

# CellPlayer™ 96-well Kinetic Angiogenesis PrimeKit Assay

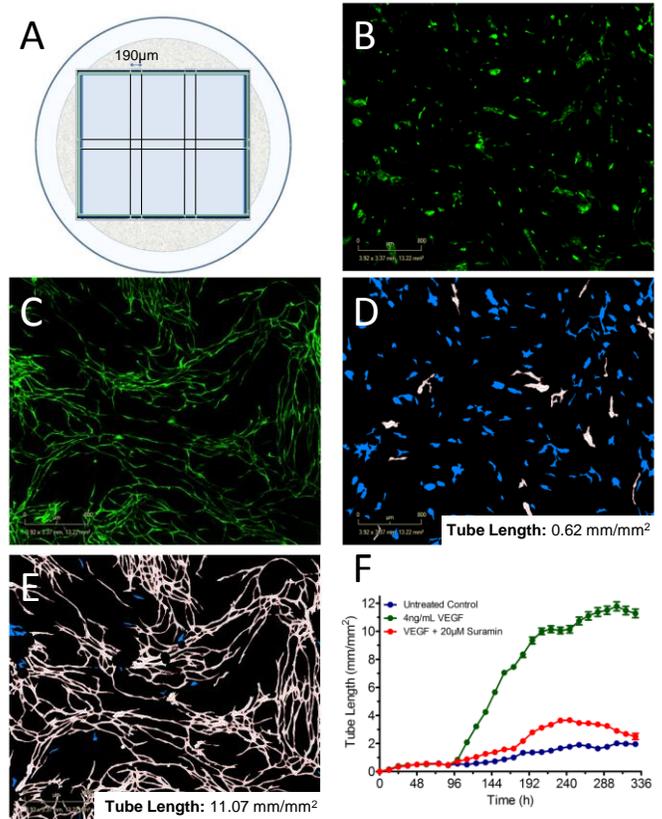
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## Introduction

Angiogenesis, the formation of new vessels from pre-existing vessels in the body, is an essential process during development, wound healing, and reproduction. In healthy tissues under steady-state conditions, the angiogenic process, consisting of endothelial cell (EC) proliferation, EC migration, and vessel morphogenesis, is tightly regulated by physiologic control of growth factors and inhibitors [1, 2]. However, under pathologic conditions, such as cancer, rheumatoid arthritis, and wet age-related macular degeneration, the balance is tipped to stimulate new vessel production providing nutrients to the diseased area (i.e. tumor growth in cancer) [1]. Clinically, anti-angiogenic therapies have proven effective at slowing disease progression [1]. However, due to either inherent or acquired resistance, these therapies have only been mildly beneficial to overall survival. Thus, advancement of *in vitro* angiogenesis models to study VEGF resistance and more complex pharmacologic paradigms are paramount to developing the next generation therapeutics.

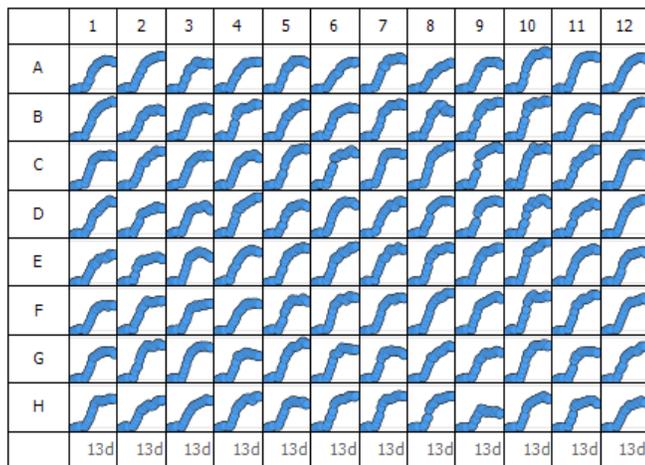
Various *in vitro* assays have been developed to study different aspects of the angiogenic process [3]. Historically, these assays tend to be highly variable, difficult to quantify, and limited in their clinical relevance. In 2009, following the early work of Bishop et. al. [4], Essen BioScience developed a kinetic, quantitative 24-well co-culture angiogenesis model, utilizing lentivirally-infected human umbilical vein endothelial cells (HUVEC) expressing GFP cultured with normal human dermal fibroblasts (NHDF). This model demonstrates all phases of the angiogenesis process, including proliferation, migration, and, eventually, differentiation and anastomosis to form complex angiogenic networks (Figure 1C). Imaging the co-culture in the IncuCyte™ FLR enabled the fluorescent identification of HUVEC CytoLight Green (a GFP variant), and allowed visualization of tube formation over time (Figure 1A-C). We then combined time lapse image acquisition with an integrated angiogenesis algorithm to measure tube length, tube area, and branch point formation. The combined capabilities enabled a critical evaluation of the stage and extent of angiogenesis throughout the assay (Figure 1D-F).

In 2010, we extended the 24-well format to a 96-well kinetic angiogenesis model providing increased throughput as well as



**Figure 1. Imaging and quantitating HUVEC CytoLight Green differentiation into angiogenic networks using IncuCyte™ FLR.** A, A cartoon depicting a single well in a 96-well plate with an overlaid tiled FOV image demonstrating the 3 x 2 tiled images with ~190µm overlap allowing for a single mosaic image 3.37 x 3.92 mm. B-C, IncuCyte™-FLR PrimeKit angiogenesis images at days 5 and 14 of the assay, respectively. Scale bar: 800µm. D-E, Computational analysis of tube formation using the IncuCyte™ Angiogenesis Analysis Module. Blue “cells” indicate events that were filtered out and not included in calculated tube length. F, Optimization of the signal to noise ratio via reduction of basal tube formation in the absence of exogenous VEGF. VEGF treatment induces a large pro-angiogenic response which can be inhibited with 20µM Suramin.

a larger signal window. We modified the culture conditions of the 24-well format to optimize our signal-to-noise ratio such that very little tube formation occurs under basal conditions, while maintaining a robust VEGF response (Figure 1F). Additionally, in order to maximize the image area in



**Figure 2. Whole plate VEGF response in the PrimeKit.** Whole plate view of tube length (mm/mm<sup>2</sup>; y-axis) versus time (d; x-axis) in a 96-well Angiogenesis PrimeKit treated with 4ng/ml VEGF.

each well of the 96-well plate, the Essen BioScience engineering group developed the Tiled Field of View (FOV) Mosaic imaging mode. This image mode enables the user to visualize a larger, more integrated view of the angiogenic network without compromising image resolution (Figure 1A). Essen has offered this assay, the **CellPlayer Angiogenesis PrimeKit**, to customers in two modes; a.) as a live-cell kit which is sent to a customer for testing at their facility or b.) as a fee-for-service where the assay is run at Essen BioScience on customer-provided compounds.

Our most recent extension (2011) is the release of a cryopreserved version of the 96-well assay that offers all of the same advantages of the live-cell assay with the additional advantages of long-term storage, user-determined assay initiation, and more flexible compound addition options. The cryopreserved **PrimeKit** allows users to genetically manipulate either cell type prior to the experiment. The flexibility and kinetic nature of the Essen BioScience angiogenesis kits, when conducted in conjunction with the IncuCyte FLR and the accompanying angiogenesis algorithm, enables an investigator to both quantitatively and qualitatively assess network development throughout the course of the assay. In this application note, we describe the **PrimeKit** as a means to kinetically study complex processes and signaling pathways that are directly relevant to *in vivo* angiogenic pharmacology.

## Approach and Methods

1. The **PrimeKit-Live** is initiated by seeding lentivirally-infected Human Umbilical Vein Endothelial Cells (HUVECs) in co-culture with Normal Human Dermal Fibroblasts (NHDF) in a 96-well microplate (Corning Life Sciences, Lowell, MA) as previously described [4]. The cell densities and growth conditions are optimized for assay performance, yielding a significant amount of VEGF-mediated tube and network formation over the 14 day time-course (Figure 2). For the **PrimeKit-Cryo**, on the day of seeding, Day 0, the NHDF are thawed, rinsed, and plated in Seeding Media into a Corning 96-well plate. The NHDF are then incubated at room temperature in a tissue culture hood for one hour to allow them to adhere to the plate. Following seeding of the HUVEC CytoLight Green, the plate is incubated at room temperature for one hour prior to placing in the IncuCyte for imaging. The cell densities for the **PrimeKit** have been optimized to conform to our strict quality control guidelines for assay performance.
2. Following seeding, co-cultures are placed in an IncuCyte FLR and images are automatically acquired in both phase and fluorescence every 6-12 hours for 8-10 days (**Cryo**) or 14 days (**Live**) using the Tiled Field of View (FOV) mosaic imaging mode (Figure 1A). In this mode, six total images (3 images wide x 2 images high) are acquired per well and merged into a single, larger image covering nearly 50% of the well (Figure 1A).
3. In the live-cell **PrimeKit**, complete media changes occur on days 0 (seeding), 3, 4, 5, 7, 10, and 12 of the assay. PrimeKits sent to customers are shipped on Day 3, for arrival on Day 4. Test reagents, such as growth factors, antibodies, or small molecules, are then typically added on Day 4 and kept throughout. As proliferation and migration are nearly complete by Day 4, test agents added at this stage are mainly affecting HUVEC differentiation. Utilizing the advantage of a kinetic read-out, we have also run tube regression assays by letting the tube network form, and subsequently adding compounds at a later time point to disrupt the stable network.

The development of the cryopreserved **PrimeKit** enables greater flexibility to study early events. With this kit, the end user has more responsibility for assay



setup, including seeding the cells. Typically, for the cryopreserved **PrimeKit**, cells are seeded on Monday (Day 0), outgrowth occurs on Day 1, with treatments including growth factors and test agents added on Day 2. Assuming Day 0 is Monday, the cells are fed on days 0 (seeding), 1 (outgrowth), 2, 4, 7. Initiation of growth factor treatment on Day 2 to Day 4 yields the maximal angiogenic stimulation (Figure 5C). These conditions will be used for the **PrimeKit** data presented below.

4. One of the more difficult aspects of running an angiogenesis assay is precisely quantifying the response. Essen BioScience's Angiogenesis Analysis Module provides multiple assay metrics, including tube length and branch point formation. These metrics are determined for each time point during the experiment, and have been optimized for use with the Essen BioScience angiogenesis kits.

In the first step of the process, the angiogenesis algorithm analyzes each fluorescent image and assigns a segmentation mask that closely resembles the *in vitro* network. From here, the mask can be refined and filtered to exclude non-tube forming events and measure angiogenesis over time. Kinetic plots of the angiogenesis metrics (tube length, tube area, and branch points) can be generated using the IncuCyte software, allowing for a direct and straightforward comparison of the wells of interest to validated control conditions. Additionally, the ability to export images and movies using the IncuCyte software enables the user to further verify and support the quantitative metrics.

## Results

### *Growth Factor Responses – Examples: VEGF, bFGF, and EGF*

Anti-angiogenic agents targeting VEGF signaling to reduce unwanted or leaky vasculature are being indicated as potential therapeutics in such diseases as cancer, macular degeneration, rheumatoid arthritis, and other pathologies, while pro-angiogenic therapies are also being developed in hind-limb ischemia and other cardiovascular diseases [2]. Thus, investigating the complexities and sensitivities of VEGF signaling is important to establishing a robust *in vitro* angiogenesis model. To this end, we examined how increasing VEGF concentrations could induce tube formation

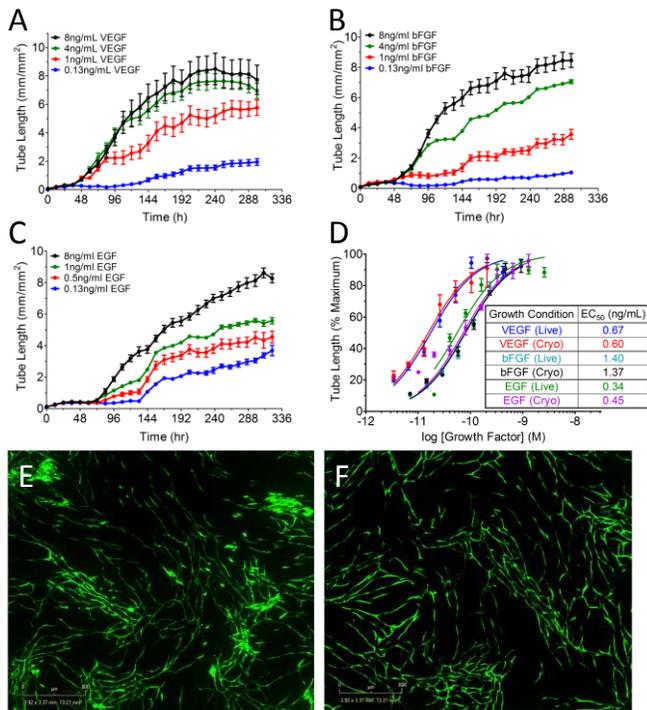
over the 14 day assay period. Using the Angiogenesis **PrimeKit**, VEGF was added on Day 2 at the indicated concentrations and maintained throughout the rest of the assay (Figure 3A). As soon as VEGF is introduced, the HUVEC respond and begin to elongate and differentiate into tubes. The extent of overall tube length that is obtained is dependent on the VEGF concentration; with 1ng/mL exhibiting a 50% response while 4ng/mL VEGF reaches maximal stimulation (Figure 3A). Regardless of the **PrimeKit** format, addition of VEGF resulted in a similar extent of network formation over the course of the assay (data not shown). Further, comparing Day 14 images from cultures treated with 4ng/mL VEGF shows no distinguishable difference between the networks formed using either **PrimeKit** format (Figure 3E-F, respectively).

We have also investigated the angiogenic effects of bFGF and EGF in the **PrimeKit**. Here, cultures were seeded and either bFGF or EGF treatment was initiated on Day 2 at the indicated concentrations (Figure 3B-C, respectively). As can be seen in Figure 3, cultures responded to bFGF and EGF in a similar manner to VEGF. Perhaps a more indicative measure of the reproducibility is to examine the growth-factor  $EC_{50}$  values for the respective assay formats. Re-plotting the data at 300h as a function of concentration revealed nearly identical  $EC_{50}$  values for VEGF, bFGF, and EGF indicating that both **PrimeKit** formats are able to respond equally well to a variety of pro-angiogenic stimuli (Figure 3D).

### *Anti-Angiogenic Response – Examples: anti-VEGF and suramin*

Due to the clinical emphasis on anti-angiogenic therapies, it was imperative to establish the ability to inhibit VEGF-mediated network formation as a means to study anti-angiogenic therapies *in vitro*. To investigate this, we employed two different strategies. First, a general tyrosine kinase inhibitor, suramin, was used to block receptor phosphorylation, thus indirectly inhibiting VEGF, as well as other receptor tyrosine kinase signaling. Suramin has been well characterized in this co-culture setting to be a potent anti-angiogenic compound. Using the **PrimeKit**, suramin treatment resulted in a concentration-dependent inhibition of VEGF-mediated angiogenesis (Figure 4A). Interestingly, the highest concentration, 160 $\mu$ M suramin, completely suppressed tube formation even below basal levels. Comparing  $IC_{50}$  values from the two **PrimeKit** formats at both 192 and 300 hours revealed no significant difference in the potency of suramin to inhibit angiogenesis (Figure 4B).



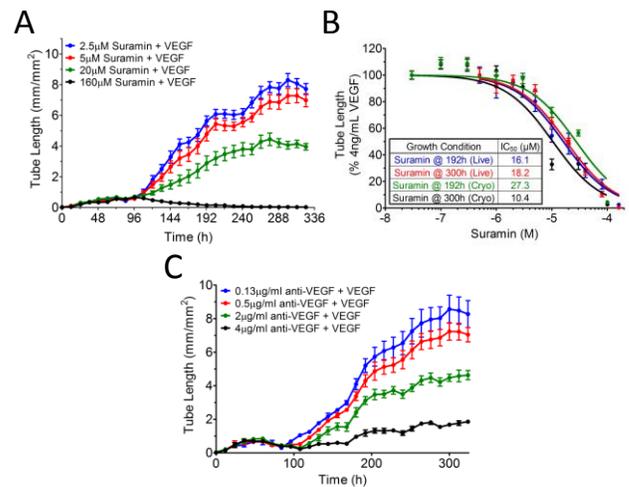


**Figure 3. Growth factor stimulation of angiogenesis in the PrimeKit.** A-C, *PrimeKit* co-cultures were seeded on Day 0 and the indicated concentration of growth factor was added on Day 2. VEGF (A), bFGF (B), and EGF (C) stimulated tube formation on a similar time-course and to a similar extent over the course of the assay. Data are representative of two separate experiments presented as the mean ± SEM (n=8). D, Plotting tube length response as a function of concentration demonstrates that the EC<sub>50</sub> value is nearly identical for each cytokine between the cryopreserved and live-cell formats of the *PrimeKit*. E-F, Representative images of 4ng/mL VEGF-stimulated angiogenic networks at 300h using the Angiogenesis *PrimeKit* – Live (E) or Cryo (F). Scale Bar is 800µm.

To look more directly at affecting VEGF signaling, an anti-VEGF antibody (R&D Systems, Minneapolis, MN) was used to inhibit VEGF from binding its receptor via sequestration of the ligand. As shown in Figure 4C, increasing concentrations of VEGF antibody exhibited a pronounced inhibition of VEGF-mediated tube formation in the *PrimeKit* (Figure 4C). Together, these data indicate that VEGF plays a significant role in stimulating angiogenesis in this *in vitro* setting.

### Network Stability

Having developed a reproducible, “kinetic” assay, enabled us to more completely characterize the role of VEGF in tube formation and stabilization. To do this, we initially investigated the effect of VEGF removal and add-back on the dynamics of tube formation. As shown below, 4ng/ml VEGF

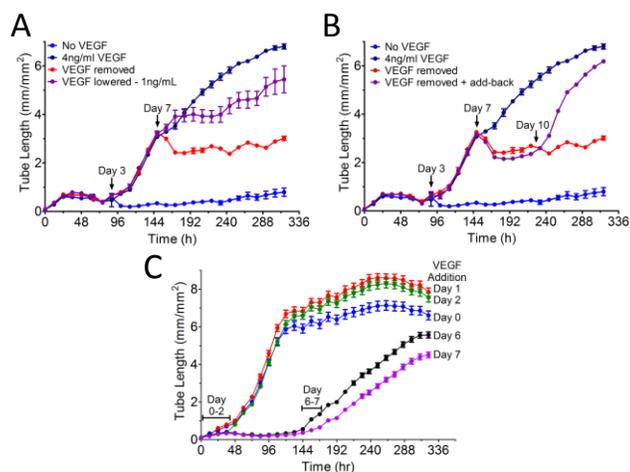


**Figure 4. Inhibition of VEGF Response.** A, Increasing concentrations of Suramin effectively inhibit and even eliminate tube formation over the course of the experiment in the *PrimeKit*. B, 192 hour (Day 10) and 300 hour (Day 14) data graphed as a function of suramin concentration in order to generate IC<sub>50</sub> data and showing the experiment-to-experiment reproducibility. Data are representative of either four (Live; n=12) or two (Cryo; n=8) separate experiments presented as the mean ± SEM. C, Increasing concentrations of anti-VEGF antibody are able to inhibit VEGF-mediated tube formation.

addition at day 3 (72 h) resulted in increasing tube formation over the complete course of the assay (Figure 5A). Lowering VEGF to 1ng/ml following four days of full stimulation slowed progression of tube formation compared to the 4ng/ml VEGF positive control. Interestingly, completely removing VEGF on Day 7 resulted in an initial reduction followed by network stabilization over the course of the assay (Figure 5A). This indicates that the initial VEGF treatment primes the HUVEC for stabilization and survival even after growth factor removal.

Once the HUVEC had stabilized, we next examined if they remained sensitive to VEGF. VEGF reintroduction after removal and stabilization resulted in additional stimulation of tube formation, demonstrating that the HUVEC remain responsive to VEGF throughout the assay (Figure 5B). Perhaps more intriguing is the responsiveness of the HUVEC exposed to serum alone (no exogenous growth factor addition).

As described above, using the Angiogenesis *PrimeKit* the co-cultures were maintained in basal media prior to the introduction of 4ng/ml VEGF at the indicated time-points (Figure 5C). Regardless of the timing of VEGF introduction, the HUVEC immediately began differentiating, eventually elongating and anastomosing into complex angiogenic networks. Interestingly, adding VEGF on day 0 resulted in a

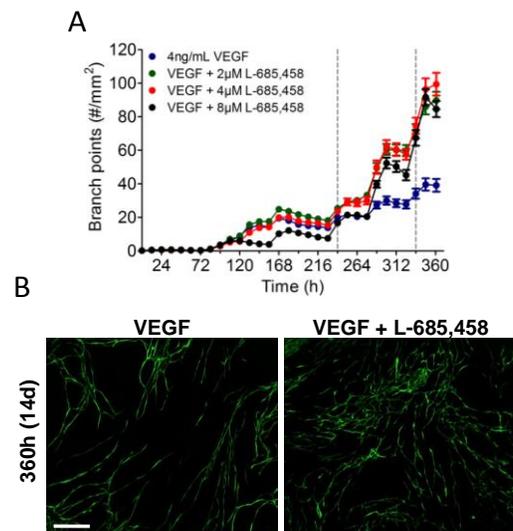


**Figure 5. Temporal Control of VEGF Response.** **A**, HUVEC differentiation was initiated on Day 4 of the *PrimeKit* via treatment with 4ng/mL VEGF. On Day 7, VEGF was either maintained (dark blue), lowered to 1ng/mL (purple) or removed, and maintained through the rest of the experiment. **B**, As in (A), tube formation was initiated in the *PrimeKit* by treatment with 4ng/mL VEGF from Day 4-7. On Day 7, VEGF was either maintained (dark blue) or removed (red and purple). On Day 10, 4ng/mL VEGF was re-introduced (purple) and maintained. Stabilization data are representative of three separate experiments each run in triplicate. **C**, Angiogenesis *PrimeKit* responds to VEGF throughout the time-course of the assay.

lower amount of tube formation than addition on Day 1 or 2. This is most likely due to VEGF's positive effects on proliferation and migration that induce HUVEC clustering and, ultimately, lower tube formation. These results show that the HUVEC remain responsive to VEGF for the duration of assay and enables extending this assay to more complex protocols and signaling pathways.

### Notch Signaling

Most *in vitro* angiogenesis assays examine changes in the angiogenic process by investigating changes in a single metric via end-point analysis. In most cases, this analysis does not reveal the entire story of a test agent's effects or mechanism of action. The advantage of the Essen BioScience CellPlayer Angiogenesis Assay Kits is that, in addition to the images generated at each time point, the automated IncuCyte angiogenesis algorithm can assess three separate metrics (tube length, tube area, and branch point formation) throughout the time course of the assay. This is important in cases where only a single aspect of the angiogenic process is significantly affected by the test agent. One example of this is the Delta-like Ligand 4 (DLL4)/Notch signaling pathway, where genetically manipulating DLL4 expression in either mice or zebrafish leads to enhanced or aberrant sprouting [5-7]. One



**Figure 6. Inhibiting Notch signaling increases branch point formation.** **A**, Treatment with the  $\gamma$ -secretase inhibitor, L-685,458, significantly increase branch point formation compared to 4ng/mL VEGF control, with only small effects on overall tube length (not shown). **B**, Images illustrate the differences in network complexity and branch point formation in representative wells treated with either 4ng/ml VEGF or 4ng/ml VEGF + 8 $\mu$ M L-685,458. These images represent data at 360h. A clear increase in network complexity and branch points was observed upon treatment with the  $\gamma$ -secretase inhibitor L-685,458 for 360h (right image). Scale bar is 800 $\mu$ m.

of the last steps in DLL4/Notch signaling is cleavage by  $\gamma$ -secretase, releasing the Notch intracellular domain (NICD) for nuclear translocation and target gene activation. In agreement with previous studies, treatment with the  $\gamma$ -secretase inhibitor, L-685,458, effectively blocking Notch signaling, resulted in an increase in overall branch point formation late in the experiment, Day 10-14, despite treatment beginning on day 4 (Figure 6A). These differences can be observed qualitatively by examining the images at time points throughout the assay (Figure 6B). Interestingly, the effect of L-685,458 on overall tube length was marginally increased compared to the VEGF control (not shown). These studies suggest three main results closely mirroring *in vivo* findings [5-7]: 1) endogenous DLL4/Notch signaling acts to attenuate VEGF signaling and tightly regulate angiogenesis; 2) blocking Notch signaling increases overall branch point formation compared to VEGF controls; and 3) this effect occurs in the later stages of tube maturation.

### Vascular Disruption

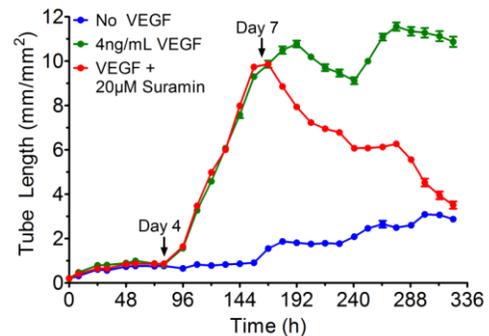
In pathologies involving the vasculature, angiogenic networks have been established to promote nutrient delivery and disease progression, for example tumor growth and metastasis.



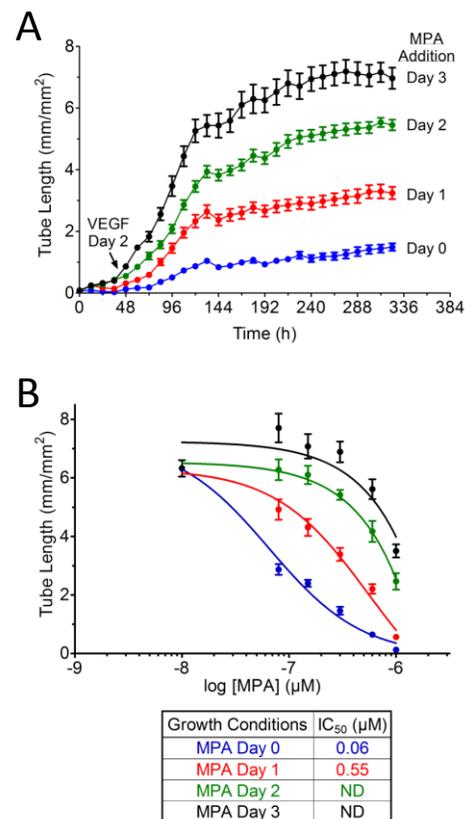
Several strategies, such as bevacizumab, aflibercept, sunitinib and sorafenib, have focused on inhibiting the angiogenic process with limited success. Next generation therapeutics, used in monotherapy or in combination with current standards of care, must demonstrate the ability to disrupt pathological vasculature, ultimately causing disease regression. Here, we investigated suramin's ability to affect established angiogenic networks. Figure 7 shows that, prior to suramin treatment, networks developed similar to control. Once suramin is introduced on Day 7, however, tubes immediately begin to regress and reach baseline levels by the end of the experiment. These results indicate that studies investigating vascular disruption can be performed using the CellPlayer Angiogenesis PrimeKit.

#### Mechanism Studies – Proliferation

One of the differences between the Angiogenesis PrimeKit assay formats is the ability to investigate compounds that affect the early angiogenic processes. For customers who choose to run the live-cell PrimeKit at their facility, the compounds are not added until day 4 of the assay. This is not a limitation for the cryopreserved PrimeKit. To demonstrate the effects of early-phase inhibitors on angiogenesis, we treated with the inosine monophosphate dehydrogenase inhibitor, mycophenolic acid (MPA). In addition to its well-characterized immunosuppressive activities, MPA has been shown to be a potent inhibitor of EC and NHDF proliferation, migration, and invasion [8]. Combined, these effects are thought to underlie the anti-tumor activity of MPA *in vivo* [8]. Initiating MPA treatment every 24 hours during the initial phases of the experiment allowed us to determine when proliferation and migration were complete, as determined by MPA's effect on overall tube formation (Figure 8A). In all cases, VEGF was added on day 2 of the assay, as this was determined to be optimal for stimulating tube formation. Our results show that, as the assay progresses, later MPA additions become less effective at inhibiting tube formation. Addition of MPA on Day 3 did not significantly differ from the 4 ng/mL VEGF control (not shown). This is confirmed by IC<sub>50</sub> analysis showing that addition at later time points makes MPA appear less potent, when, in fact, HUVEC proliferation and migration are nearly complete and differentiation is becoming more prominent (Figure 8B). Thus, treatment with a test agent altering cell proliferation and/or migration/invasion can affect overall tube formation, but is highly dependent on when the test agent is introduced.



**Figure 7. Measuring vascular disruption.** HUVEC CytoLight Green were stimulated on Day 4 with 4ng/mL VEGF and allowed to differentiate until Day 7. Addition of 20µM suramin on Day 7 results in an immediate and prolonged regression of VEGF-mediated angiogenic networks.



**Figure 8. Mycophenolic acid inhibition of VEGF-mediated tube formation.** A, 0.3µM MPA treatment initiated every 24h for the first 3 days, and maintained in the presence of 4ng/mL VEGF, indicates that proliferation and migration become less prominent and are complete by Day 3. B, Concentration response curves for MPA demonstrate its anti-angiogenic potential is dependent on timing of MPA treatment addition.



## Conclusion

Given a highly reproducible, quality controlled format, an *in vitro* angiogenesis assay can prove to be a useful tool in studying complex mechanisms and therapeutic interventions of vascular formation. Combining the CellPlayer Angiogenesis **PrimeKit** with the IncuCyte FLR provides a robust, quantitative assay that can be used to investigate multiple angiogenic processes, including proliferation, migration, and HUVEC differentiation. As shown in this application note, the pharmacological and physiological effects obtained using both assay formats are highly translatable to previous *in vivo* characterizations. The kinetic nature of the assay, in conjunction with high-resolution images, increases the depth of knowledge obtained from a single experiment. This is exemplified by the DLL4/Notch studies, where the increase in branch point formation occurs late in the angiogenic process and closely mimics effects observed in both chemically treated and genetically modified *in vivo* models [6]. With the introduction of the cryopreserved CellPlayer Angiogenesis **PrimeKit**, the end-user now has maximal flexibility in terms of cell storage, assay setup, and test agent initiation. Finally, the design of the cryopreserved assay format is such that it lends itself to more detailed studies, including genetic manipulation via siRNA or miRNA to investigate a gene's effect on angiogenesis. Together, the CellPlayer Angiogenesis family of assays, in combination with IncuCyte FLR, provides a powerful approach for studying angiogenesis *in vitro*.

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### About the IncuCyte™ Live-Cell Imaging System

The Essen BioScience IncuCyte™ Live-Cell Imaging System is a compact, automated microscope. The IncuCyte™ resides inside your standard tissue culture incubator and is used for long-term kinetic imaging. To request more information about the IncuCyte™, please visit us at [www.essenbioscience.com](http://www.essenbioscience.com).

