**IZ Extraction**

*Modified from Jardon et al. Euk. Cell 2008*

Dilute saturated culture back 1:100 and in 10ml (2x5ml) and grow to OD 0.6-0.8.

Spin cells out of media at 4°C 5 min at 1,500 rpm.

Remove media, resuspend in 1ml H2O and move to 1.5ml tube. Spin out of water at 4°C 5 min at 1,500 rpm.

Wash 2x with 1ml 20mM IZ buffer. Pellet with same conditions as above.

Resuspend in 500ul IZ buffer. Add full scoop of glass beads.

Vortex at max speed 1 minute. Incubate on ice 1 minute. Repeat total of 5 times.

Centrifuge homogenate at 4°C 15 minutes at 13,000 rpm.

Pull off supernatant. Use immediately or store at -80°C.

**Reagents**

IZ Buffer

*20mM imidazole*

*HCl to pH 7*

**KCl extraction**

*Modified from Zelle et al. AEM 2008*

Dilute saturated culture back 1:100 and in 10ml (2x5ml) and grow to OD 0.6-0.8.

Spin cells out of media at 4°C 5 min at 1,500 rpm.

Remove media, resuspend in 1ml H2O and move to 1.5ml tube. Spin out of water at 4°C 5 min at 1,500 rpm.

Wash 2x with 1ml 20mM KHPO buffer. Pellet with same conditions as above.

Resuspend in 500ul KHPO buffer. Add a full scoop of beads.

Vortex at max speed 1 minute. Incubate on ice 1 minute. Repeat total of 5 times.

Centrifuge homogenate at 4°C 15 minutes at 13,000 rpm.

Pull off supernatant. Use immediately or store at -80°C.

**Reagents**

KHPO buffer

*100mM potassium phosphate*

*2mM MgCl2*

*1mM dithiothreitol*

*HCl to pH 7.5*

**FPBase assay**

Start with cell pellets equivalent to at least 2ml saturated culture.

Extract total protein in IZ buffer.

Transfer 1 ml of pure extract into a fresh tube. Keep on ice.

Mix enzyme reactions in 50 mM imidazole pH 7. (Include a set of negative controls).

Mastermix = n =samples x 2 x replicates (1 negative control for each sample)

**1 ml reaction volume mastermix**

5 *M* KCl | 20 ul

0.5 *M* MgCl2 | 20 ul

0.5 *M* EDTA | 2 ul

120 *mM* NADP | 2 ul

PGI Z | 0.5 ul

G6PDH Z | 1 ul

50 mM imidazole | 855 ul

**100 ul reaction volume mastermix**

5 *M* KCl | 2 ul

0.5 *M* MgCl2 | 2 ul

0.5 *M* EDTA | 0.2 ul

120 *mM* NADP | 0.2 ul

PGI Z | 0.05 ul

G6PDH Z | 0.1 ul

50 mM imidazole | 85.5 ul

**For 1ml RXN’s**

Distribute 900 ul MM to 1.5ml tubes on ice. Add 100 ul IZ-extracted supernatant for each sample to respective tubes (include negative control).

Still on ice - add 4 ul of FDP (25*mM*) to all positive rxns. Do **not** add FDP to negative controls.

Distribute 200 ul of RXN to 4 UV-compatible 96well plate per reaction to generate 4 technical replicates.

**For 100ul RXN’s**

On ice - distribute 90 ul of RXN to wells of UV compatible 96 well plate.

Still on ice - add 0.4 ul of FDP (25*mM*) to all positive rxns. Do **not** add FDP to negative controls.

**For all**

Start timer.

Spec all samples at 340 nm (NADPH).

Incubate at appropriate temperature and spec all samples at 5 minute intervals.

**Reagents**

5 *M* KCl 0.5 *M* MgCl2

*7.5 g KCl in 20 ml H2O 952 mg in 20 ml H2O*

120 *mM* NADP 0.5 U/μl G6PDH Z (glucose 6-phosphate dehydrogenase)

*100 mg/ml H2O .5 mg/ml in* 5 mM NaCit buffer pH 7.4

*25.8 mg sodium citrate*

1.0 U/μl PGI (phosphoglucose isomerase) *20 ml H2O*

*100 mg/ml H2O*

**Beer’s Law and pathlength correction to find units ezyme**

(1)

*ε* = NADPH extinction coefficient = 6220 M-1 / cm-1

*d* = pathlength (see below)

*C* = concentration = mol/L

Equation 1 requires a pathlength. In order to use a standard 1cm pathlength, measurements taken in a plate reader need to be corrected for pathlength. Below is the pathlength correction for 100ul reaction volumes in 50mM IZ in a flat-bottom UV compatible Corning 96-well plate.

K-Factor = Abs(cuvette)975nm - Abs(cuvette)900

K-Factor (50 mM IZ) = 0.173 - 0.002

K-Factor (50 mM IZ) = 0.171

corrected Abs = Abs(original) \* (K-Factor/(Abs(well)975nm - Abs(well)900nm))

corrected Abs = Abs(original) \* (K-Factor/(0.0825 - 0.031))

corrected Abs = Abs(original) \* (K-Factor/0.0515)

corrected Abs = Abs(original) \* (0.171/0.0515)

corrected Abs = Abs(original) \* 3.32

After correcting pathlength to a standard 1cm pathlength, I can assign ***d* = 1cm.**

Therefore, to find concentration substrate in mols/L, I can use equation 2.

(2)

I then correct the concentrations to umols/100ul by multiplying by 100 (multiplying by 106 to convert umols and then dividing by 104 to convert L to ul).

I then use the change in absorbance over time (selecting an optimal time window) to find Units Enzyme as follows:

1 Unit = 1umol substrate per 1 minute

(3)