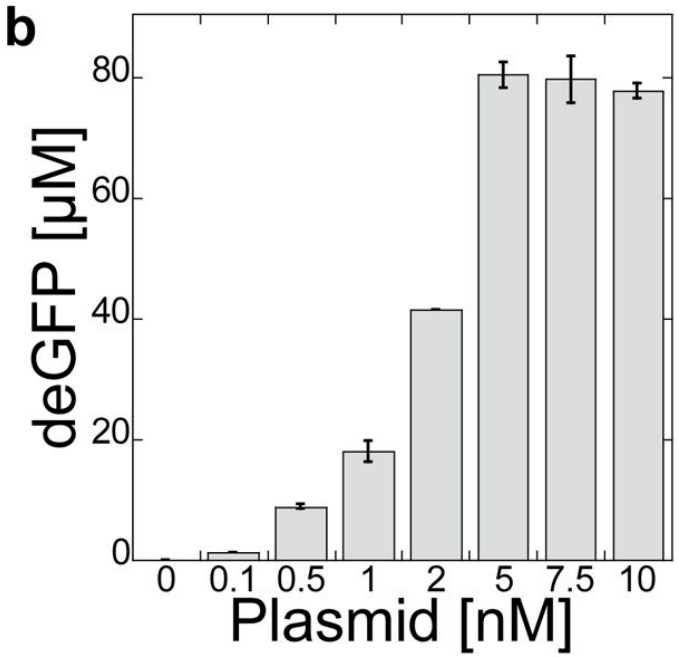
Biocircuits (Murray) Lab, April – June 2018
TX TL Life Extension Project – General Notes

The goal of the project was to increase the longevity of a TX TL reaction with output (total protein produced) also being assessed. Longevity of a TX TL reaction is considered as the time of linear or close to linear protein production. The strategy was to replicate things reported in the literature and then improve on that. For more detailed info on the literature check out the research folder in the life extension folder on Benchling.

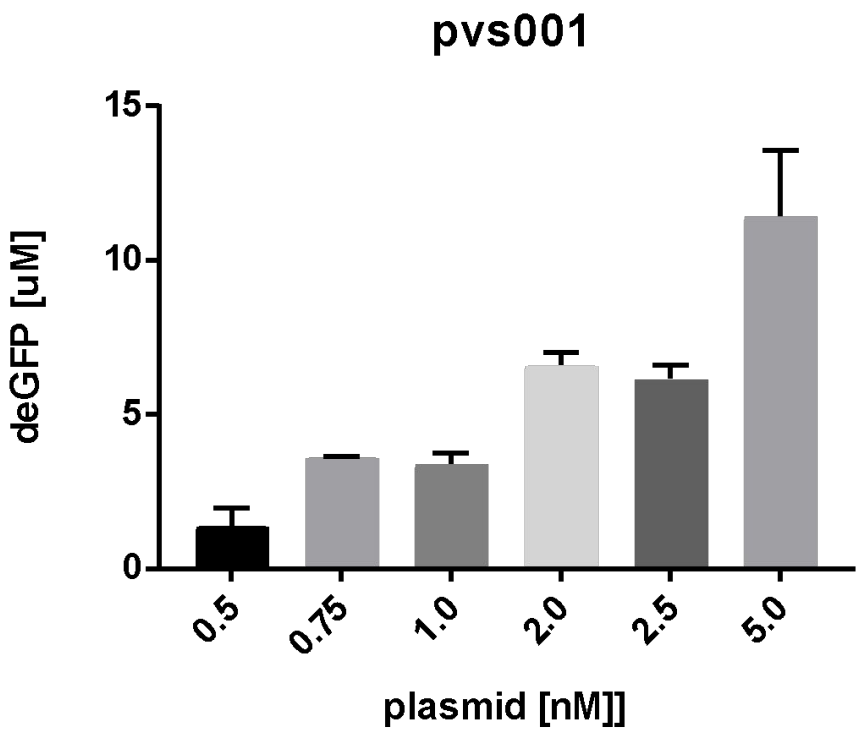
- All reactions were carried out in 10ul volumes for 12-20 hours at 29 degrees. The buffer + extract made up 6.6 ul of the final volume (10ul), leaving 3.3 ul for DNA and reagents.
- The construct used is the same as in the JoVe paper (*Sun et al* 2013), Sigma 70 promoter with lambda-phage operators.
    - Plasmid: pvs001: pBEST - OR2 - OR1 - Pr1 - UTR1 - deGFP - MG15 - T500

- Energy solution was prepared the same as in the above paper, as was the extract. Extract was made and calibrated by Shaobin. Calculations for the reagents tested and the modified Energy Solution are on the lacZ drive.
- All biotek raw data is saved on the lacZ drive with the biotek number indicated. All data was collected at gain 60.
- Benchling has a record of discussions, background research, an online notebook and notes related to methods and materials
- Day to day TX TL template data sheets were printed and some calculations were done by hand. There are physical notes regarding most experiments that are not saved in an online format, see Miki for the project folder if interested.
    - The lacZ contains TX TL JovE template files with the reaction conditions for each experiment organised by date
- The only difference between our protocol (as far as I know) and Vincent's is that his plates were incubated with constant rotation, however when we tried this we saw no difference on the performance of the system.
- Some slides have notes that provide extra details/information regarding the results on the slide.

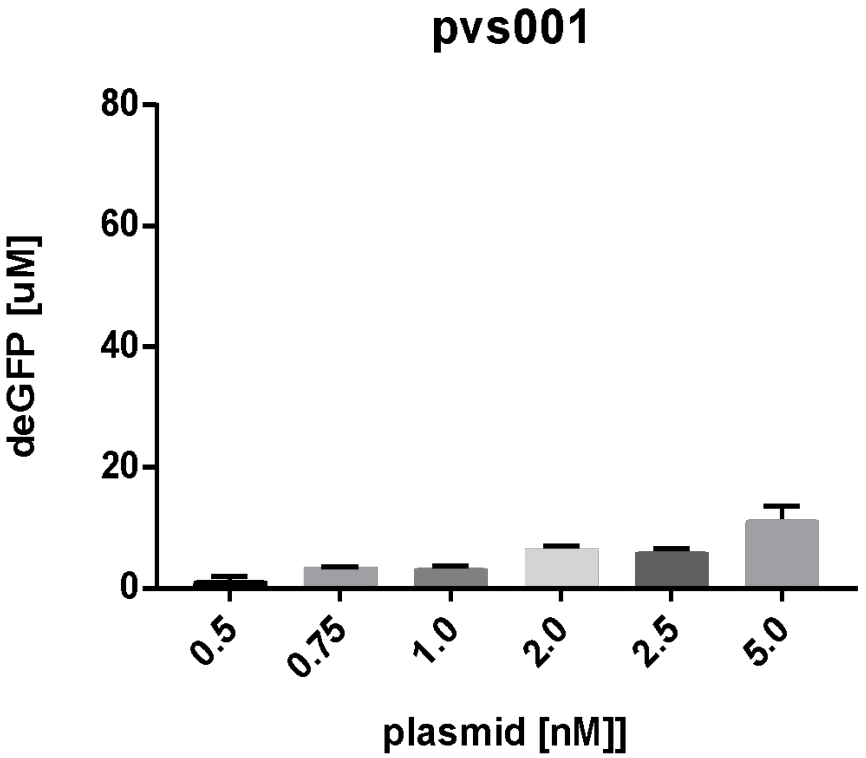
Comparing system output between 2.0 All *E. coli* toolbox paper and biocircuits system



DNA titration from Vincent's paper, batch TX-TL reaction



DNA titration conducted in biocircuits lab



Same graph but scaled to Vincent's graph.

Unfortunately I was never able to replicate Vincent's results and consistently got lower expression, I have no explanation for this. The reaction also saturated above 5.0nm [DNA], although not shown.

General TX TL Observations

Range of DNA concentration:

- A range of 0.5nM to 5.0nM of plasmid DNA was used and resulting expression was found to be fairly linear as reported by Vincent and thus was used as the useable range during the project.
- As seen in the previous slide, the output (uM of deGFP) of TX TL in the biocircuits lab was lower than that reported by Vincent. There was also much more variability from run to run than Vincent's. This could have been due to error in preparation or setup. There is little difference in output between 0.75nM and 1.0nM DNA as well as 2.0nM and 2.5nM DNA.

Effects of changing reaction conditions:

- Adding reagents to the TX TL reaction only had a positive effect (output and longevity) when using lower DNA concentrations. When working at 5.0nM [DNA], effects tended to have no effect or negatively affect the reaction.
- **Total output:** When working at 0.5nM [DNA], the total output was increased. Working at 2.0nM [DNA], using HEPES saw little to no increase in overall output. Working at 5.0nM [DNA] saw little to no change to output.
- **Longevity:** Vincent's system expresses for 8-9 h with 4-6h of close to linear protein production while the conditions tested increased this to **10-15h of expression with linear protein production lasting 8-10h** depending on the buffer.

The role of air (O2) on TX TL:

- The chromophore contained within deGFP requires oxygen for maturation. When using deGFP or any other fluorescent protein, the availability of oxygen can affect the rate of and final amount of reporter maturation.
- Oxygen is required for oxidative phosphorylation (make ATP) which has been shown to be active and thus power TX TL
- When using a fluorescent over an enzymatic reporter, it is difficult to detangle the above two factors (energetics and reporter maturation). If your goal is to increase total output or longevity, increase circulation of air and thus amount of available oxygen. This can be done by poking holes or by using a breathable membrane.

Starting point? Although we are working with a cell free system, the components are naturally tuned for an intra cellular environment, thus as a starting point we looked at what is naturally occurring in a cell. The table below (modified) lists some of the important cellular metabolites.

Table 1 Intracellular metabolite concentrations in glucose-fed, exponentially growing *E. coli*

Metabolite	mol l^{-1}	Metabolite	mol l^{-1}
Glutamate	9.6×10^{-2}	Deoxyribose-5-P (58)	3.0×10^{-4}
Glutathione	1.7×10^{-2}	AMP	2.8×10^{-4}
ATP	9.6×10^{-3}	Inosine monophosphate (61)	2.7×10^{-4}
UDP-*N*-acetylglucosamine (29)	9.2×10^{-3}	Succinyl-CoA (63)	2.3×10^{-4}
UTP (30)	8.3×10^{-3}	Inosine triphosphate (64)	2.1×10^{-4}
GTP (31)	4.9×10^{-3}	Guanine (65)	1.9×10^{-4}
dTTP	4.6×10^{-3}	Phosphoenolpyruvate	1.8×10^{-4}
Glutamine	3.8×10^{-3}	*S*-Adenosyl-L-methionine (66)	1.8×10^{-4}
6-Phosphogluconate	3.8×10^{-3}	NADPH	1.2×10^{-4}
CTP (33)	2.7×10^{-3}	Fumarate (71)	1.2×10^{-4}
NAD^+	2.6×10^{-3}	Phenylpyruvate (72)	9.0×10^{-5}
Glutathione disulfide (36)	2.4×10^{-3}	NADH	8.3×10^{-5}
Citrate	2.0×10^{-3}	Malonyl-CoA (81)	3.5×10^{-5}
UDP (38)	1.8×10^{-3}	Cyclic AMP (82)	3.5×10^{-5}
Malate (39)	1.7×10^{-3}	dCTP (83)	3.5×10^{-5}
Coenzyme A (41)	1.4×10^{-3}	Inosine diphosphate (85)	2.4×10^{-5}
Glucosamine-6-phosphate (43)	1.2×10^{-3}	GMP (86)	2.4×10^{-5}
Acetylphosphate (44)	1.1×10^{-3}	Acetoacetyl-CoA (87)	2.2×10^{-5}
GDP (46)	6.8×10^{-4}	Riboflavin (88)	1.9×10^{-5}
Acetyl-CoA (47)	6.1×10^{-4}	dATP (91)	1.6×10^{-5}
ADP	5.6×10^{-4}	Cytosine	1.4×10^{-5}
α-Ketoglutarate	4.4×10^{-4}	dAMP (98)	8.8×10^{-6}
dTDP (55)	3.8×10^{-4}	Cytidine (105)	2.6×10^{-6}
Dihydroxyacetone phosphate	3.7×10^{-4}	$NADP^+$	2.1×10^{-6}
Homocysteine (56)	3.7×10^{-4}	Guanosine (106)	1.6×10^{-6}
		Adenine (107)	1.5×10^{-6}
		Adenosine (109)	1.3×10^{-7}

Table 1. modified from Bennett *et al.* see notes for citation

This table explains why calibrating the amount of glutamate is necessary when making new extract as it is the most common metabolite found in the cell.

This shows the importance of glutathione (reduced environment of the cell must be maintained) and cysteine (listed as homocysteine)

Both NADH/NAD+ and NADPH/NADP+ are important co-factors but note the ratios between them:
NAD+ > NADH while NADPH > NADP+

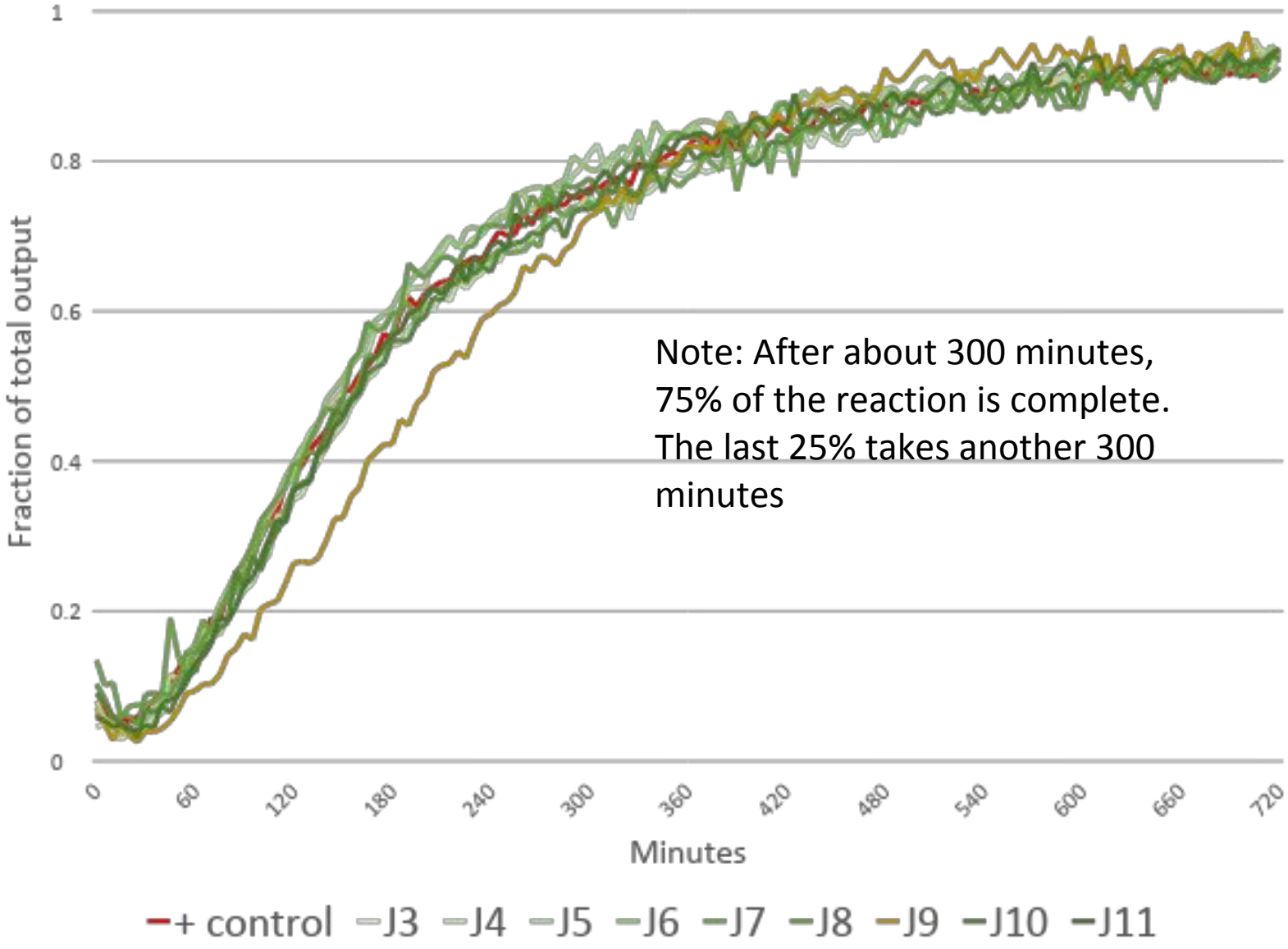
High levels of ATP are required for normal functions

High levels of TCA cycle intermediates: citrate, malate, alpha-ketoglutarate etc. are present

High levels of GTP, UTP, CTP and the deoxy versions dTTP, dGTP etc. are present

This was used as a starting point for testing different reagents that could be added to increase performance of the system. (next slide)

Controls: substrates listed in legend were added in isolation to TX TL reactions with 0.5nm of pvs001 plasmid as a control. The positive control (all data on all slides) was always pvs001 at the concentration listed with nothing added.



<u>Controls for conditions tested</u>

+ control: 0.5nm of pvs001

J4: 2 holes poked in membrane
J5: 25mM maltodextrin (mdx)
J6: 2.5x $NaHCO_3$ (sodium bicarbonate)
J7: 1.25mM L-cysteine + 2.5mM glutathione
J8: 15mM hexametaphosphate (HMP)
J9: 12.5mM maltose (mal)
J10: 1mM NADP+
J11: 1.5mM NAD+ (final [], addition of 1.17mM)

As you can see, adding the above reagents in isolation have no visible difference on the reaction, except the addition of maltose (J9).

Initial Screening / Results

The reagents added (controls from previous slides and listed on the right) did not seem to have much of an effect individually, however when combined there was some synergistic effect. This was optimised into what I refer to as the 'base conditions', which are the following:

+ 25mM mdx + 12.5mM mal + 2.5x $NaHCO_3$ + 1.5mM L-cysteine + 2.5mM glutathione

The above conditions, consistently improved the consistency of the protein production rate when compared to the controls.

The following reagents/conditions were then assayed to further enhance the effects of the above conditions:

- NAD+
- NADP+
- HMP
- Increasing air circulation by poking holes

Combinations of the above reagents/conditions were tested to see if they could further improve on the 'base conditions'.

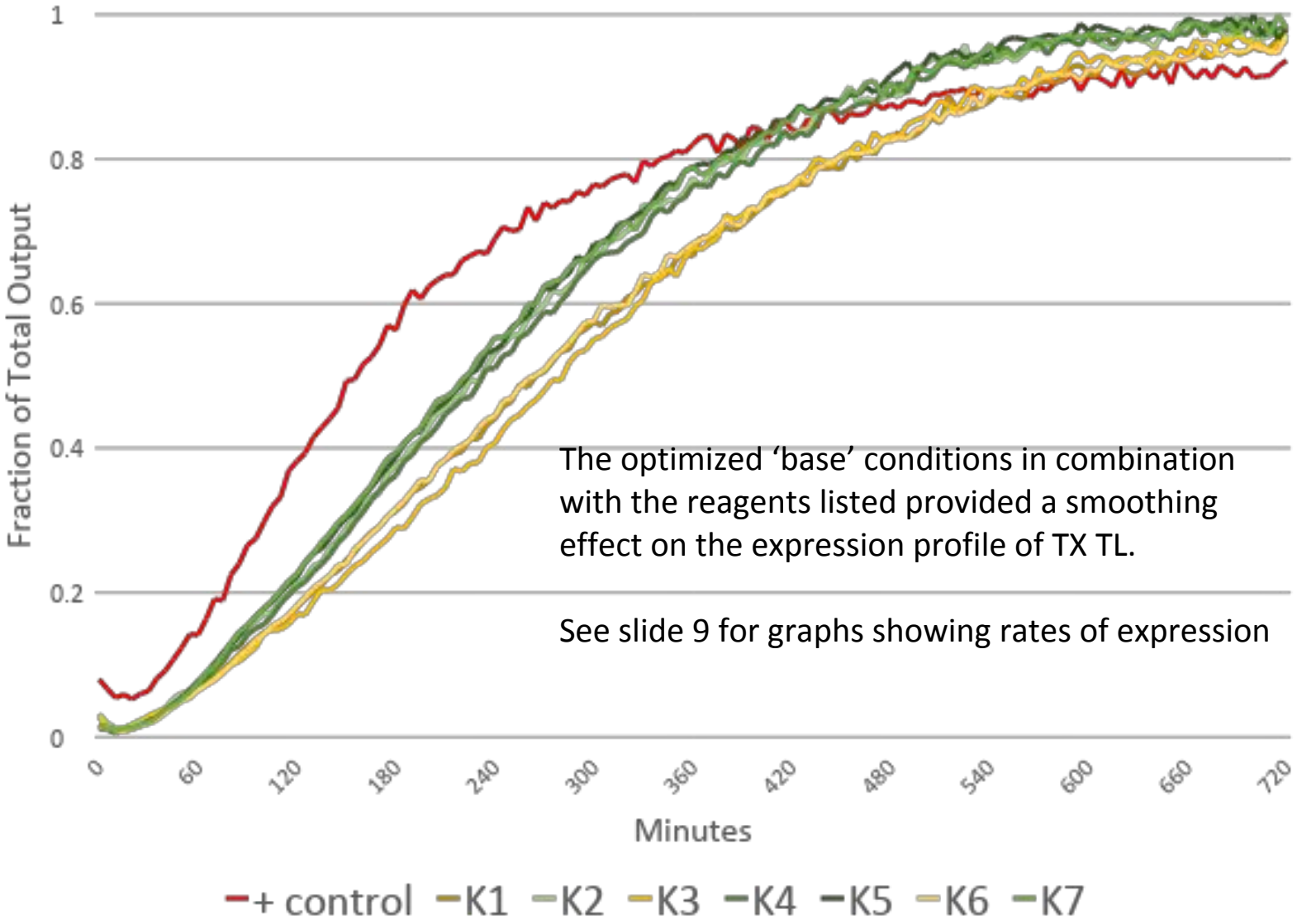
This is what is in the slides below.

Controls for conditions tested (previous slide)

+ control: 0.5nm of pvs001 (see notes above)

J4: 2 holes
J5: 25mM maltodextrin (mdx)
J6: 2.5x $NaHCO_3$ (sodium bicarbonate)
J7: 1mM L-cysteine + 2.5mM glutathione
J8: 15mM hexametaphosphate (HMP)
J9: 12.5mM maltose (mal)
J10: 1mM NADP+
J11: 1.5mM NAD+ (final, addition of 1.17mM)

Results: K1 – K7 all have the same base condition as described, and the reagents listed are what were added in addition to the base conditions. These results are with **no holes**. The next slide is the same but with holes.



The optimized 'base' conditions in combination with the reagents listed provided a smoothing effect on the expression profile of TX TL.

See slide 9 for graphs showing rates of expression

+ control: 0.5nM of pvs001

Each of the following was added (final [ ])to the base condition:

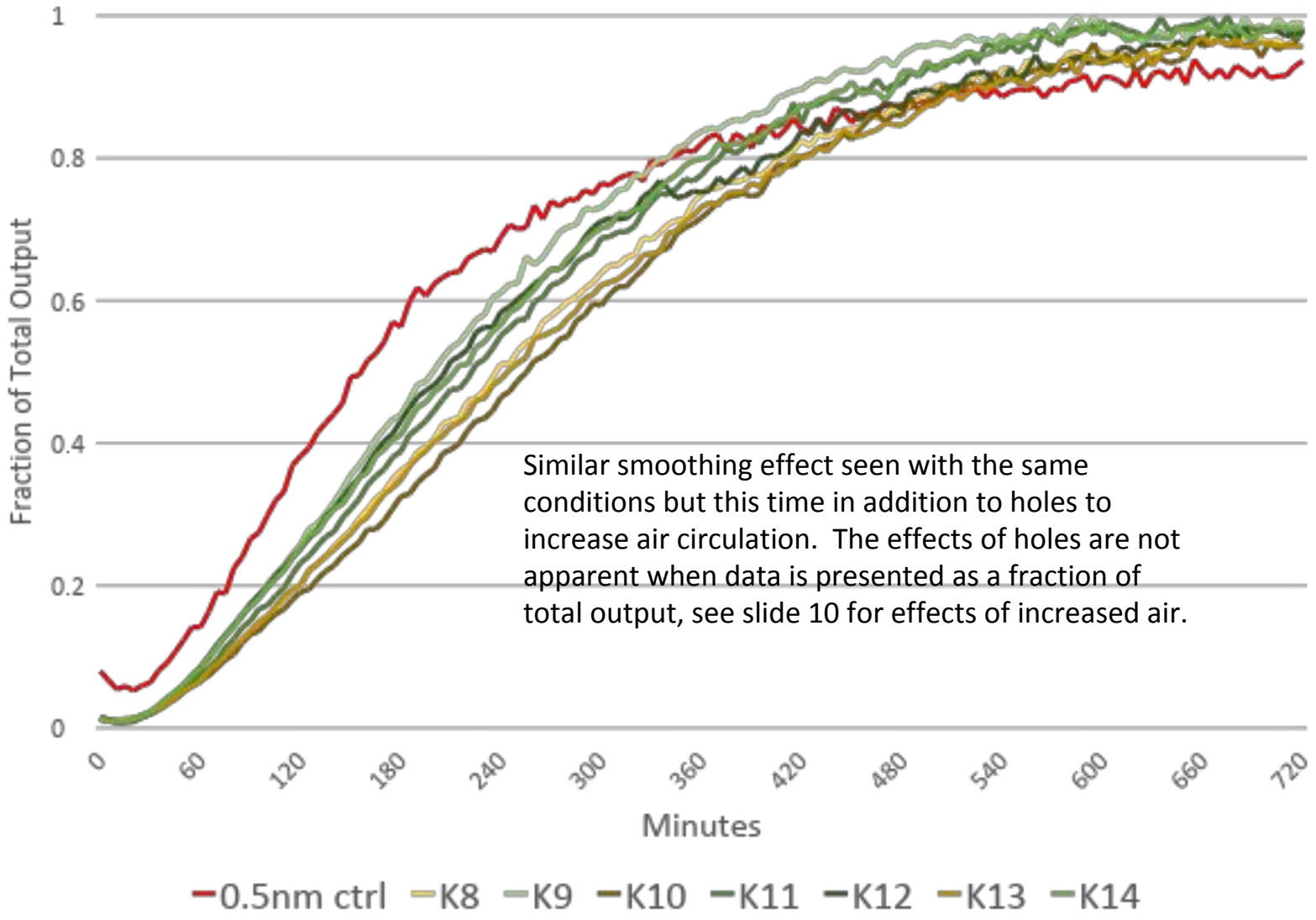
K1: 1mM NADP+
K2: 1.5mM NAD+
K3: 15mM HMP
K4: 1.5mM NAD+ + 1mM NADP+
K5: 1.5mM NAD+ + 15mM HMP
K6: 1mM NADP+ + 15mM HMP
K7: 1.5mM NAD+ + 1mM NADP+ + 15mM HMP

In terms of total [uM] of deGFP expressed:

+ control: 0.5
K1: 1.77
K2: 1.11
K3: 1.24
K4: 2.03
K5: 4.22
K6: 2.53
K7: 2.20

Base condition: 0.5nM DNA + 25mM mdx + 12.5mM mal + 2.5x $NaHCO_3$ + 1.25mM L-cys+ 2.5mM gluta

Results: K8 – K14 all have the same base condition as described, and the reagents listed are what were added in addition to the base conditions. These results are **with holes** poked in the membrane.



Similar smoothing effect seen with the same conditions but this time in addition to holes to increase air circulation. The effects of holes are not apparent when data is presented as a fraction of total output, see slide 10 for effects of increased air.

+ control: 0.5nM of pvs001

Each of the following was added (final [ ])to the base condition:

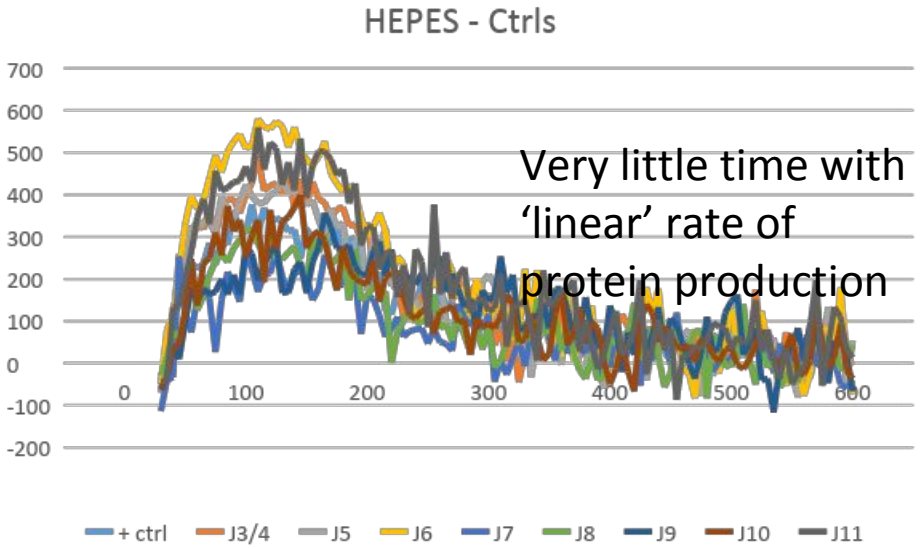
K8: 1mM NADP+
K9: 1.5mM NAD+
K10: 15mM HMP
K11: 1.5mM NAD+ + 1mM NADP+
K12: 1.5mM NAD+ + 15mM HMP
K13: 1mM NADP+ + 15mM HMP
K14: 1.5mM NAD+ + 1mM NADP+ + 15mM HMP

In terms of total [uM] of deGFP expressed:

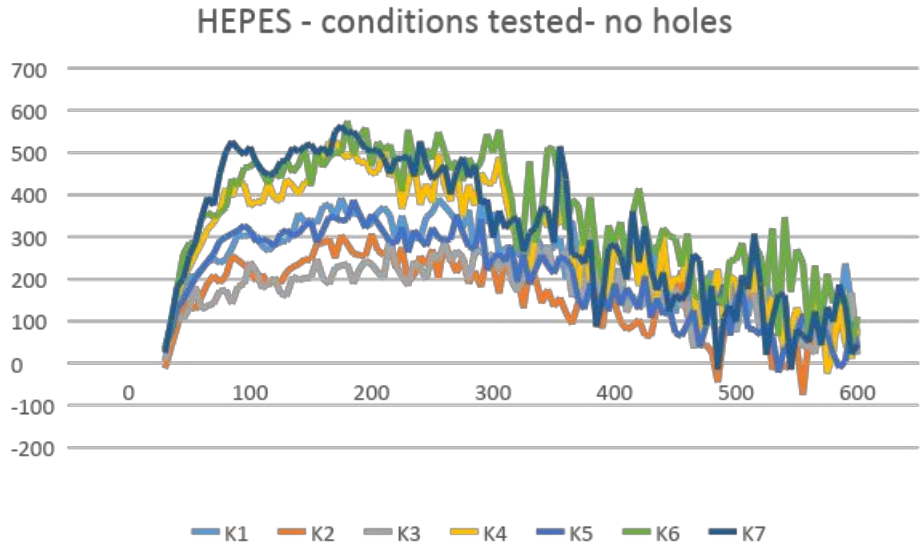
+ control: 0.5
K8: 3.25
K9: 3.20
K10: 2.74
K11: 3.15
K12: 4.00
K13:5.10
K14: 5.00

Base condition: 0.5nM DNA + 25mM mdx + 12.5mM mal + 2.5x $NaHCO_3$ + 1.25mM L-cys+ 2.5mM gluta

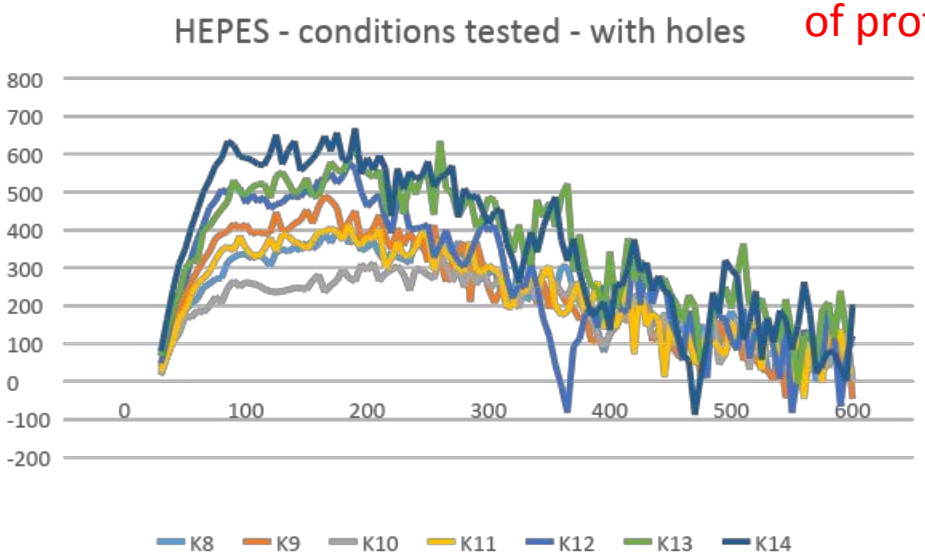
Results: effects of the conditions on the rates of expression of the system, changes in fluorescence (RFUs) are expressed as a function of time, over the duration of the entire run and then scaled for easy comparison. Effects of holes (not yet discussed) are shown on the following 2 slides.

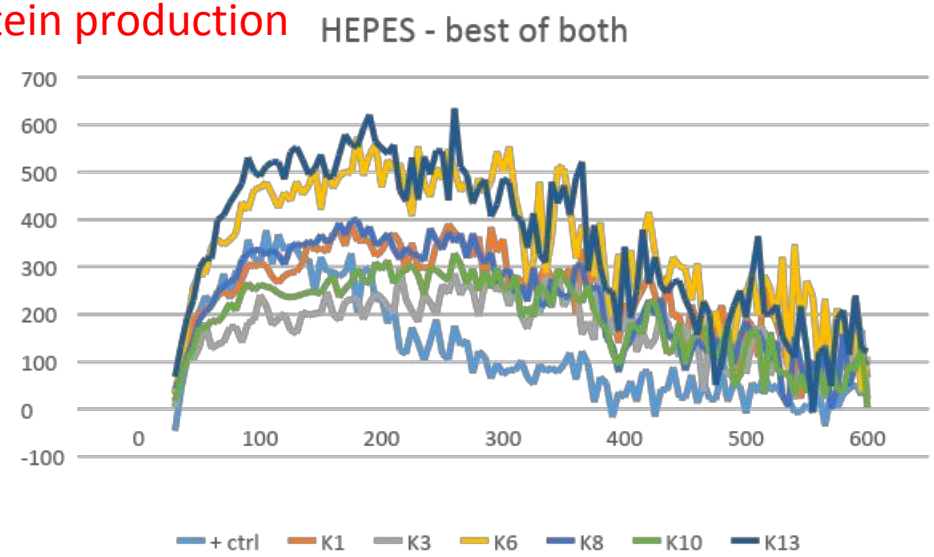


Very little time with 'linear' rate of protein production



Conditions produce more consistent rates of protein production



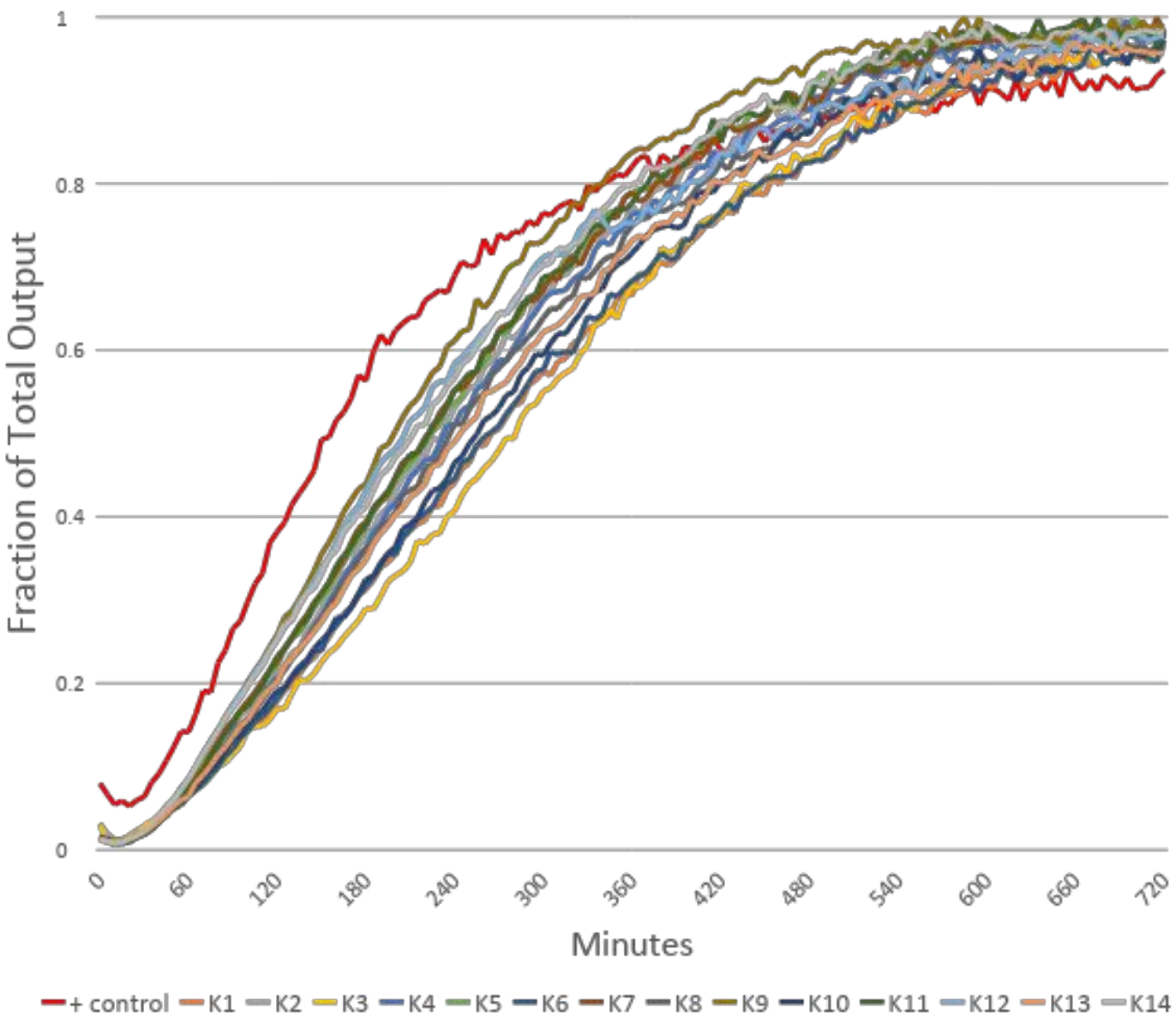


Previous slides displayed data as a fraction of the total output. Here rates of expression of system are shown for the controls (slide 5), reagents with no holes (S7) and with holes (S8) and finally, the best of both.

While adding reagents does not greatly enhance the overall longevity of TX TL, a much more consistent expression level is maintained throughout the assay.

In the controls, after 200 minutes, expression becomes muted, while expression remains elevated after 400 minutes in the conditions tested.

Summary graph: These are the same results that are on slides 7 and 8 but all in one graph.



In summary: in slides 7 and 8, 3 reagents are added in different combinations to the base conditions: NAD+, NADP+ and HMP

These combinations were then tested with and without holes.

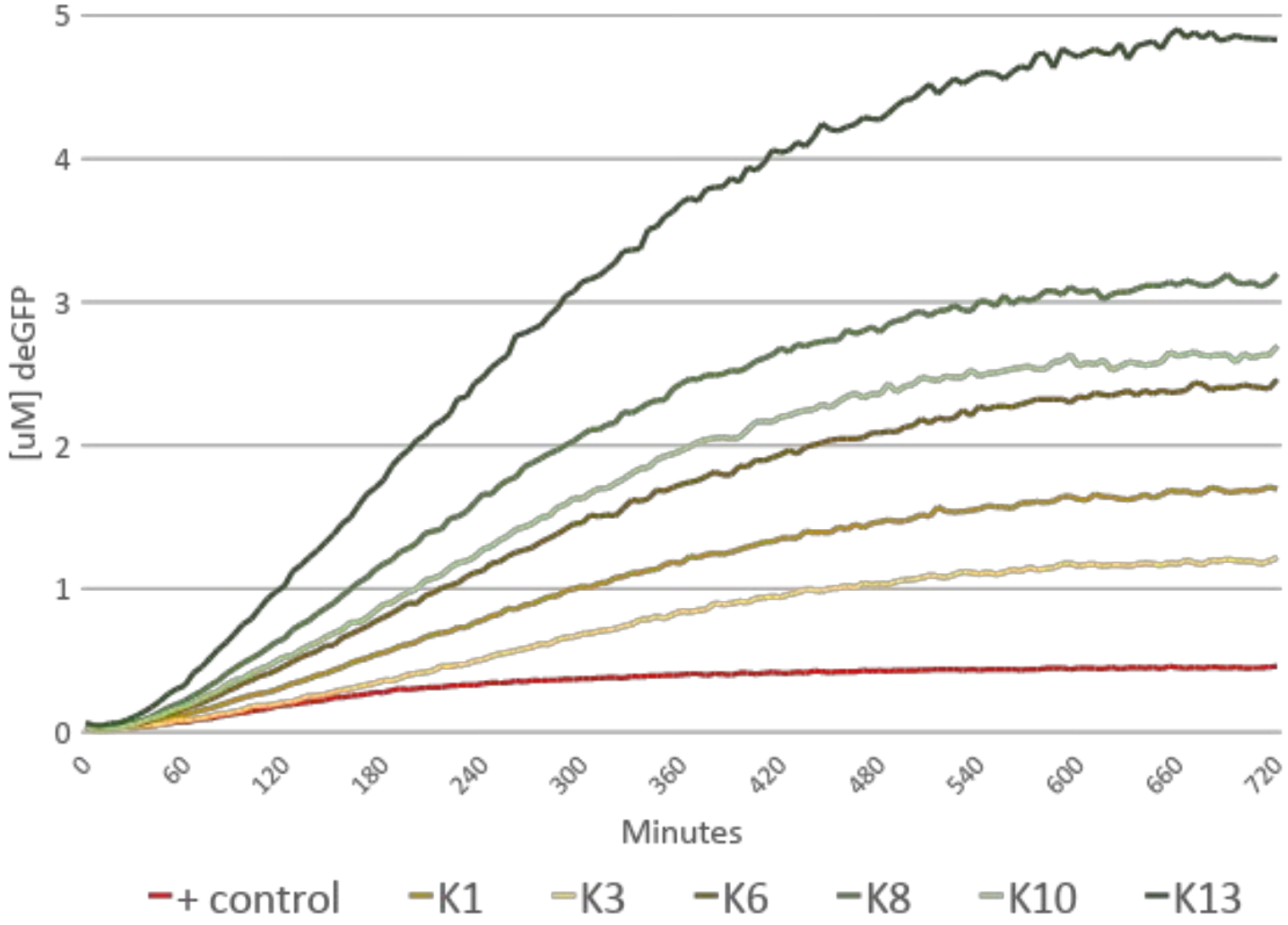
The conditions that perform best are those that have either NADP+ or HMP or both.

Note that with the best conditions you get approximately 450-500 minutes of fairly linear expression.

Effects of holes:

While the longevity (shape of curve) of the reaction does not change with or without holes, the effects are seen with the total output of the system. Higher concentrations of reporter are seen with holes. Next slide compares the total output of the best conditions with (K1, K3 and K6) and without (K8, K10 and K13) holes.

Results: Comparing the best conditions from previous experiments in term of total output in [uM] of reporter. This is to illustrate the effects of holes on the total output (protein production) of the reaction.
Recall this is at 0.5nM of DNA.



Effects of holes:
Recall that K1, K3 and K6 are without holes and K8, K10 and K13 are with holes.

All 3 conditions with holes outperform those without holes in terms of [uM] of reporter.

From the graph:
No holes: K6 > K1 > K3
With holes: K13 > K8 > K10

Recall that conditions K1 = K8, K3 = K10 and K6 = K13. Thus the order is the same, ie. The performance is the same with or without holes.

This suggests that the best overall conditions in terms of longevity AND output are K6 and K13, which contain the same combination of reagents:
base conditions with NADP+ and HMP

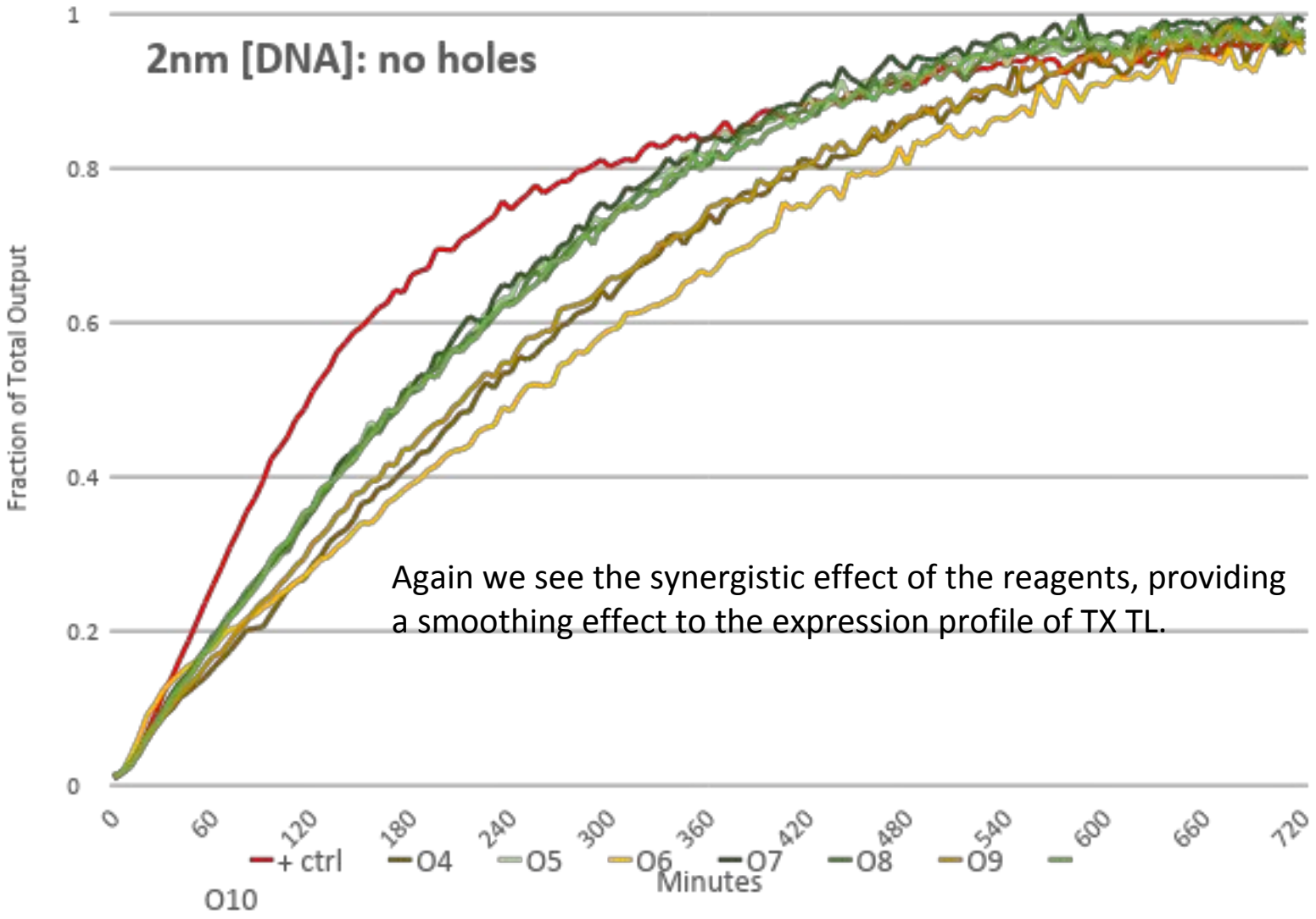
Base condition: 0.5nM DNA + 25mM mdx + 12.5mM mal + 2.5x $NaHCO_3$ + 1.25mM L-cys+ 2.5mM gluta

Quick summary…

- Previous slides detailed different combinations of reagents that were added to the TX TL reaction to try and enhance the performance, specifically longevity but also looked at rate of protein production and total output of the system.
    - N.B. Previous slides show data carried out at 0.5nM of [DNA].
- First showed how individual reagents had little effect – the controls
- Then showed how combining these reagents or conditions had synergistic effect
- Once initial 'base' conditions were optimized (data not shown), effects of HMP, NAD+, NADP+ and extra air were assayed. Extra air was introduced into the system by poking holes into membrane.
- Expressed data as a fraction of total to compare controls to conditions tested: No large difference in longevity of system.
- When looking at the rate of expression: System produced a much more consistent rate of protein expression with conditions tested. Time of close to linear protein production was increased.
- Addition of extra air via holes had little to no effect on the longevity or the rate of protein production of the system. Effects of holes were seen in the total output of the system. Extra air increased the total amount of protein produced.
- Data suggested that at 0.5nm [DNA], addition of NADP+ and HMP to the base conditions gave the best result in terms of total output and most consistent rate of protein production.

- The next few slides repeat the above but at a higher [DNA], instead of 0.5nM, we **will investigate 2.0nM [DNA].** Recall the system saturated at or above 5.0nM [DNA]

Results: Previous data was all at 0.5nm [DNA], next few slides deal with testing the same conditions but at 2nm [DNA]



+ control: 2.0nM of pvs001

Each of the following was added (final [ ])to the base condition:

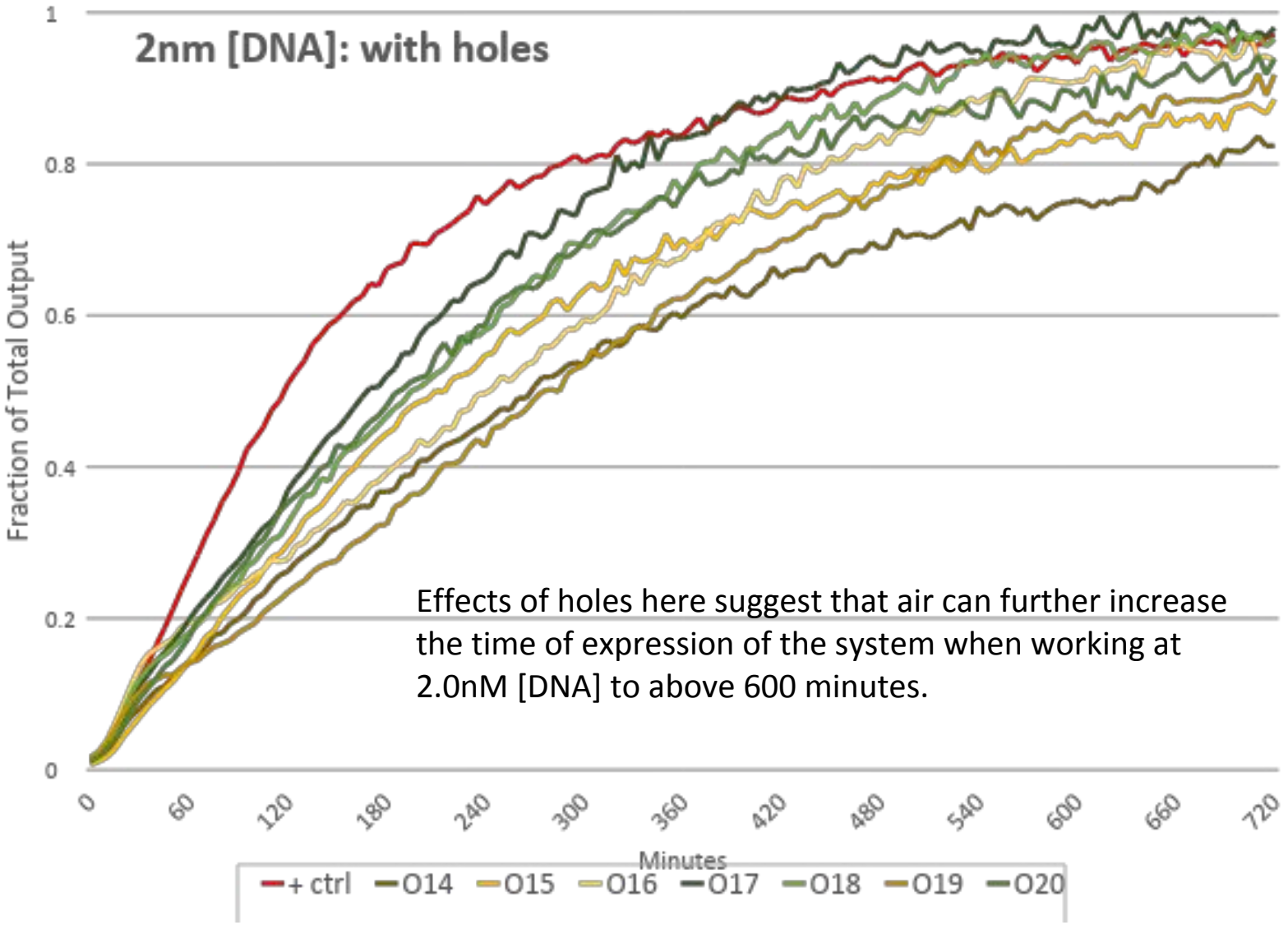
O4: 1mM NADP+
O5: 1.5mM NAD+
O6: 15mM HMP
O7: 1.5mM NAD+ + 1mM NADP+
O8: 1.5mM NAD+ + 15mM HMP
O9: 1mM NADP+ + 15mM HMP
O10: 1.5mM NAD+ + 1mM NADP+ + 15mM HMP

In terms of total [uM] of deGFP expressed:

+ control: 6.39
O4: 6.54
O5: 7.45
O6: 5.59
O7: 6.46
O8: 7.18
O9:7.68
O10:7.03

Base condition: 2.0nM DNA + 25mM mdx + 12.5mM mal + 2.5x $NaHCO_3$ + 1.25mM L-cys+ 2.5mM gluta

Results: Again this data is the same conditions as the previous slide, but with holes – at 2nM [DNA]



Effects of holes here suggest that air can further increase the time of expression of the system when working at 2.0nM [DNA] to above 600 minutes.

+ control: 2.0nM of pvs001

Each of the following was added (final [ ])to the base condition:

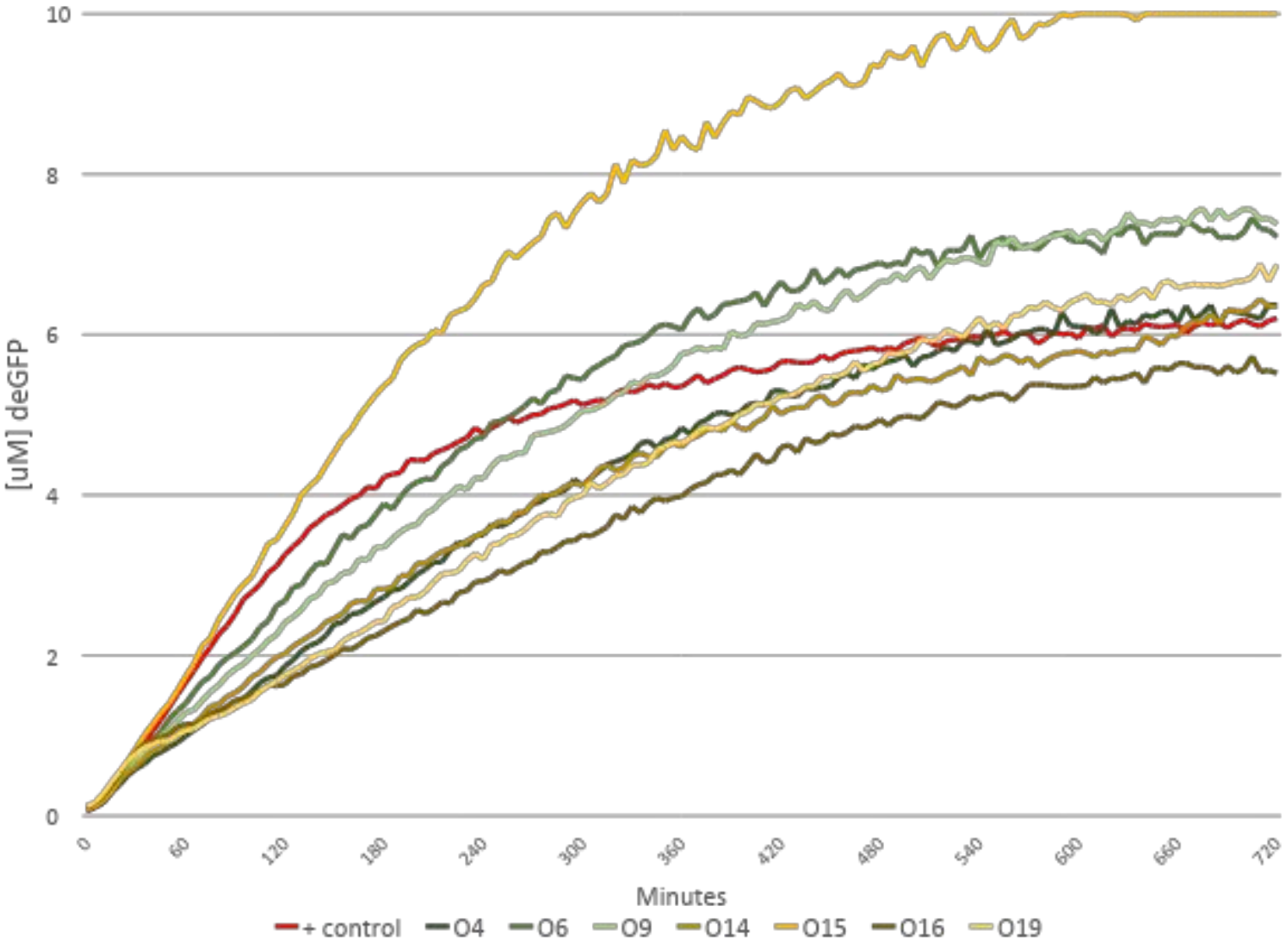
O14: 1mM NADP+
O15: 1.5mM NAD+
O16: 15mM HMP
O17: 1.5mM NAD+ + 1mM NADP+
O18: 1.5mM NAD+ + 15mM HMP
O19: 1mM NADP+ + 15mM HMP
O20: 1.5mM NAD+ + 1mM NADP+ + 15mM HMP

In terms of total [uM] of deGFP expressed:

+ control: 6.39
O14: 6.54
O15: 7.45
O16: 5.59
O17: 6.46
O18: 7.18
O19:7.68
O20:7.03

Base condition: 2.0nM DNA + 25mM mdx + 12.5mM mal + 2.5x $NaHCO_3$ + 1.25mM L-cys+ 2.5mM gluta

Results: Again, let's take the best results from the previous two slides, those conditions with HMP, NADP+ or both, with and without holes and compare total output.



+ control: 2.0nM of pvs001

At a higher [DNA], the effects of air are not as obvious. You actually see higher total output without air at 2.0nM [DNA] whereas at 0.5nM [DNA] you saw increased output. That said the curves are more linear with air, thus protein production is more consistent and close to 10h.

I believe O15 is an outlier. Although I have seen good results with NAD+ before, just not consistently.

Similarly as to the results at 0.5nm [DNA], as stated on the previous slide, the results seem to correlate, that adding HMP or NADP+ or both to the base conditions seem to give the best results.

This result here suggests that the protein output of the system is more difficult to 'juice' (ie improve) when working at higher [DNA].

Base condition: 2.0nM DNA + 25mM mdx + 12.5mM mal + 2.5x $NaHCO_3$ + 1.25mM L-cys+ 2.5mM gluta

What about the buffer? Is HEPES the best choice?

- pH has been shown to affect translation significantly (see background literature on benchling) and thus buffering of the TX TL reaction has been shown to be important. Different groups report different buffers to work best.

- The TX TL 'buffer' is made up of an amino acid solution and an energy solution. Because TX TL buffer is made up in bulk, to replace the buffer in the system requires to remake the buffer from scratch.

- The energy solution contains the buffer responsible for buffering the TX TL reaction, which in our case is HEPES pH 8 at a final concentration of 50 mM.

- There is some literature that reports that Bis-Tris is superior to HEPES (not conclusive and not agreed by all of the literature)

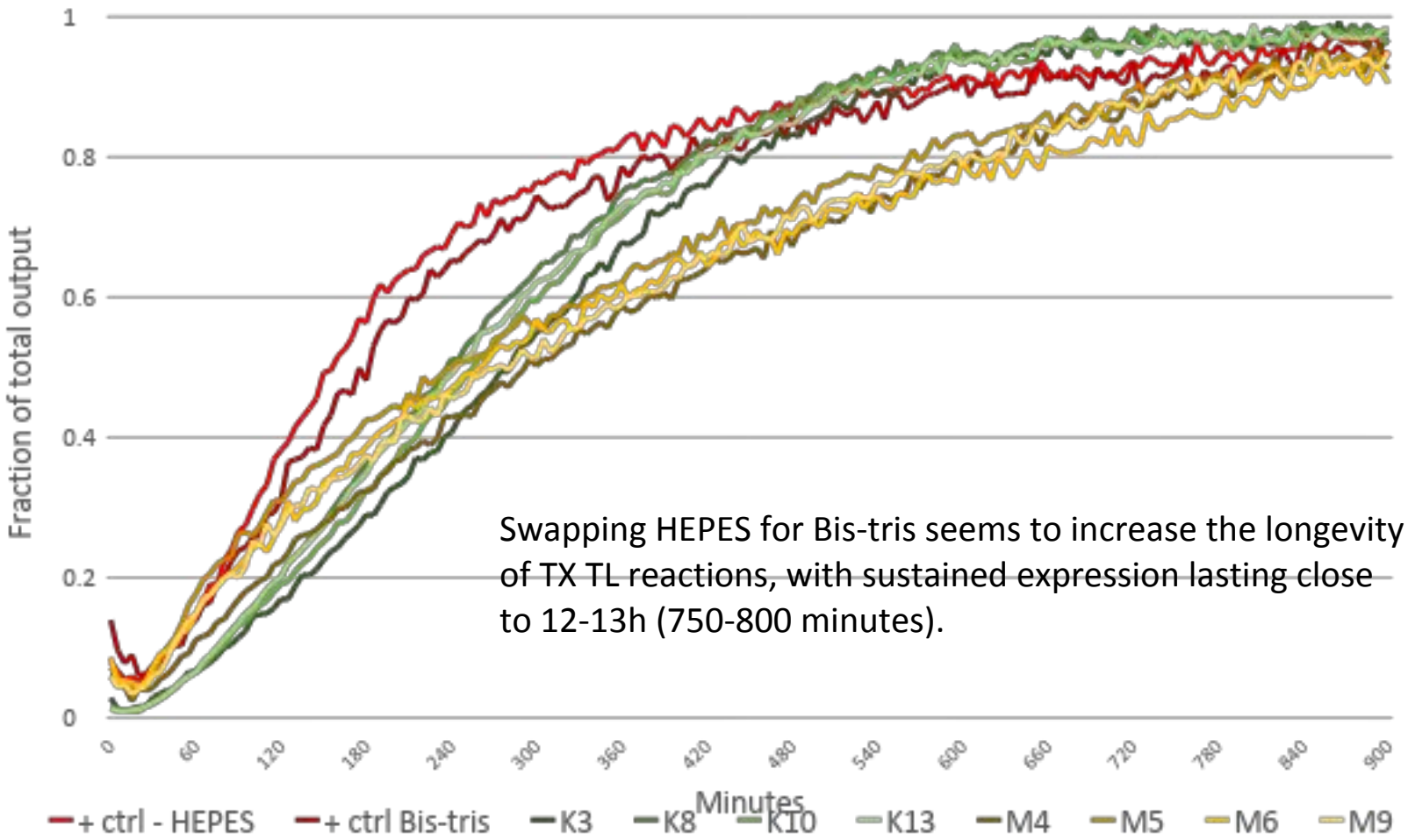
So we wanted to test if the type of buffer had an effect. Is HEPES superior to Bis-Tris?

- The following slide compares the best results obtained with HEPES (from above slides) and plots them against the best results obtained with Bis-Tris buffer to crudely assess the effects on longevity of TX TL, our main concern.
  - The tests using Bis-Tris as the buffer instead of HEPES was only completed at 0.5nM [DNA] and so I have no results for higher DNA concentrations.

- Rates of protein production between TX TL run with HEPES vs. Bis-Tris are then shown (slide 18)

- The slide after then compares the total output of the system in [uM] deGFP between all conditions to see if there is a pattern (slide 19).

Swapping buffer results: Remade the TX TL buffer with Bis-Tris instead of HEPES and ran the same conditions as shown on previous slides. Best results from holes or no holes are summarized below and compared to the best results obtained with HEPES buffer. Buffer [ ] 50mM for all.



Swapping HEPES for Bis-tris seems to increase the longevity of TX TL reactions, with sustained expression lasting close to 12-13h (750-800 minutes).

+ ctrl – HEPES 50mM: 0.5nM pvs001

K3: 15mM HMP
K8: 1.5mM NADP+
K10: 15mM HMP
K13: 1.5mM NADP+ + 15mM HMP
No holes = K3
Holes = K8, K10, K13

+ ctrl – Bis-Tris 50mM: 0.5nM pvs001

M4: 1.5mM NAD+ + 1.5mM NADP+
M5: 1.5mM NAD+ + 15mM HMP
M6: 1.5mM NADP+ + 15mM HMP
M9: 1.5mM NAD+
No holes = M4, M5, M6
Holes = M9

Interestingly, when using Bis-Tris, having holes is less important and the combination of secondary reagents (NAD+, NADP+ and HMP) that produce the best results differs slightly. This is why different combinations of these reagents was always assayed. I am unsure exactly why the different combinations produce different results.

While HEPES provides a more linear time of protein production, the longevity of TX TL can be extended by using Bis-Tris.

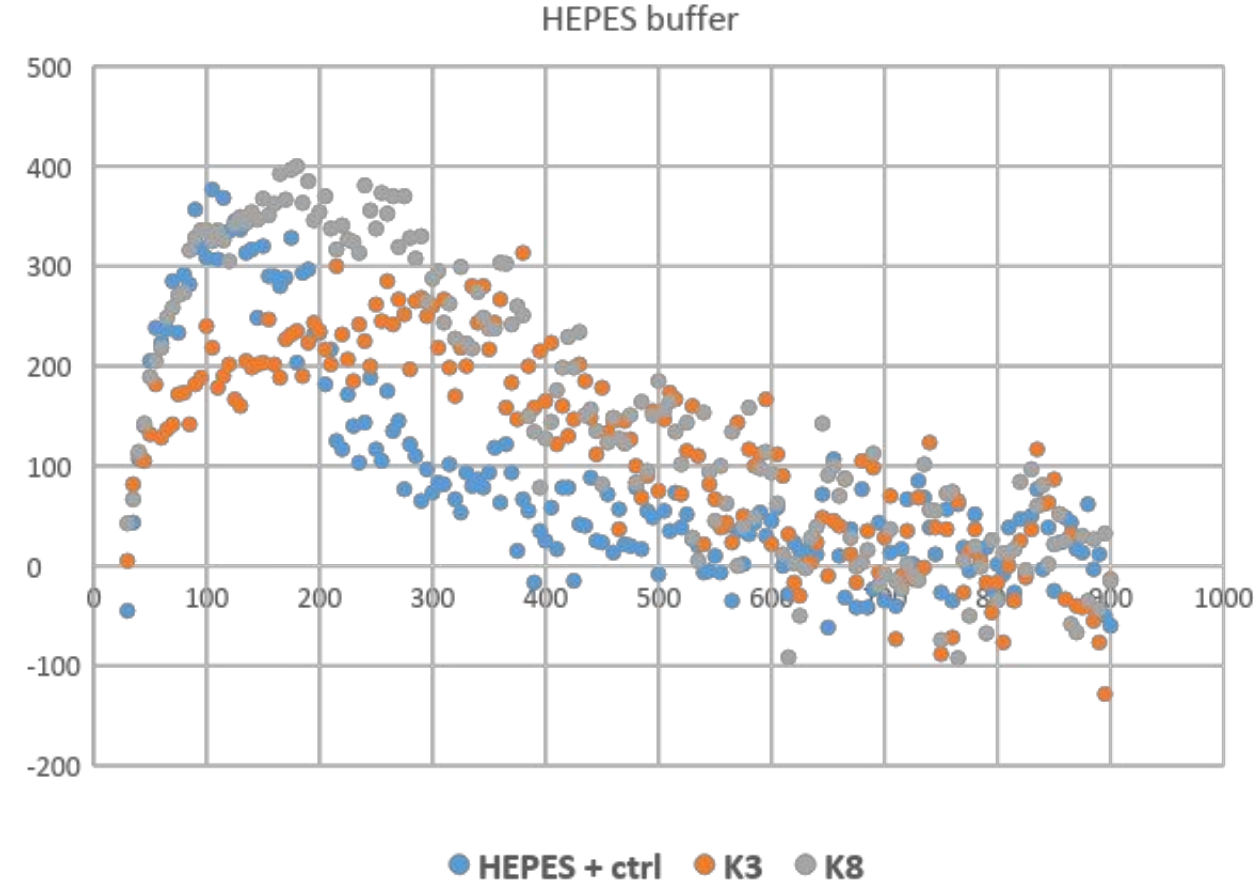
90% of total output is reached at approximately:

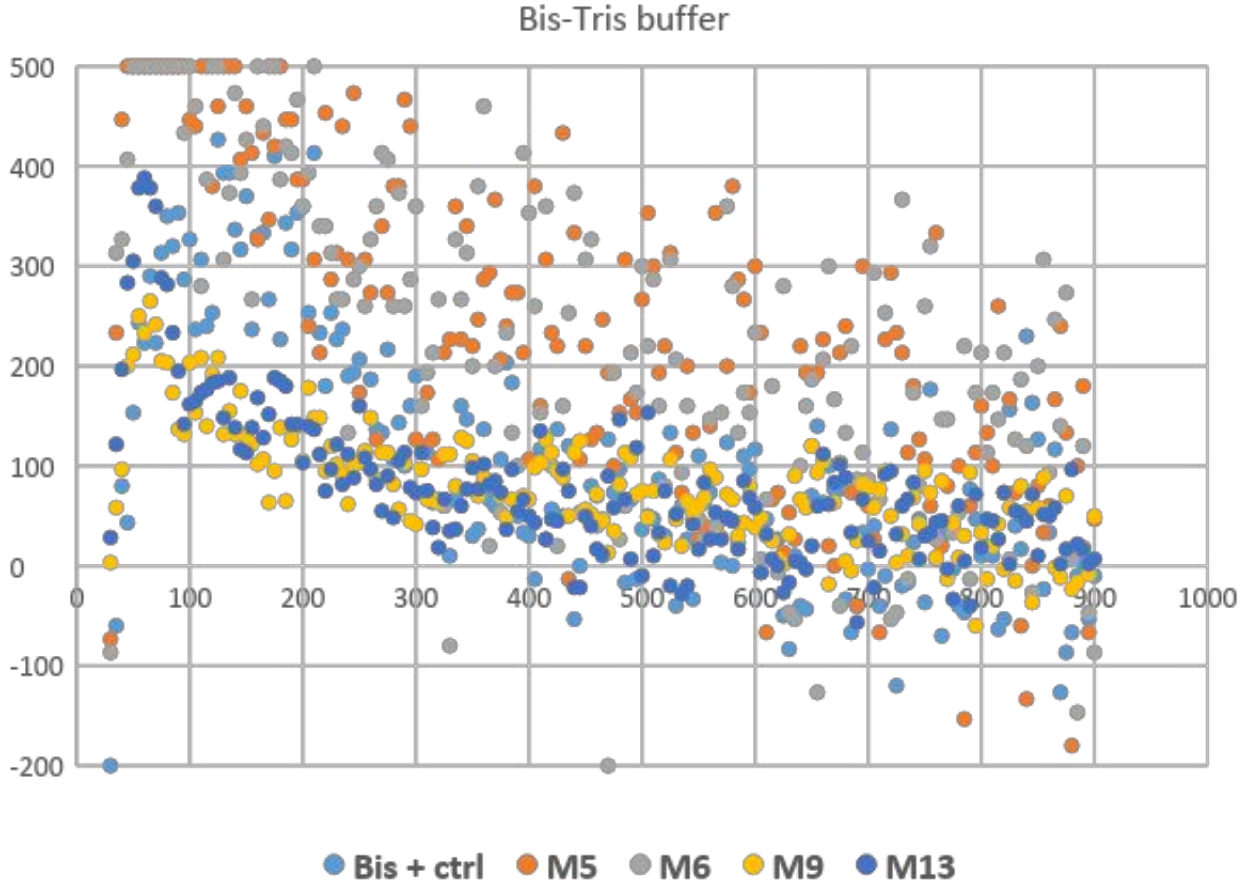
Controls: 500 minutes; HEPES: 500 minutes albeit with more linear expression; Bis-Tris: 750 minutes, less linear expression

Results: comparing the effects of the buffer on the rates of expression of the system, changes in fluorescence (RFUs) are expressed as a function of time, over the duration of the entire run and then scaled for easy comparison.

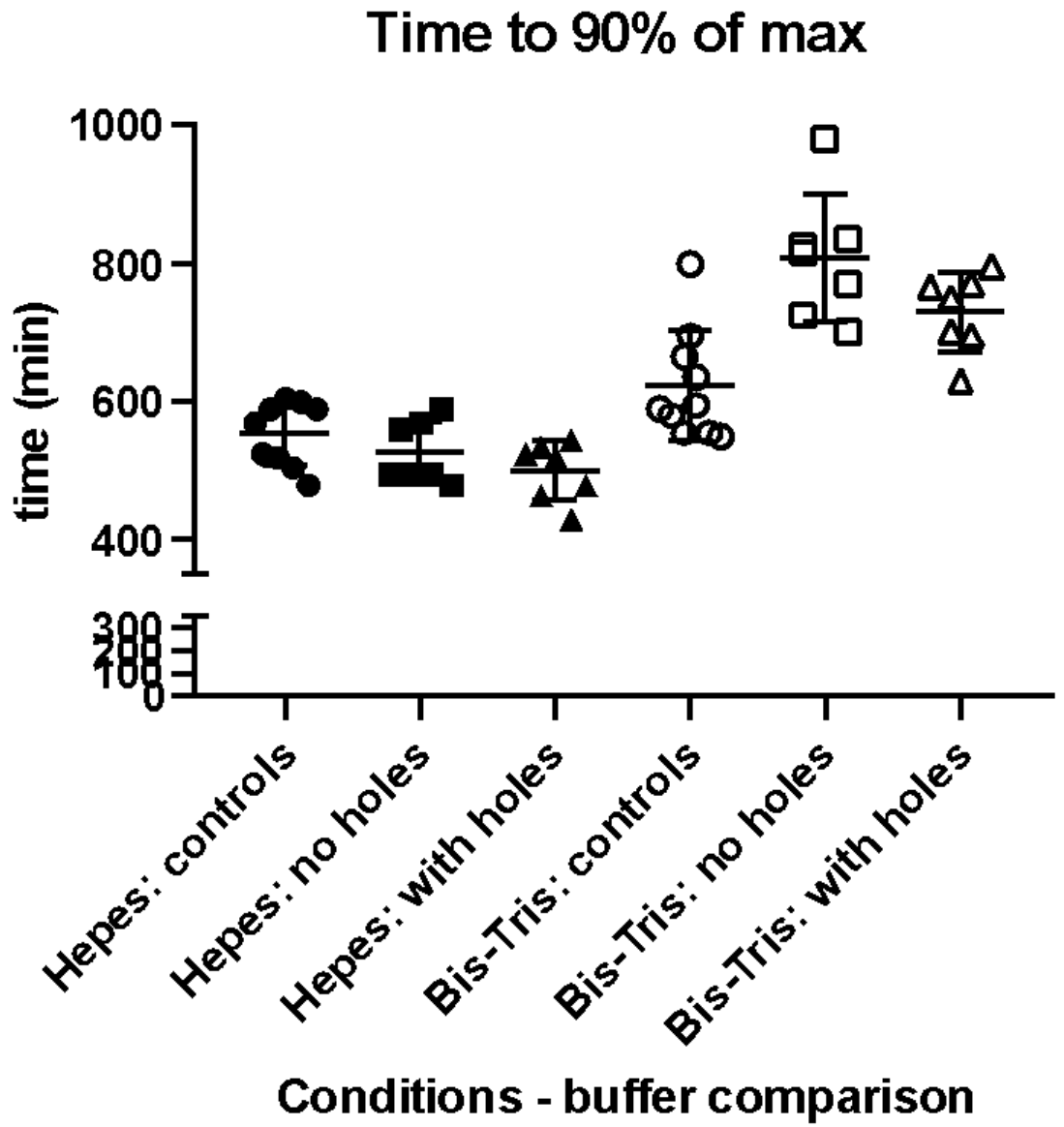
Rates of protein expression using HEPES and Bis-Tris are compared below (best results). While the conditions tested led to a more constant rate of protein production (as seen on slide 9), switching HEPES for Bis-tris helped prolong TX TL further. While using Bis-tris does not prevent TX TL from dying, higher rates of protein production are seen later in the run (>600 minutes), suggesting that Bis-tris is a superior buffer to HEPES in terms of longevity.

Note that the x axis extends to 900 minutes, where the previous graphs comparing rates only showed to 600 minutes.





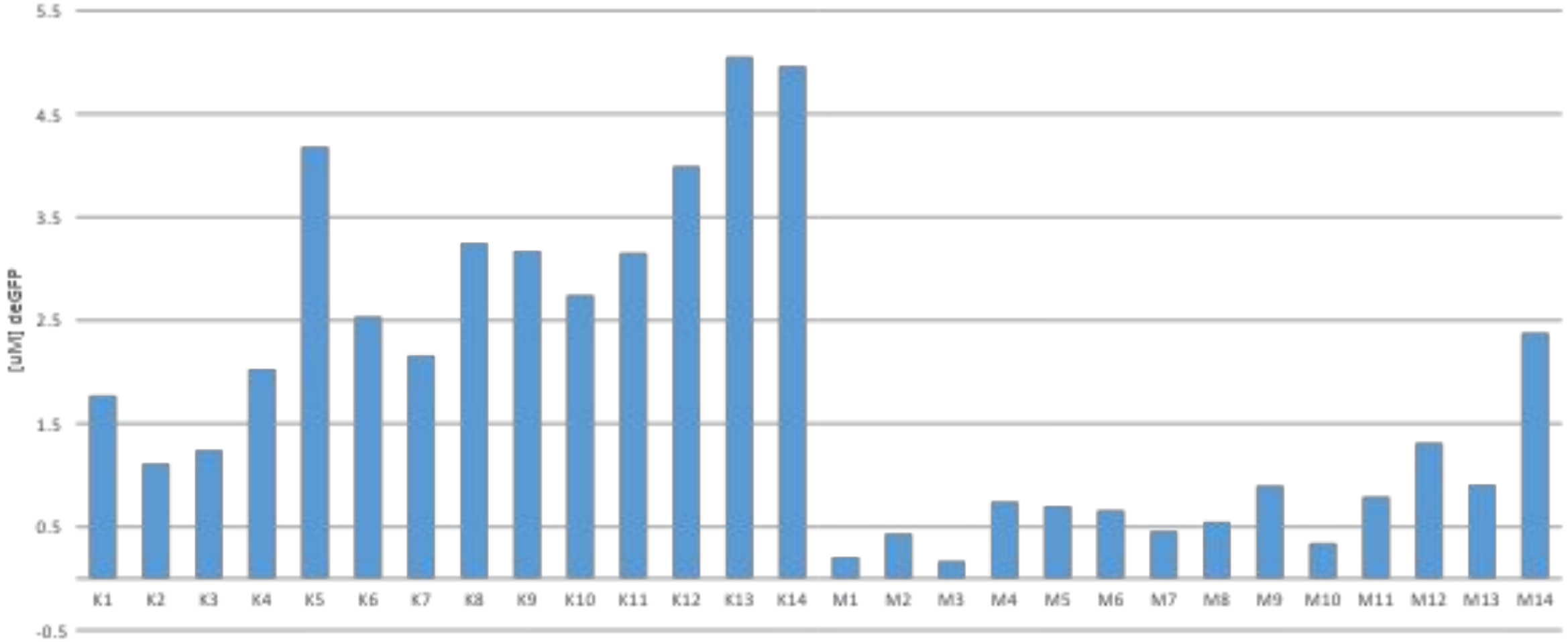
Comparing longevity: Previous slide showed the deGFP expression kinetics curve for comparing the different base buffers, HEPES vs Bis-Tris with the different reaction conditions tested, and then with and without holes. Data here is based on the previous slide, except only plotting the time it takes to get to 90% of max.



Data suggests that Bis-tris supports TX TL reaction longevity better than HEPES.

Next slide compares total output of the System in terms of [uM] deGFP.

Comparing total system output in [uM] of deGFP between using HEPES or Bis-Tris as the buffer in TX TL



Conditions K1 – K14 = HEPES Buffer

Conditions M1 – M14 = Bis-Tris Buffer

While you could make the case that using Bis-tris provides a better expression profile, perhaps extending the reaction from 500 to 750 minutes before reaching 90% of the total output, the absolute amount of protein production is reduced. Which to use then? I would leave that to the user, if you need higher amount of proteins stick with HEPES. If you want longer time of expression then Bis-Tris may be better. If sensitivity is a concern, since Bis-Tris produces lower amounts of protein, you could use gain 100 instead of gain 60 to increase your range. All of this data was done using gain 60.

Other things tested but now shown…

- The amount of primary energy source, 3-PGA, is 30mM in a standard TX TL reaction, see JoVe paper. We also tested increasing this to 40mM but performance was decreased. Lower concentration (10mM and 20mM) were also tested and in combination with other carbon sources, but again, performance was decreased.

- Different concentrations of Bis-tris buffer were tested. So although data for 50mM were shown, this was to compare it directly to 50mM of HEPES that is found in our standard TX TL reaction. Lower and higher amounts of buffer were also tested but found to either have no effect or decrease performance, 50mM seems to be a sweet spot.

- As mentioned in the first few slides, shaking the plate over the course of the reaction (20h), is standard as described in Vincent's papers. However when tested this had no effect on the performance of the system. This was tested at different DNA concentration and with other reagents and with and without holes.

- The best carbon source is not a clear cut answer as different results were obtained at different DNA concentrations. For example, at higher concentrations, combining glucose and maltodextrin was found to be slightly better than combining maltose and maltodextrin, while at lower concentrations the opposite was true. I imagine that different carbon sources produce different ratios of co-factors when metabolised, stressing the energetics of the system differently, which in turn when combined with the different transcription and translational demands of the system at different DNA concentrations, ultimately results in slightly different expression levels of reporter. This is however speculation.

Recommendations for future users

- The use of HEPES as the buffer component seems to work well so I would keep as is, unless longer expression times are very useful to the user, in which case it may be worth while to switch to Bis-Tris.
- If making a large batch of buffer, incorporating the 'base' conditions reagents such as maltodextrin, glucose etc., glutamine, L-cysteine, NAD+ or NADP+ and HMP at the time of making would make preparation easier on the day and reactions more consistent between days.
    - Baking soda (sodium bicarbonate) is difficult to incorporate when making up batches as it is difficult to measure and pipette. Probably easiest to add it on the day of use with the echo. See benchling material and methods to see how I calculated/pipetted 2.5x $NaHCO_3$ into reactions.

- Working at lower concentration of DNA seems to stress the system less in the first few hours and allows for more manipulation of the reaction. Even though your total output in uM may be lower at lower DNA concentrations, the range can be expanded by working at a higher gain. You also save DNA material.

- **Attempting to further increase the longevity of TX TL by manipulating the reaction conditions will probably be a fruitless endeavour. Better results will probably be attained by manipulating the biochemical /metabolic pathways that are active during TX TL. This will require genome engineering:
    - Removal of pta and ackA genes and thus abolish production of acetate (which drives pH acidification)
    - Removal of ldh genes (there are 2 or 3 in E. coli) and thus abolish lactate production, another cause of pH acidification.
    - One could also introduce acid consuming pathways (see ABE fermentation) and turn them into solvents, thus alleviating pH stress.

- Another method would be to incorporate the synthetic biochemistry modules from the Bowie lab.
    - Addition of NoxE to recycle NADH (could potentially work or inhibit energetics of system)
    - Addition of GapM6 would allow for continuous reduction of NADP+ to NADPH.
    - Addition of PHB pathway enzymes to allow for a carbon sink as PHB is a polymer that falls out of solution, creating a 'pulling' force.

The final slide is speculation on my part as to what is causing the 'death' of TX TL – the cessation of protein production.