a basic cell culture lab set-up (like CO_2 incubator, phase contrast microscope, culture plates, − 20 and − 80 °C freezers 4 °C refrigeration system etc.). Further changes in costing can vary

Table 4 Companies providing CRISPR-Cas tools

Company	Product description
Applied StemCells (Menlo Park, California)	Genome engineering
	Gene editing
	Knock-in cell lines
GenScript (Piscataway, New Jersey)	gRNA design
	Transfection and cell pool evaluation
	Single-cell clone generation and validation
Horizon Discovery	Gene-editing tools
	Validated gRNAs
	Cas9 vectors
	Cell line generation kit
	Delivery vectors
Thermo Scientific	CRISPR Nuclease Vector Reporter Kit
	Genome-CRISPR sgRNA design
	Cloning services
Origene	CRISPR cloning kits
	CRISPR-Cas9 custom services
Addgene	Cas9 plasmids

depending on type of experiment planned and need for cell line/*CRISPR-Cas9* engineered mouse models. The overall approximate costs as visible from the table appear to be within reasonable range of a basic molecular laboratory doing research in a LMIC.

Current CRISPR-*Cas9* Status in Hematology-Oncology Research

With the advent of CRISPR-*Cas9* genome editing technology in last decade or so, many research groups in West have focussed on targeting inherited genetic diseases especially monogenic disorders and a wide number of studies have been conducted in various benign and malignant hematological disorders. In addition three improtant review articles have been published on role of CRISPR-*Cas9* in hematology research in international peer reviewed hematology journals by Zhang and McCarty [15], Hoban and Bauer [16] and Lucas et al. [17]. These review articles in detail highlight the successful research done in various hematological conditions using the CRISPR-*Cas9* tool. In concise, CRISPR-*Cas9* has been shown to correct β thalassemia mutations in induced pluripotent stem cells (iPSCs) derived from patient fibroblasts [18–20]. Park et al. [21] similarly reverted the inverted chromosomal lesions in hemophilia A patient derived cells and showed their in vitro efficiency to produce factor VIII afer endothelial differentiation. Osborn et al. [22] showed correction of a

Table 5 Estimated costs of reagents/consumables required for CRISPR-Cas9 experiment in laboratory

S. no.	Item	Cost (INR)
1.	pSpCas9(BB)-2A-GFP	7800.00
2.	pUC19	4355.00
3.	Oligos	920.00
4.	Herculase II	3685.00
5.	Taq polymerase with std. buffer	5259.42
6.	dNTPs mix	8925.83
7.	$MgCl_2$ 25 mm	6159.59
8.	Gel extraction kit	12,000.00
9.	Spin column	6050.00
10.	TBE buffer 10x	3844.38
11.	Agarose low EEO	7121.25
12.	BbsI	4225.00
13.	DTT	5294.83
14.	T7 DNA ligase with buffer	4355.00
15.	T4 Polynucleotide kinase	3752.00
16.	T4 DNA ligase reaction buffer 10x	1340.00
17.	ATP 10 mM	2144.00
18.	PlasmidSafe ATP dependednt DNase	5025.00
19.	OneShot Stbl3 *E.coli*	NA
20.	LB medium	14,083.80
21.	LB agar	7452.88
22.	Ampicillin 100 mg/ml	9229.15
23.	HEK 293FT cell line	NA
24.	K562 cell line	NA
25.	DMEM high glucose	515.75
26.	DMEM high glucose	3474.19
27.	Dubelco's PBS	1788.87
28.	FBS, heat inactivated	7383.00
29.	Opti-MEM reduced	3401.00
30.	Pencillin–streptomycin	1628.00
31.	TrypLE express, no phenol red	1784.00
32.	Lipofectamine	25,922.00
33.	Geltrez LDEV free reduced growth factor basement membrane matrix	3683.00
34.	Normocin	9179.00
35.	Accutase cell detachment solution	4265.68
36.	Rho-associated protein kinase inhibitor	40,803.00
	Total	102,156.12

FANCC gene mutation in Fanconi anemia fibroblasts via CRISPR-*Cas9* mediated homology directed repair mechanism while Ablain et al. [23] successfully inactivated p53 in a zebrafish DBA (Diamond Blackfan Anemia) model thereby restoring normal erythropoiesis. Research in various hematological malignancies has also yielded successful results like silencing of Jak-2 signalling in various CMPDs

[24] and that of oncogenes like MUC1-C in multiple myeloma [25].

In addition to above studies we wish to highlight that in our own laboratory at PGIMER, Chandigarh, we are carrying out an in vitro study to target the chimeric fusion gene of BCR-ABL1, which is the driving mechanism behind CML and Ph positive ALL. We have designed our own SgRNA and constructed a CRISPR-*Cas9* DNA plasmid to silence the chimeric gene. The same has been successfully transfected with good efficiency in both secondary K562 cell line and in patient derived cells, though the results are yet to be published. We are currently noting downstream effects of BCR-ABL gene silencing and looking forward to carry further our work with CRISPR-*Cas9* into mouse models and patient derived iPSCs.

Conclusion

CRISPR-Cas9 gene editing has taken giant strides since its inception and is one of the most hot areas of research involving all specialities of medicine namely hematology, oncology, neurology and genetics. However, despite gene editing being a high-end research field, *CRISPR-Cas9* system has revolutionized our approach and brought gene editing research to basic laboratories, even in LMIC. But we need to rationally plan our experimental designs with proper addressal of ethical issues to maximize efficient results in a resource constraint setting.

Compliance with Ethical Standards

Conflict of interest The authors declare that they have no conflict of interest.

Human and Animal Rights This article does not contain any studies with human participants performed by any of the authors.

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