

# Design of High-Throughput Drug Screening Assay for Genetic Skin Fragility Disease

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**Abstract**— Epidermolysis Bullosa Simplex (EBS) is an inherited skin fragility disorder caused by mutations in keratin intermediate filament proteins. A robust and effective assay is needed to expedite the discovery of protease inhibitor drugs for EBS treatment. In this study we evaluated and compared the detection speed, sensitivity, and specificity of various fluorescence-based enzyme substrates which have potential application in the development of screening assay for EBS therapeutics. Cell and drug based experiments were conducted to determine the optimal assay conditions and fluorescence excitation/emission wavelengths.

## I. INTRODUCTION

Keratins are cytoskeletal filament-forming proteins found in skin and other epithelia tissues. Two main types of keratins, type-II keratin K5 and type I keratin K14, are major components of the intermediate filament (IF) network in basal cells of epithelia. These IFs are crucial in providing mechanical stability within a single cell, between neighboring cells and to the basement membrane [1].

EBS, the first-reported skin disease caused by keratin mutations, sets pattern for the diagnosis and treatment to all the other keratin disorders as well as several non-keratin genetic skin diseases [2]. It is characterized by the formation of blisters and erosions after minor traumatization, thereby significantly compromising patients' quality of life. Genetically, EBS is a heterogeneous disease caused by pathogenic dominant mutations in the K5 and K14 genes, resulting in IFs with misfolded proteins that are sensitive to mechanical stress. Upon trauma, these filaments disrupt and the keratinocytes lyse, leading to intra-epidermal blistering [3].

Current therapeutic approaches to EBS include (i) bone marrow transfusion, (ii) genetic ablation of mutant proteins, and (iii) small-molecule therapies to stabilize the keratin network, all of which are temporary solutions that merely focus on symptom relief. Although so far no effective treatment for EBS is known, MMP-9, a cellular biomarker over-expressed in diseased cells, has been found to be a

potential therapeutic target [4]. Assay detecting MMP-9 would thus have potential for effective screening of EBS drug candidates.

A potential starting point for EBS drug discovery is the identification of protease inhibitors by high-throughput screening (HTS) approach. Large numbers of compounds might be tested for their inhibitory potential in preliminary HTS before being selected for further testing [5]. The HTS strategy requires robust and sensitive assay, and protease-based assay with fluorescence readouts is a promising approach. In this study, we discuss the optimization of fluorescent assay that can detect activity of MMPs biomarkers over-expressed in EBS keratinocytes.

## II. PROCEDURE

### A. Materials

#### Cell Lines

Wild Type	NTERT (keratinocytes immortalized by transfection to express TERT, genetically similar to naturally derived NEB cell line but show higher stability upon many passages)
Mutant	NTERT R125P mutation (contain Green Fluorescence Probe (GFP), and is immortalized by viral transfection)
Control	NTERT (contain neither mutation nor GFP)

#### Enzymes

MMP-2 and MMP-9 enzymes reconstituted in HEPES buffer at 1 $\mu$ g/10 $\mu$ l, stored under -80°C; working concentration 0.1 $\mu$ g/well of 200 $\mu$ l.

#### Fluorogenic Enzyme Substrates

Dye I	MMP2/MMP9 Specific*	Dissolved at 10mM in DMSO, stored under -20°C;
Dye II	MMP2 Specific*	When in use, reconstituted to 1mM in PBS then to corresponding working concentration.
Dye III	MMP13 Specific*	corresponding working concentration.

(\* Specificity as described by vendor)

<b>Reagents</b>	
Cell Culture Media	K-sfm (1* 500ml), Bovine Pituitary Extract (25µg/ml), Epidermal Growth Factor (0.2µg/ml), Penicillin/Streptomycin (10,000u/ml), CaCl <sub>2</sub> (0.4mM)
Trypsinization & Cell Counting	Trypsin, RM+ ready mix serum rich media, K-sfm serum free media, Trypan Blue

### **Microplate**

96 well plate, polystyrene, TC-treated, clear flat bottom wells, sterile, w/lid, black, 48/ea.

## *B. Experiment Design*

### *1) Fluorescence dye spectra analysis*

#### **Objective**

To select the best dye (fluorogenic enzyme substrate) used in the screening assay.

Criteria for selection:

1. Speed of detection: time taken for assay to show positive fluorescence signal
2. Sensitivity: highest signal strength for positive result
3. Specificity: greatest difference in signal between positive and negative results

#### **Method**

When the peptide-based fluorescent probe is intact, no fluorescence is emitted due to the proximity of the donor and quencher molecules at the two ends of the probe. When the probe is digested by its specific enzyme, the peptide chain will break and fluorescence signal from fluorescence resonance energy transfer can be observed.

### *2) Cell autofluorescence analysis*

#### **Objective**

To compare the auto-fluorescence property between mutant and wild type cells, thus decide the detection mechanism of screening assay.

If there is a difference between mutant and wild type cells, auto-fluorescence may be used as a marker for detection;

If there is no significant difference, cells' auto-fluorescence factor may be assumed as constant for both cell types. Other detection mechanism such as measuring digestion by enzymes secreted from certain type of cells may be used.

In addition, cell density was also varied to determine the threshold value for a detectable difference.

#### **Method**

Both R125P mutant cells and wild type cells have been genetically engineered to contain genes that produce Green Fluorescence Probes (GFP). The level of expression of GFP may vary between different types of cells and can be checked by fluorescence emission test.

To conduct fluorescence emission test, cells were transferred in 96-well microchip in a process called cell seeding, where specific amount of cells are placed in wells of cell culture media and incubated at all-time except during signal measurement.

Experiments that involve cells were harder to control and more likely to be affected by the environment. Thus, more replicates (at least six) were used for reliable results.

Emission and excitation wavelengths setting were also determined by both literature reference and spectra analysis.

### *3) Drug intrinsic fluorescence*

#### **Objective**

To check if the drug candidates emit any intrinsic fluorescence when being read by fluorescence reader, thus eliminating the possibility of false positive results due to property of the drugs.

#### **Method**

The drug candidates have different chemical make-up, and some of the chemicals may emit fluorescence when light shines on them.

The check was conducted by placing drugs in their working concentration in wells of microplate, then comparing the fluorescence emission spectrum reading from fluorescence reader to check for any unusual peak.

## III. RESULTS & DISCUSSION

### *A. General assay optimization*

#### **Plate Selection**

Solid black plate were chosen over white plate to amplify fluorescence signal and reduce crosstalk of signals between adjacent wells; the bottom of wells were chosen to be flat to enable even readout of signals and attachment of cells, instead of round or V-shaped bottom generally used for washing or removal of well content.

The chosen plate, however, was not UV-transparent (no UV-transparent plate available for purchase with black-wall and transparent-bottom). Literature has confirmed that MCA/Dnp dye pair has excitation maximum in the ultraviolet range [5], thus the non UV-transparent plate may result in false positive signals when excited at wavelengths near UV range.

### Edge Effect

Wells on the edge of the plate tend to produce signals that are statistically different from those in the center, mainly because evaporation happened to a larger extent at the edge, reducing solution volume in edge wells.

This edge effect has been significantly reduced by incorporating a perimeter buffer zone (leaving the outermost wells empty or fill with sterile water). Drawback of this method is the lower throughput and efficiency due to wastage of microplate wells.

### Excitation/Emission Wavelengths

Three target dyes are attached with different fluorescence probes (active agents) and thus have different optimal excitation and emission wavelengths. Dye I with active agent Tryptophan has excitation/emission peaks at 280/360nm [7]; both Dye II and Dye III with active agent MCA have peaks at 328/393nm [8]. Final setting of excitation wavelength 323 was chosen to prioritize reading of non-silent Dye II and Dye III with reference to literature value [9], and emission wavelength 384nm for greatest signal readout.

As for GFP in mutant and wild type cell lines, emission peak checked by experiment as shown in Figure 9 coincides with literature value of 500nm [10].

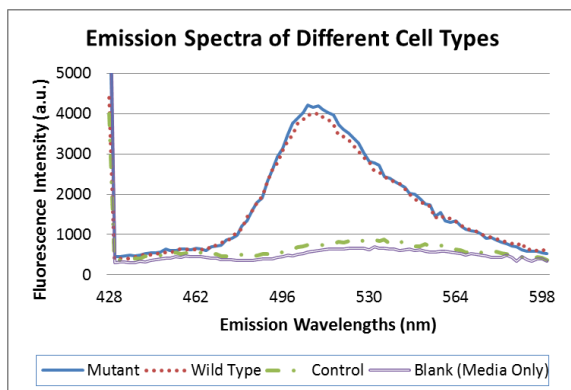


Figure 1: Fluorescence emission spectra of different cell types at excitation wavelength of 400nm and gain of 100a.u.

### Assay Turnaround Time

Figure 3 shows that assay readout of all dyes for target enzyme MMP9 have reached maximum value after 48 hours and did not further increase

until 72 hours. Thus, incubation time for assay could be equal or shorter than 48 hours.

As for the microplate reader setting, 'number of flashes per well', it could be reduced from default value 25 to 5, thus reducing reading time from around 1 hour to 10 minutes without significantly sacrificing readout quality.

### B. Fluorescence Dye spectra analysis

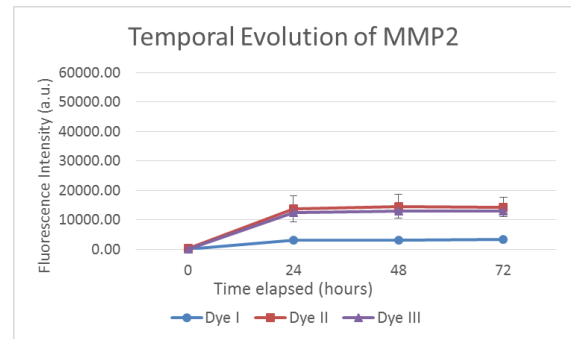


Figure 2: Temporal evolution of fluorescence intensity of different dye substrates before and after addition of MMP2 enzyme

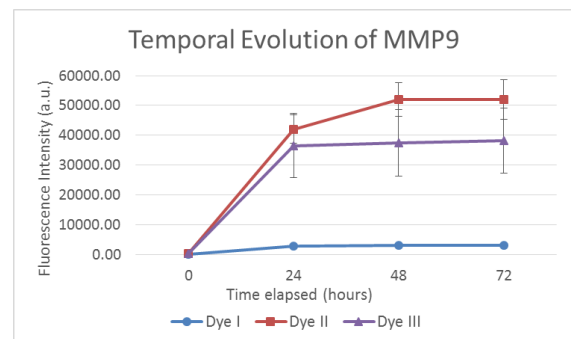


Figure 3: Temporal evolution of fluorescence intensity of different dye substrates before and after addition of MMP9 enzyme

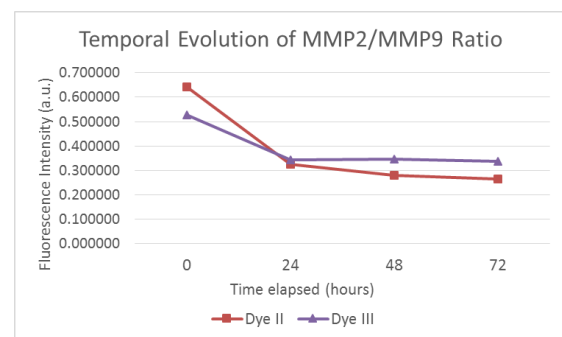


Figure 4: Temporal evolution of fluorescence intensity ratio of different dye substrates

(For figures 2, 3, 4: Excitation wavelength was 323nm, emission wavelength were chosen at 384nm for greatest signal readout, gain was 100a.u.; error bars were for internal variance.)

Dye II is not specific to MMP2 enzyme only as specified by the vendor, as it shown strong positive signal when MMP9 was added (Figure 3).

Likewise, Dye III is not specific to MMP13 as it shown signals when either MMP2 or MMP9 is added (Figures 2 & 3). As for Dye I, it was not MMP2/MMP9 specific as described by the vendor as it shown negligible signal in both enzymes (Figures 2 & 3).

A possible explanation for the discrepancy could be that in exopeptidases such as MMP2 and MMP9, substrate binding is structurally constrained so that only one or two amino acid residues of the substrate can specifically bind the protease [5].

For dye selection, Dye I is out of consideration due to its silence to both enzymes. As for Dye II and Dye III, although Dye III shown a higher detection speed (24 hours, compared to 48 hours for Dye II), Dye II has shown higher signal when detecting MMP9 and has a lower MMP2/MMP9 ratio, thus Dye II is the best choice for assay due to its sensitivity and specificity.

### C. Cell autofluorescence analysis

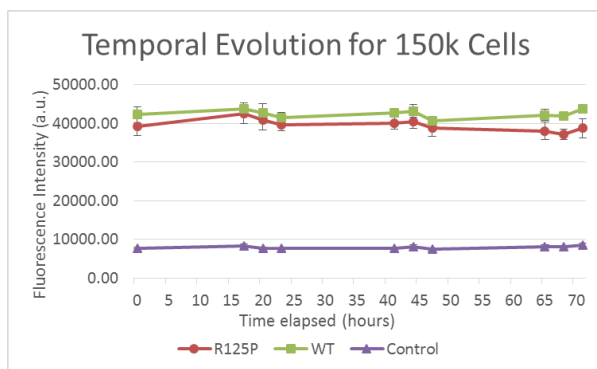


Figure 5: Temporal evolution of fluorescence intensity of different cell suspensions at density 150k/200 $\mu$ l media in a well

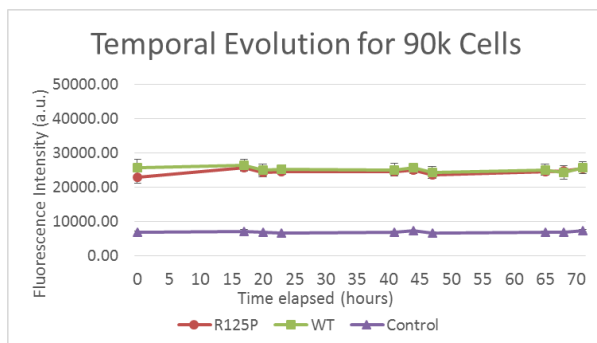


Figure 6: Temporal evolution of fluorescence intensity of different cell suspensions at density 90k/200 $\mu$ l media in a well

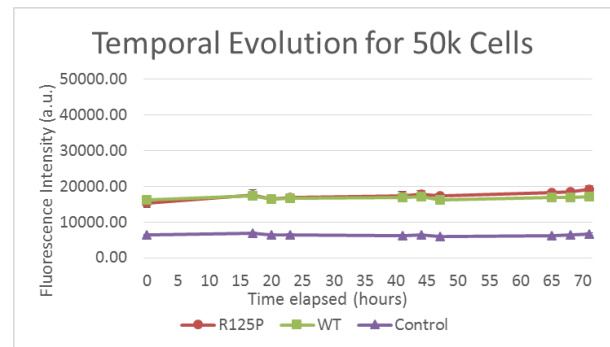


Figure 7: Temporal evolution of fluorescence intensity of different cell suspensions at density 50k/200 $\mu$ l media in a well

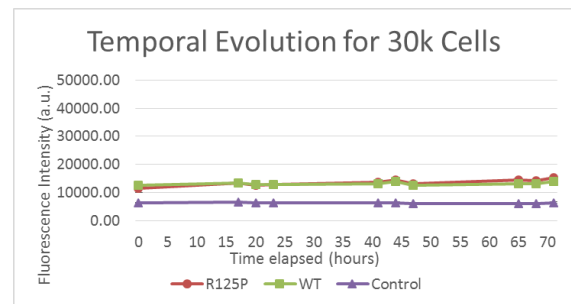


Figure 8: Temporal evolution of fluorescence intensity of different cell suspensions at density 30k/200 $\mu$ l media in a well

(For figures 5~8: Excitation wavelength was 400nm, emission wavelength was 500nm, and gain was using optimal gain; error bars were for internal variance)

From Figures 5~8, it is observed that auto-fluorescence difference between mutant and wild type cells are negligible, although difference in auto-fluorescence caused by the presence of GFP genes indeed exists when compared with GFP-free control cells, and when cell concentration varies. Thus, we may need to add in background signal control of 'cell only' wells when performing the assay, or only collect supernatant of well content for reading to eliminate the effect of auto-fluorescence from cells.

### D. Drug intrinsic fluorescence

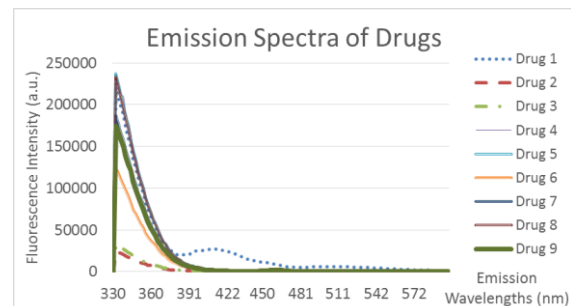


Figure 9: Emission fluorescence spectra of solutions of drug candidates in their respective working concentrations (Error bars for internal variance only)

From Figure 9, only Drug 1 shows fluorescence emission peak at around 410nm which may

coincide with emission wavelength used for assay readout.

For drugs with intrinsic fluorescence property such as Drug 1, assay with non-fluorescence-based mechanism may be used, or a control with drug only may be needed to serve as background signal guide. Alternatively, excitation wavelength could be carefully selected to avoid the fluorescence emission peak (for example, the peak from 386~470nm for Drug 1 curve in Figure 9).

#### IV. CONCLUSION

A protease assay based on fluorescence readouts has been designed and developed, with potential application in HTS strategy for drug discovery in treating EBS. The assay has been developed by optimizing fluorescence dye substrate selection, drug intrinsic fluorescence check, as well as wavelength settings among others.

Further testing of assay with cell lines, MMP inhibitors, and drug candidates are needed. Dose response curve and drug toxicology analysis should be conducted either as part of assay or independently as future work.

The completed assay will enable rapid screening of libraries of small molecule drugs to identify potential candidates for further development, as well as for pharmaceutical companies to identify therapeutic agents for EBS diseases.

#### V. REFERENCES

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