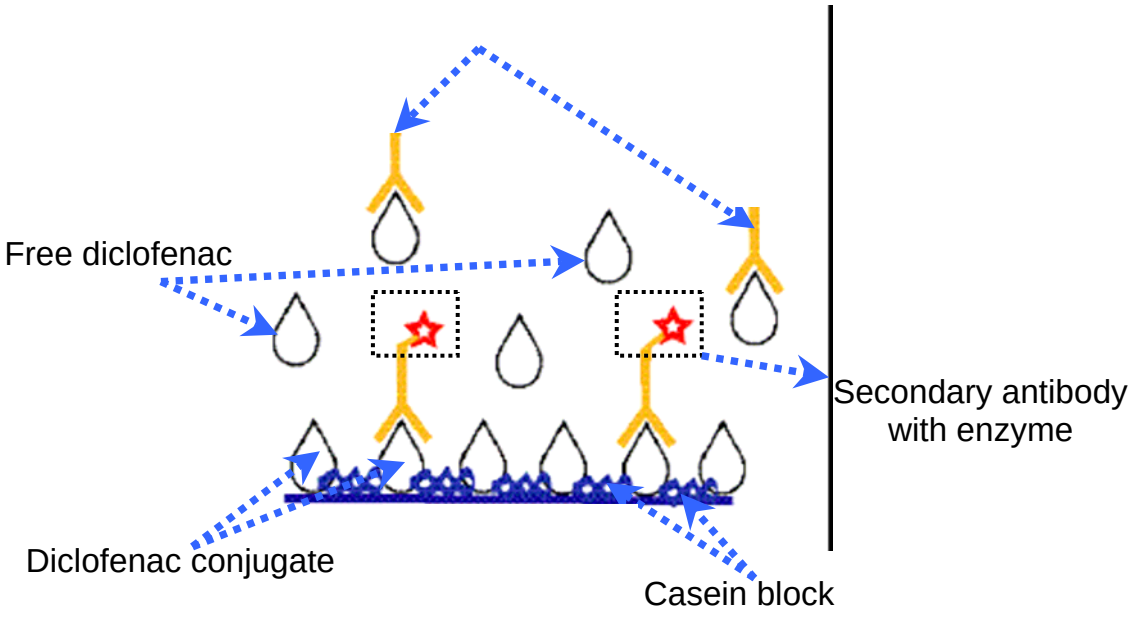
# PROCEDURES FOR EXTRACTING AND ANALYSING TISSUE SAMPLES FOR DICLOFENAC USING ELISA.




**Dr Mark Taggart, Dr Mohini Saini, Prof. Dietmar Knopp**

## ***EXTRACTION:***

Before analysing our tissue sample for diclofenac we must first extract the drug from the tissue. In this case we will use a simple solvent-based extraction technique. At all stages, please bear in mind we are working at the part per billion detection level and as such it is extremely easy to cause cross contamination of samples. We must take every possible step to avoid this or we could have false positives within our data.

1. Take the tissue you wish to extract. Commonly this will be liver tissue provided by Dr Kalu Ram Senacha from field collected livestock carcasses. However this extraction has and can be applied to any other tissue you wish. Dessicated tissue or fibrous/fatty tissue is not particularly suitable, and if you wish to use plasma (from blood samples), simply replace the homogenisation step with mixing using a vortex mixer.

2. Take 0.5g (0.45-0.55g) of tissue and weigh this accurately (ideally to 4 places using a well shielded balance) into a glass test tube. This test tube can only be used once for this purpose and is unlikely to be useful for other work as it will be very difficult to get completely clean after the extraction is complete. I would advise using a fairly thick walled test tube approximately 75mm long. Take the test tube, tare this on the balance, add the tissue and record the weight. Also record the field sample code (GJ-RJ-69, for example) and give the test tube/extract another sequential code (i.e, IVRI-2008-1 or similar). Using Excel, generate a set of sample sheets as such:

| Test tube No. | Sample Code | Tissue weight | Lab Code | Comments, if any |
|---|---|---|---|---|
| 1 | GJ-RJ-69 | 0.455 | IVRI-2008-1 | |
| 2 | GJ-RJ-86 | 0.523 | IVRI-2008-2 | |
| 3 | GJ-RJ-102 | 0.550 | IVRI-2008-3 | |

Print the sample sheets and record the above information in writing as you extract. This hard record should be kept for the long term but the data recorded should also be kept in Excel. It is important that we record all of this information and that we track the order in which the samples were extracted so that we can check for cross contamination effects in the laboratory (which will happen). Tissue should be cut from the bulk sample using a clean scalpel, transferred to the test tube using clean forceps, and I would advise using a stone/granite cutting block which is much easier to keep clean than a wood or plastic cutting board. Before transferring the 0.5g of tissue to the test tube, cut it up using the scalpel to aid in the homogenisation. In between cutting each tissue sample, clean down very thoroughly. Use tissue and acetone to clean the scalpel, the tweezers and the cutting block. Work in batches of 8-16 depending on how many tubes your centrifuge will take.

3. With 8-16 test tubes filled with sample tissue, move to a fume cupboard or a well ventilated location. Then to each test tube, add 2ml of HPLC grade acetonitrile to each tube. Take the IKA homogeniser and blend sample 1. This should take around 30-45

seconds to fully blend. Work at the highest speed of the homogeniser. If the tissue is cut into small pieces at the weighing stage, 30-45 seconds of homogenising should be enough to fully blend the sample. Having blended the sample please take the time to fully strip down the homogeniser and give it a very good clean with tissue and acetone. Take care not to lose the 2 white plastic washers located inside the homogeniser as these are difficult to replace, and in one case perhaps impossible. Having re-assembled the instrument use three 250ml baths of acetone to wash the homogeniser cutting-tool whilst it is running. Use 500ml jars with lids, fill each with 250ml of acetone and keep the lids on loosely between each wash to minimise evaporation. Maintain an order to the three baths so that you wash in bath 1 first, then in 2, then in 3. When bath 1 seems quite dirty (after 30 extracts maybe), make bath 2 into 1, bath 3 into 2, and create a new, clean, bath 3. This way we should minimise cross contamination on extraction which is our biggest source of laboratory based cross contamination. If done carefully, these procedures can, and have, almost eliminated carry over of diclofenac from one extract to the next.

4. Having homogenised all the 8-16 extracts, move to the centrifuge. These extracts need only be centrifuged gently (1-2000rpm) for 5 minutes. Just to create a plug of solid at the bottom of the tube.

5. Next, take two batches of 8-16 2ml LC screw top glass vials. Also take 2 plastic vial boxes. Label one vial box as "Concentrated extract for LCMS", and a second box as "1:100 dilutions for ELISA". Take the vials and label both sets with the respective laboratory code for the extract (i.e, IVRI-2008-1, or, just 1, since space is limited for writing on these vials). As long as we store the vials in sequence (as recorded on the data sheets) in the vial boxes, there should be no way that they can get mixed up or out of sequence, even if the labels fade or are removed by acetonitrile by spillage on a few vials.

6. Take a disposable 5ml syringe and a 0.45micron disposable filter unit. Fit together and place the unit above the first LC vial. Pour in the supernatant from test tube 1 and syringe filter the extract directly into the vial. These vials take 2ml and since we have added 2ml of solvent to 0.5g of wet tissue, we may have over 2ml of free liquid in the test tube. Fill the LC vial to just below the neck (do not fill to the very top) and discard the remaining supernatant with the syringe unit. Take 10µL from the LCMS stock solution and add this to the second LC vial for 1:100 dilution and ELISA analysis, making the 10µL up to a final volume of 1ml with 990µL of water. Seal both the LC vials tightly with a screw top lid. Make sure the lids are tight to prevent both evaporation and leakage of the LC vials during future airfreight.

**NOTE:**

The "timing" of when to make up the 1:100 dilutions depends on how you decide to manage the analysis. The stock LCMS solutions are stable in the short term in the fridge but should be stored in the long term in the freezer. If ELISA analysis is to occur within days of the extraction, make up the 1:100 dilutions at the extraction stage and store these in the fridge until analysis. Alternatively if the ELISA analysis is not scheduled to occur for several weeks or months, make up the LCMS extracts, store these in the freezer, then

make up dilutions the day before ELISA analysis. The LCMS extracts will probably freeze only partially (i.e, the water component from the tissue will freeze). Hence, prior to making up the 1:100 dilutions, remove the LCMS stock from the freezer, bring them up to room temperature, invert the vial box several times to mix the extract (there may well be some protein precipitation), then allow them to settle in the fridge for 24 hours. After this, make up the 1:100 dilutions as required.

**So, to summarise:**

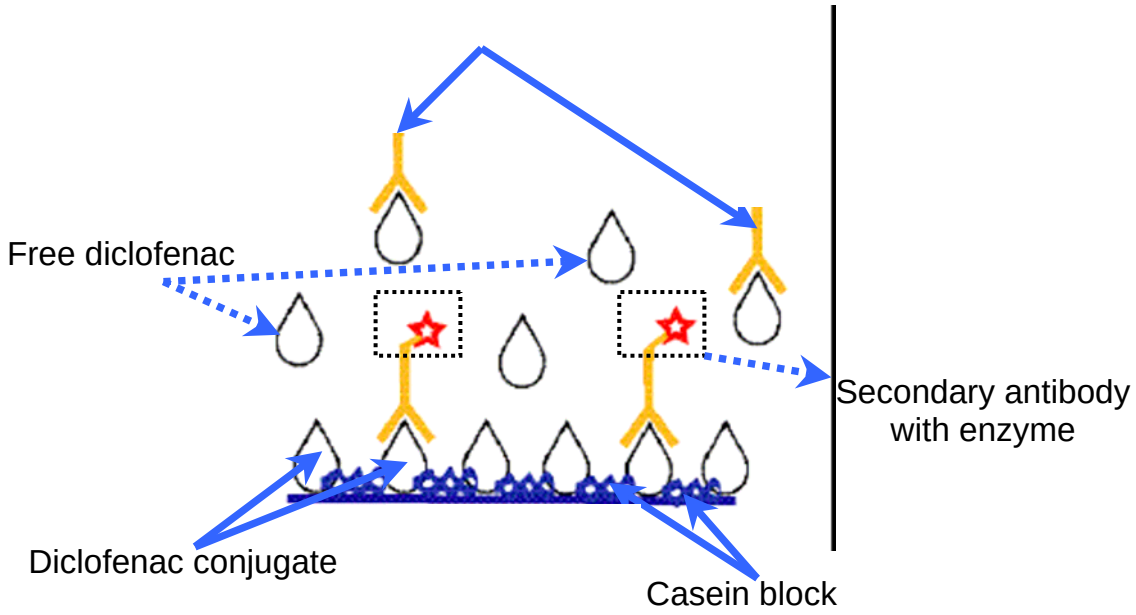
1. **Add 0.5g of well cut up tissue to a new clean test tube and record the tissue weight.**
2. **Add 2ml of HPLC grade acetonitrile to each test tube.**
3. **Homogenise the mixture.**
4. **Centrifuge the mixture.**
5. **Filter the mixture into a screw top LC vial.**
6. **Store the primary extracts in the freezer.**

**AT ALL STAGES, CLEAN DOWN THE CUTTING AND HOMOGENISATION EQUIPMENT THOROUGHLY BETWEEN SAMPLES.**

***ELISA ANALYSIS:***

In order to analyse the diclofenac in our 1:100 diluted extracts we will use an ELISA (enzyme linked immuno-sorbant assay). In this case we will use an indirect competitive ELISA where we do not measure the diclofenac directly but instead the competition between free diclofenac and plate bound diclofenac for free antibodies to diclofenac.

**THE PRINCIPLE:**




First of all, let us remind ourselves of the principal behind this test using the above diagram (NOTE: there is a second "version" of this diagram at the end of the manual). Firstly, we use plastic plates with 96 wells that each hold 300µl of fluid. The above diagram shows what is happening on the surface plastic of each well. The plastic is designed to have a high tendency to bind organic compounds. I will go through the specific laboratory steps in detail below, but first, the principal. The procedure adds various reagents to the plate in sequence which then bind or react with compounds that are bound to the plate. In between each step we wash unbound compounds out of the well before adding something else. Our first step (**COATING STEP**) is to add a diclofenac-thyroglobulin conjugate (diclofenac bound to a protein) to the plate. This compound "sticks" to the plate creating a surface layer with exposed surfaces of diclofenac. However, not all binding sites on the plate are used up, many potential binding sites remain and these must be "blocked". Hence, secondly, we add a solution of casein (a phosphoprotein from milk). This acts to "block" all the other sites on the plate that do not have the diclofenac conjugate attached (**BLOCKING STEP**). Thirdly we add free diclofenac either in the form of a standard with a known concentration, or in the form of a sample containing an unknown amount of diclofenac. Immediately after this

(without having washed the plate), we add the free antiserum (containing the diclofenac antibody) to diclofenac that has been synthesised using rabbits. This antibody is obviously highly prone to bind to diclofenac. In the plate it can therefore bind to the diclofenac coated on the plate OR to that which is in solution (standard or sample). Obviously if there is a lot of diclofenac in solution (a high standard or sample with high diclofenac) then the antibody binds to this and remains free in the liquid/is not bound to the plate. If, alternatively, there is no diclofenac free in the liquid (as is the case for a blank standard or sample containing no diclofenac), all the antibody will bind to the plate (**COMPETITION STEP**). We then wash the plate free of liquid again leaving us with a lot of primary antibody bound to the plate in our wells to which we added NO diclofenac, and proportionally less antibody bound to the plate in our wells where diclofenac was added (i.e, for standards containing diclofenac). Finally we add a commercially available secondary antibody which ONLY binds to the sites where there is already primary diclofenac antibody on the plate (**LABELLING STEP**). This secondary antibody also has an enzyme (horseradish peroxidase, HRPO) attached to it. We then add a final solution (called TMB substrate) which is converted by the the action of the enzyme (as it oxidises the TMB) into a soluble blue coloured pigment (**DEVELOPING STEP**). Again, on plates where no free diclofenac was added, there will be a lot of primary antibody, and therefore a lot of secondary and the enzyme, hence this reaction will occur to a greater extent/more rapidly. As the reaction proceeds the liquid in the well turns blue. The intensity of that blue gives a measure of how much this reaction has taken place and therefore a measure of how much diclofenac we added as a standard or unknown sample. The enzymes (no matter how many there are) will continue to "eat" the TMB as long as there is TMB in solution, hence, if we leave the plate indefinitely we will simply have a strong blue colour in all wells. Hence, we finally add acid to the wells to stop the reaction progressing when we can visibly see good blue colour difference in the well, i.e, intense blue in wells where no diclofenac was added, much lighter blue where we added a lot. When we stop the reaction (**STOPPING STEP**) with acid the well colour changes to yellow. We can then measure the intensity of this colour, and therefore the diclofenac level, using an ELISA plate reader which calculates the absorbance of light as it passes through the well at 450nm.

So, in detail, the **PROCEDURE:**

1. Make up a stock of COATING BUFFER. This solution should be stored in dark glass at room temperature. We will make up 2 litres, enough for 80 plates. Ingredients:

3.18 g sodium carbonate
5.86 g sodium hydrogencarbonate
0.4 g sodium azide (CARE – TOXIC!)
2 litres of HPLC grade/milli-Q water

2. Take two 96 well flat-bottomed high binding ELISA plates. We must then coat the plate with DILUTED diclofenac coating conjugate. This compound has been provided

by a laboratory in Germany specifically for this purpose. Stocks of this compound are stored in the freezer and should always be kept frozen (FURTHER DRY STOCK WILL BE SENT FROM GERMANY WHEN SYNTHESISED). One 50µl aliquot can be removed from the freezer and used to do the test but this should be kept in the fridge at all times. Do not return this working aliquot to the freezer, once it is defrosted, it should remain so, and be used. Using a 10µl pipette and sterile tips, take 10µl of the diclofenac conjugate stock and add this to 50ml of COATING BUFFER. This is enough for 2 plates (I would advise that we do no more than 2 plates per day). Add just 200µl of this solution (using a multi-channel pipette) to each of the 96 wells. Cover the plates with plate sealing film and store them in the fridge overnight to coat.

3. Use the premixed PBS (phosphate buffered saline) powder (in foil packs) to make up a 10x concentrate PBS solution in 1 litre of HPLC grade/milli-Q water. Dilute 100ml of this in 900ml of HPLC grade/milli-Q water to make up a final working PBS solution (our PBS BUFFER).

4. Make up a solution of 1% w/v casein. Add 1g of casein powder to 100ml of PBS BUFFER. Make this up in glass since the solution will need to be heated and stirred to make it dissolve. After the casein is dissolved, cool the solution down to room temperature in the fridge (for about 15 minutes) or using running cold water to cool the outside of the glass and therefore the liquid.

5. Remove the plates from the fridge, remove the film, and wash the plate 3 times using an automatic plate washer. To wash the plates, use a solution made up from the PBS with Tween 20 tablets provided. Two of these tablets should be added to one litre of HPLC grade/milli-Q water to make the WASH SOLUTION.

6. Next, we must BLOCK the plates with the casein solution. To do this we take our casein solution at room temperature and add 300µl to each well on the plate using the multi-channel pipette. Plate seal the plates and leave these at room temperature for AT LEAST 1 hour with shaking. Longer is fine.

7. Now, create a set of diclofenac standards. It is important that EVERY plate is "calibrated", i.e, that we construct a standard curve using known concentrations of diclofenac on the plate. EVERY plate will be slightly different because this technique has so many steps and errors/differences can easily occur. Our standard curve on every plate tells us whether the plate as a whole has worked well, and if we are going to quantify unknowns on the plate we need a plate specific calibration curve to do this. To create the standards you will need Diclofenac sodium salt (Sigma-Aldrich, D6899). Take a 50ml PP centrifuge tube and add 53.9mg of diclofenac sodium salt. Add 25 ml of HPLC grade acetonitrile and make up to the 50ml mark with HPLC grade/milli-Q water. This will give us a solution containing 50mg/50ml or 1000mg/l (1000ppm) of diclofenac. From this we can dilute down to make standard concentrations between 10µg/l and zero. Take the 1000mg/l stock, take 100µl of this and dilute to 50ml using a 50:50 mix of water:acetonitrile as above. This will give us a 2mg/l stock. Take 1ml of this stock into 50ml of water (no acetonitrile) and we will have a 40µg/l working solution. From this we

can make up our standards (in water only). Make the standards up in glass and store them in the fridge (and the stocks). On each plate we will use 400µl of each standard, hence, if we make up 10ml (or similar) of each concentration this should last a reasonable time. Use serial dilution in µg/l, i.e, make up a 10, 5, 2.5, 1.25, 0.625, 0.3125, etc (or 0, 0.01, 0.05, 0.1, 0.5, 1, 5, 10µg/l). Make up a series of 11 standards (or less) with diclofenac, and 1 blank (free of diclofenac), i.e, 12 concentrations.

Suggested Standard dilutions

| Mix No | Concentration µg/l | Volume of Standard solution | Volume of Water | Total volume of the mix |
|---|---|---|---|---|
| 1 | 10.000 | 50µl of 2mg/l stock | 9.95 ml | 10 ml |
| 2 | 5.000 | 5 ml of mix 1 | 5 ml | 10 ml |
| 3 | 2.500 | 5 ml of mix 2 | 5 ml | 10 ml |
| 4 | 1.250 | 5 ml of mix 3 | 5 ml | 10 ml |
| 5 | 0.625 | 5 ml of mix 4 | 5 ml | 10 ml |
| 6 | 0.313 | 5 ml of mix 5 | 5 ml | 10 ml |
| 7 | 0.156 | 5 ml of mix 6 | 5 ml | 10 ml |
| 8 | 0.078 | 5 ml of mix 7 | 5 ml | 10 ml |
| 9 | 0.039 | 5 ml of mix 8 | 5 ml | 10 ml |
| 10 | 0.020 | 5 ml of mix 9 | 5 ml | 10 ml |
| 11 | 0.010 | 5 ml of mix 10 | 5 ml | 10 ml |

8. Take the plates, remove the plate seals and wash 3 times on the plate washer. Now we must add our standards and our unknown samples.

| | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Std10 | Std5 | Std2.5 | Std1.25 | | | | | | | | Blank |
| Std10 | Std5 | Std2.5 | Etc… | | | | | | | | Blank |
| Std10 | Std5 | Std2.5 | | | | | | | | | Blank |
| Std10 | Std5 | Std2.5 | | | | | | | | | Blank |
| Sam1 | Sam2 | | | | | | | | | | Blank |
| Sam1 | Sam2 | | | | | | | | | | Blank |
| Sam1 | Etc… | | | | | | | | | | Blank |
| Sam1 | | | | | | | | | | | Blank |

Above is a suggested plate design where we use all of column 12 for blanks and the first 4 rows for our standards (Std10, 5, 2.5, etc), and the bottom 4 rows for our unknowns (Sample1, Sample 2, etc). Every sample and standard should be added in 4 replicate wells so that we get 4 readings for each concentration. Here we can therefore do 11 unknowns on a plate. This design would be fine for +ve/-ve screening of sample extractions. For quantification we may need to adjust the design, drop down to 10 standards, do the blank 8 replicates in the upper 4 wells of the last 2 columns, and leave all 12 columns free for samples on the bottom 4 rows. This way we can work on 4 unknowns at 3 dilutions (1:100, 1:250 and 1:500). So, take 100µl of sample or standard and add it to the appropriate well.

9. Next, we must add our primary antiserum dilution to the well which will then compete for binding with the diclofenac that is bound and that is free in solution. Again, there are stocks of antiserum in the freezer in 50µl aliquots. One of these should be stored in the fridge to use on the plates (the remainder MUST remain frozen!). Take 1.25µl of antiserum stock and add this to 25ml (1:20000 dilution) of PBS BUFFER. Shake this well and then add 100µl of this solution to every well using a multi-channel pipette. Plate seal and stand for 1 hour at room temperature whilst shaking. **NOTE:** *At IVRI Dr Mohini has now successfully used incubation at 37°C (in an incubator) in order to "normalise" the conditions of room temperature in India which varies a great deal from place to place and from season to season. Hence, where incubation at room temperature is mentioned below, this could be replaced by incubation at 37°C instead.*

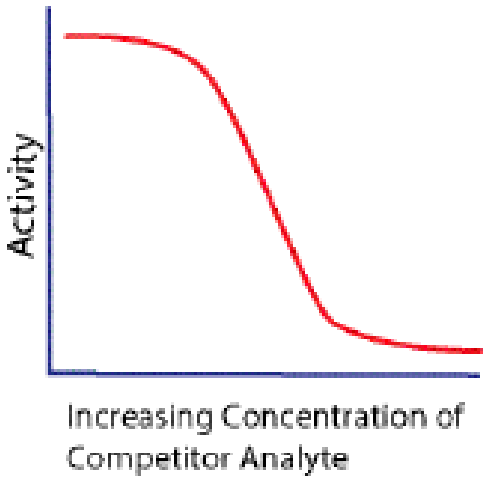
10. After 1 hour, remove the plate seal and wash the plates again 3 times in the plate washer. Next, we must add the secondary antibody. This is commercially available through Sigma (A6154). This antibody is stored in the freezer at ALL times but is nevertheless in a liquid state, allowing us to pipette from the stock without having to store it in the fridge. Take 5µl of the secondary antibody and add this to 50ml of PBS BUFFER. Give this a good shake then add 200µl to all plate wells. Plate seal and leave for 1 hour at room temperature (or 37°C) whilst shaking.

11. We must then make a STOP SOLUTION. This is simply diluted sulphuric acid. Take 1 litre of HPLC grade/milli-Q water and VERY SLOWLY add 100ml of sulphuric acid. Do this in a thick walled glass vessel, and ideally do it with the vessel sitting in a water + ice bath. This is a highly exothermic mixture which gives off a lot of heat on mixing. Add the acid very slowly whilst continually mixing the solution and keeping it cool (TAKE CARE!!). Having added all the acid, finally, make the solution up to 2 litres final volume with HPLC grade/milli-Q water (again, do this slowly).

12. After 1 hour, remove the plate seal and wash the plates 3 times again. Next, we must add the TMB which the enzyme on the secondary antibody will "eat". TMB is light and temperature sensitive and it should be stored in the fridge at all times. It can be made from a powder but I suggest we use a ready made substrate liquid such as that available from Sigma (T0440). Take the TMB substrate and add 200µl to each well. Plate seal and set the plate to shake gently at room temperature (or 37°C).

13. Watch the plate whilst it is shaking. If the test is working you should see a blue colour develop in the wells. It is purely subjective and takes practice, but watch for a strong blue in the blank wells and a light blue in the wells with a lot of diclofenac. Once a "range" is clearly visible across the plate we must add our STOP SOLUTION. Add 100µl of the STOP SOLUTION to each well. Because the colour changes with time it is important that we do steps 10 and 11 quite quickly when we are pipetting and that we do each step at the same rate. If we start our pipetting on column 1 and finish on column 12 in step 10, we must do the same on step 11 so that each well has approximately the same reaction time. When we add the stop solution the fluid in the wells will turn yellow.

14. Place the plate in the plate reader and "read" the plate. If the plate works well we should see a curve like this when we plot the concentration on the x-axis and the mean absorbance readings (of 4 replicates remember) on the y:




**DATA INTERPRETATION:**

In order to process your data, please use the spreadsheet template provided. This will automatically take means of your readings. It will also tell you your %RSD for the 4 replicates of each solution. This is important and should ideally be below 10%. If it is >10%, I suggest that you identify the outlier in the 4 replicates and delete this data point. Ideally work towards achieving %RSDs of <5%. The template will also tell you what the mean blank reading is minus 3SDs, and what the mean sample reading is plus 3SDs. This can then be used to at least assess your data on a +ve and –ve basis. The graph will plot the standard curve. When copying and pasting this template please be aware that you will still have to change the cells that the graph uses to plot the curve, every time. I have also attached below an abbreviated protocol generated by Prof. Knopp which includes the ingredients needed to make the solutions mentioned above from scratch if you do not use pre-mixed solutions (i.e, for TMB substrate).

If you are using this technique and encountering problems please contact Dr Mark Taggart (Mark.Taggart@uclm.es) or Dr Mohini Saini (praveenmohini@rediffmail.com) in the first instance. Alternatively if these individuals are not available then this test was developed by Prof. Dietmar Knopp in Germany who can also be contacted at dietmar.knopp@ch.tum.de.

**REFERENCES:**

There is a paper and a book chapter published on the technique, the references are:

Knopp, D., Deng, A., Letzel, M., Taggart, M.A., Himmelsbach, M., Zhu, Q., Perobner, I., Kudlak, B., Frey, S., Sengl, M., Buchberger, W., Hutchinson, C., Cunningham, A., Pain, D., Cuthbert, R., Raab, A., Meharg, A., Swan, G., Jhala, Y., Prakash, V., Rahmani, A.,

Quevedo, M., Niessner, R., 2007. Immunological determination of the pharmaceutical diclofenac in environmental and biological samples. In: Rational Environment Management of Agrochemicals: Risk Assessment, Monitoring, and Remedial Action. Edited by Kennedy, I.R., et al., American Chemical Society Symposium Series, Oxford University Press, Chapter 13, pages 203-226.

Deng, A., Himmelsbach, M., Zhu, Q.Z., Frey, S., Sengl, M., Buchberger, W., Niessner, R., Knopp, D., 2003. Residue analysis of the pharmaceutical diclofenac in different water types using ELISA and GC-MS. Environmental Science and Technology, 37, 3422-3429.

# Diclofenac ELISA protocol generated by Prof. Dietmar Knopp (Germany)

**Coating buffer, 50 mM, pH 9.6**
3.18 g sodium carbonate
5.86 g sodium hydrogencarbonate
0.4 g sodium azide
2 litres of pure water

**PBS-buffer, 80 mM, pH 7.6**
3.12 g sodium dihydrogen phosphate
24.92 g disodium hydrogen phosphate dihydrate
17 g sodium chloride
2 litres of pure water
**NOTE: Dr Mohini is also going to test a 10mM version of this solution!**

TMB solution
0.375g of 3,3',5,5'-tetramethylbenzidine
5ml dimethylsulfoxide
20ml of methanol

Substrate buffer, pH 3.7
92.08 g potassium dihydrogen citrate
0.2 g sorbic acid
2 litres of pure water

$H_2O_2$ solution
1.5 ml $H_2O_2$ (30%)
ad 50 ml with pure water

**Final TMB substrate solution**
25ml substrate buffer (see above)
500µL TMB solution (see above)
100µL $H_2O_2$ solution (see above)

**Stop solution**
100ml of sulfuric acid
2 litres of pure water

**Plate washing buffer (60 times concentrate)**
5.89 g potassium dihydrogen phosphate
65.32 g dipotassium hydrogen phosphate
51 g sodium chloride
30mL Tween 20
1 litre of pure water

## ELISA PROTOCOL:

**Important: use plate sealing film between the different steps to avoid evaporation:**

**1. Coating of plates with coating antigen**
Take 5µL of coating antigen (conjugate) solution and add to 25mL of Coating Buffer (enough for 1 plate); add 200µL to each well; coat overnight in the fridge at 4°C.

**2. Wash the plate with PBS Tween/plate washer**

**3. Block**
Add 300µL of casein solution (1%, w/v) in PBS buffer to each well and block at room temperature (RT).

**4. Wash the plate with PBS Tween/plate washer**

**5. Competitive step**
Add 100µL of standard (or sample)
*THEN ADD*
100µL of primary antiserum dilution (between 1:15000 and 1:20000); incubate for 1 hour under shaking at RT.

**6. Wash the plate with PBS Tween/plate washer**

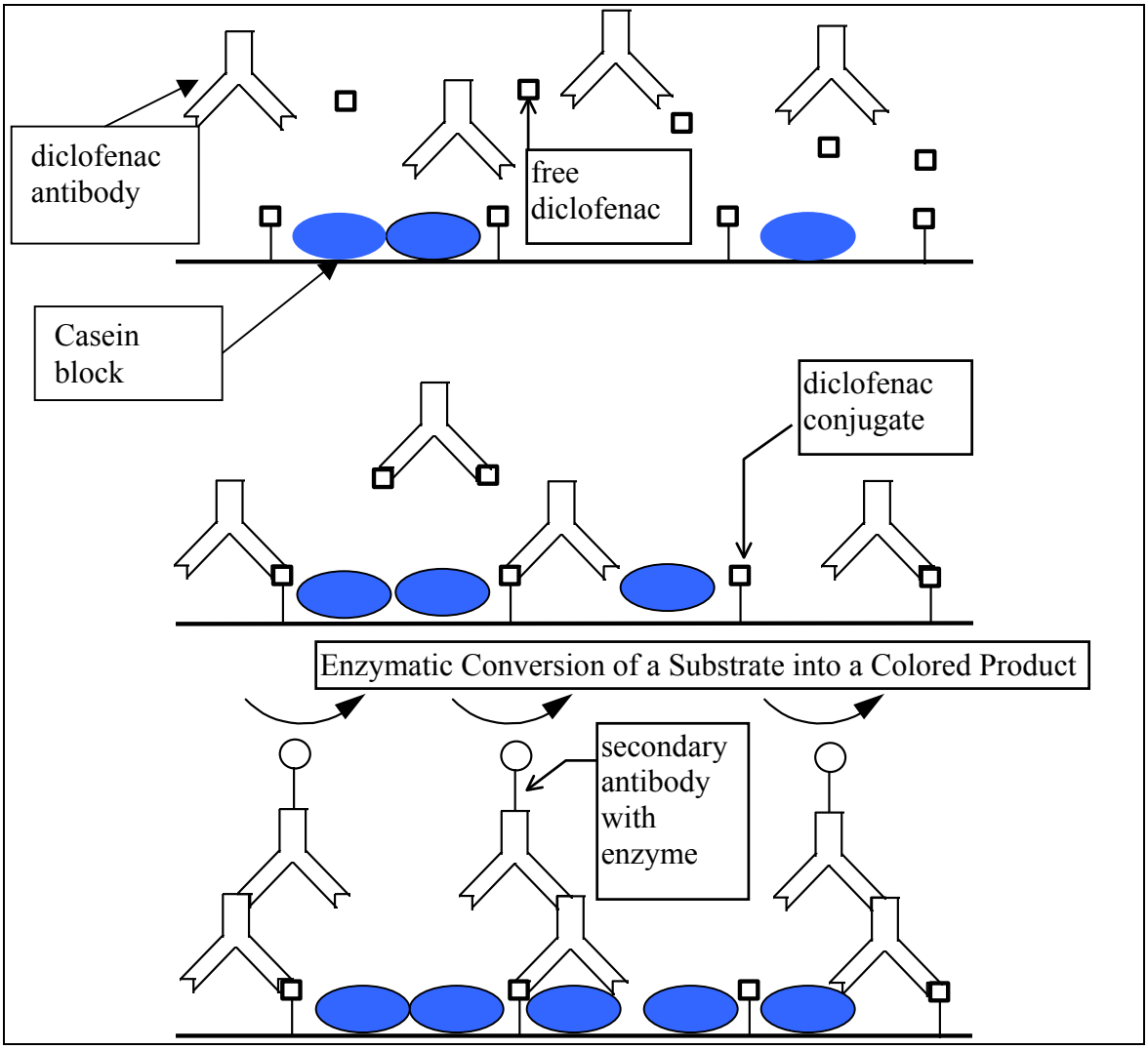
**7. Labelling**
Add 200µL per well of secondary antibody dilution (1:8000 – 1:10000); incubate at RT under shaking for 1 hour.

**8. Wash the plate with PBS Tween/plate washer**

**9. Substrate addition**
Add 200µL of TMB substrate solution; wait up to 20 minutes (until good blue colour has developed) then add 100µL of stop solution.

**10. Measure the plate at 450nm**

**Principle of diclofenac immunoassay:**



## Requirements:

### Equipment

Homogeniser
ELISA reader with 450nm filter
Shaker incubator
Automatic plate washer
Centrifuge
Fridge
Freezer
Balance (4 place, digital)

### Plasticware

96 well high binding ELISA plates
Plate sealing film
Pour boats or large weigh boats
Multichannel pipette 20-300µl
One channel 1-5ml pipette
One channel 0.5-10µl Pipette
One channel 0-100µl Pipette
10µl Tips
200µl/300µl Tips
15ml Centrifuge tubes
50ml Self-standing plastic tubes (for preparing dilutions of Antibody and Antigen)
13mm 0.45µm disposable syringe filters, non sterile
5 ml syringes
Pipette tip boxes (1ml/200µl/10µl)
Racks to store 2ml LCMS vials
Latex gloves

### Glassware

Glass LCMS vials (and plastic lids)
Hard glass test tubes for extraction
2 litre glass bottles
500ml glass bottles
Measuring cylinders
Glass vials

### Reagents

Coating antigen (conjugate)

Primary antiserum (anti-diclofenac antibody)
Secondary antibody (antirabbit-HRPO conjugate)

## Chemicals

HPLC grade or MilliQ water
Diclofenac sodium salt
Acetonitrile (HPLC Grade)
Acetone (GR)
Sodium carbonate
Sodium hydrogencarbonate
Sodium azide
Casein
PBS 10x solution (OR sodium chloride, sodium dihydrogen phosphate and disodium hydrogen phosphate dihydrate)
PBS tween tablets (OR sodium chloride, Tween 20, potassium dihydrogen phosphate and dipotassium hydrogen phosphate)
TMB substrate solution ready to use (OR 3,3',5,5'-tetramethylbenzidine (TMB), dimethylsulfoxide, methanol and potassium dihydrogen citrate)
$H_2O_2$ (30%)
Sorbic acid
Sulfuric acid

## Miscellaneous

Tissue rolls
Scalpels
Tweezers
Granite-cutting block
Marker pens